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Nicotinic Cholinergic Receptors Labeled by [^{3}H]Acetylcholine in Rat Brain

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SUMMARY

On the basis of its ability to bind to nicotinic cholinergic receptors in electric tissue and at the neuromuscular junction, α-bungarotoxin has been widely used to identify central nicotinic receptors. However, the suitability of this ligand as a probe for nicotinic cholinergic receptors in the central nervous system has been questioned. The use of [^{3}H] acetylcholine of high specific activity to measure cholinergic binding sites in rat brain is reported here. In the presence of a cholinesterase inhibitor to prevent hydrolysis and atropine to block muscarinic cholinergic receptors, [^{3}H]acetylcholine bound rapidly, reversibly, and with high affinity to rat brain membranes (K_D = 12.3 ± 0.8 nM, B_{max} = 4.6 ± 0.1 pmoles/g of tissue). Kinetic analyses revealed a half-time for association of 3.2 min and a half-time for dissociation of 2.5 min. The K_D of 12.0 nM calculated from the kinetic experiments was in excellent agreement with that calculated from equilibrium experiments. Subcellular distribution studies indicated that these binding sites were located primarily in the synaptosomal fraction. In competition studies, nicotinic agonists were more than 1000 times more potent than ganglionic and neuromuscular blocking drugs in displacing [^{3}H]acetylcholine binding. Cytisine and (−)-nicotine had the highest affinity (K_I = 1–6 nM) for the [^{3}H]acetylcholine binding site. The highest levels of binding were found in the thalamus, cortex, superior colliculus, and striatum, whereas the lowest were found in the pyriform cortex and hippocampus. The K_D values in several of these areas were found to be similar, whereas the density of binding sites varied. A lack of correlation between the regional distribution of sites labeled by [^{3}H]acetylcholine and those labeled by ^{125}I-labeled α-bungarotoxin suggests that these two binding sites are not located on the same molecule.

INTRODUCTION

Nicotinic cholinergic receptors have been studied extensively in electric organs of fish and eel (1, 2) and at the mammalian neuromuscular junction (3, 4). Much less is known about the nicotinic cholinergic receptor in the central nervous system. Radioactive α-bungarotoxin has been useful for characterizing the nicotinic cholinergic receptor in electric organs and muscle. However, in the central nervous system, α-bungarotoxin may bind to sites distinct from the nicotinic cholinergic receptor binding site (5–7). The evidence for this statement is that nicotinic cholinergic drugs such as nicotine, carbamylcholine (carbachol), and acetylcholine itself have relatively low apparent affinities (10^{-5}–10^{-6} M) for the α-bungarotoxin binding site in brain (8–11), and in the case of acetylcholine, the inhibition of α-bungarotoxin binding appears to be noncompetitive (12). In addition, α-bungarotoxin does not block cholinergic functions in certain neuronal systems (13–17). The α-toxin of *Naja naja* also has been used to investigate nicotinic cholinergic receptors in brain, and the characteristics of the binding site are similar to those of α-bungarotoxin (18).

[^{3}H]Nicotine binds with high affinity to brain membranes, and the binding is stereospecific (5, 6). The distribution of [^{3}H]nicotine binding sites among different brain regions is similar to that of ^{125}I-labeled α-bungarotoxin (19). Interestingly, however, α-bungarotoxin is a weak displacer of [^{3}H]nicotine binding in brain (5, 6), and the affinity of the cholinergic agonist carbachol (1–5 μM) at [^{3}H]nicotine binding sites is relatively low (5, 6). Values for displacement of [^{3}H]nicotine by acetylcholine itself have not been reported (5, 6, 19). From behavioral studies and receptor binding data, Abood *et al.* (5, 20) concluded that [^{3}H]nicotine binds to a non-cholinergic site in brain. However, Romano and Goldstein (6) have presented data that suggest that [^{3}H]nicotine does label a nicotinic cholinergic receptor under their assay conditions.

[^{3}H]Acetylcholine has been used to measure cholinergic receptors in mouse brain (21). The assay method employed was equilibrium dialysis which required 36 hr to reach equilibrium. The [^{3}H]acetylcholine binding sites appeared to represent both nicotinic and muscarinic re-

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ceptors. However, the specific radioactivity of the [3H] acetylcholine used was very low (0.29 Ci/mmole), and it was not clear how specific binding was defined.

The use of [3H]acetylcholine to study nicotinic receptors in *Torpedo* electric tissue recently has been reported (22, 23). The kinetics of [3H]acetylcholine binding was studied by using a rapid filtration technique. It was determined that [3H]acetylcholine binds to a single population of receptors that exists in two interconvertible conformations in the absence of agonist, i.e., one that binds the agonist with high affinity and one that binds the agonist with low affinity. The high-affinity conformation was characterized, and it was concluded that this conformation represented the agonist-stabilized, desensitized state of the receptor at equilibrium.

We report here the use of high specific radioactivity [3H]acetylcholine to measure cholinergic binding sites in brain. In the presence of a cholinesterase inhibitor to prevent hydrolysis and atropine to block muscarinic cholinergic receptors, [3H]acetylcholine binds rapidly, reversibly, and with high affinity to sites in brain with characteristics of nicotinic cholinergic receptors. Because the density of these nicotinic receptors is substantially lower than that in *Torpedo* tissue, [3H]acetylcholine of high specific radioactivity was needed for these studies. A simple, rapid method for its synthesis also is reported.

MATERIALS AND METHODS

Synthesis and purity determination of [3H]acetylcholine. [3H]Acetylcholine of high specific radioactivity was synthesized by esterification of [*methyl*-3H]choline (77 Ci/mmole; Amersham Corporation; Arlington Heights, Ill.) to yield [3H]acetylcholine at a specific radioactivity of 77 Ci/mmole. For each synthesis, 1 mCi of [*methyl*-3H]choline in ethanol was evaporated to dryness under a stream of N_2 at room temperature in a 10 × 75 mm disposable culture tube. To the dried residue, 100 μl of ethyl acetate, 10 μl of acetic anhydride, and 10 μl of triethylamine were added. The mixture was incubated at room temperature for 1 hr, followed by the addition of 1 ml of 95% ethanol to destroy any remaining acetic anhydride. The whole mixture was then evaporated to dryness under N_2, redissolved in 1 ml of 95% ethanol, and stored at 4° under N_2.

The extent of conversion of [3H]choline to [3H]acetylcholine was determined by using a previously described procedure (24, 25). The method makes use of the ability to separate phosphorylcholine from acetylcholine using a liquid/liquid cation-exchange process. Aliquots of the newly synthesized [*methyl*-3H]acetylcholine were incubated in 0.25 ml of a 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)-buffered salt solution for 15 min at 37° in the presence or absence of ATP (10 mM), $MgCl_2$ (10 mM), and choline kinase (10 mU/ml; Sigma Chemical Company, St. Louis, Mo.). During this time, choline is quantitatively converted to phosphorylcholine but acetylcholine is unchanged. The reaction was terminated by adding 2 ml of 3-heptanone containing tetraphenylboron (10 mg/ml) and vigorously vortexing the mixture. After centrifugation to separate the phases, 1.7 ml of the upper organic phase were transferred to a scintillation vial, and the 3-heptanone was evaporated at 60° under a stream of air. Liquid scintillation solution (Liquiscint) was added to dissolve the residual tetraphenylboron and allow determination of radioactivity by liquid scintillation spectrometry. Under these conditions, acetylcholine is quantitatively extracted into the organic phase whereas the phosphorylcholine formed from residual, nonesterified choline quantitatively remains in the aqueous phase. The authenticity of the [3H]acetylcholine was demonstrated by the ability of acetylcholinesterase from electric eel to convert quantitatively the acetylated material back to choline, which then was phosphorylatable by choline kinase.

Four different batches of [3H]acetylcholine were used in the experiments presented here. The percentages of material which behaved as [3H]acetylcholine from each synthesis were 97.7, 93.3, 97.5, and 85.0. The purity of Batches 1 and 3 was virtually unchanged during storage for 1–2 months. The purity of Batch 2 decreased to about 70% after 1 month of storage and therefore was not used further. In the fourth acetylcholine synthesis, the conversion of choline to acetylcholine was initially only 85% efficient. However, reacetylation of the product produced [3H]acetylcholine of 97% purity, indicating that the simple acetylation reaction can be performed more than once to achieve higher purity. This material produced specific binding indistinguishable from that observed with [3H]acetylcholine produced from a single acetylation step. Analysis of the [3H]acetylcholine after incubation with tissue, as in a binding assay, showed less than 4% hydrolysis during 40-min incubations at either 0° or 37°.

Binding assays. Sprague-Dawley rats (males, 250–350 g) were decapitated, and the brains and spinal cords were removed and dissected into various regions. The tissues were homogenized with a Brinkmann Polytron in 50 mM Tris-HCl buffer containing 1.5 μM atropine sulfate, 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$ (pH 7.4 at 0°). The tissue was washed twice by centrifugation at 49,000 × *g* for 10 min with intermediate homogenization in fresh buffer. The final pellet was suspended in buffer containing approximately 100 μM DFP.[1] For routine assays the reaction was started by the addition of 200 μl of tissue (10 mg of original weight, or about 650 μg of protein) to tubes containing [3H]acetylcholine in the presence or absence of 100 μM carbachol (total volume of reaction fluid equaled 500 μl). The reaction mixture was incubated at 0° for 40 min and then filtered under reduced pressure through Whatman GF/C glass-fiber filters which were previously wet with a 0.05% PEI solution to eliminate specific binding to the filter. The filters were washed three times with 4-ml aliquots of cold buffer, placed in vials containing Liquiscint scintillation fluid, and counted in a Searle Mark III liquid scintillation spectrophotometer at a counting efficiency of 35–40%. All assays were run in triplicate. Nonspecific binding was defined as that in the presence of 100 μM carbachol whereas total binding was determined in the absence of carbachol. Specific binding was defined as the difference between total and nonspecific binding. [3H]Choline did not bind to cortical tissue under these conditions.

[1] The abbreviations used are: DFP, diisopropyl fluorophosphate; PEI, polyethyleneimine.

Subcellular fractionation. Subcellular fractions were prepared according to the method of Whitaker and Barker (26). Fresh cortex was homogenized in 0.32 M sucrose containing 10 mM Tris-HCl buffer (pH 7.2). The homogenate was centrifuged at 1,000 × *g* for 10 min and the pellet was discarded. An aliquot of the supernatant (S_1 fraction) was removed for determination of "total" homogenate specific binding. It was diluted with 50 mM Tris-HCl buffer (pH 7.4 at 0°) and prepared for the binding assay as described above. The remaining S_1 supernatant was centrifuged at 13,500 × *g* for 20 min. The pellet (P_2 fraction) containing mitochondria, synaptosomes, and myelin was resuspended in 4 ml of 0.32 M sucrose and was layered on top of 4 ml of 0.8 M sucrose which was layered on top of 1.2 M sucrose. The gradient was centrifuged at 48,000 × *g* for 120 min, using a fixed angle rotor. After centrifugation the uppermost fraction containing myelin was removed first, followed by the synaptosomal fraction and then by the mitochondrial fraction. Each fraction was diluted and washed with 50 mM Tris-HCl buffer. They were then prepared for the binding assay as described above. Aliquots from the total homogenate and the mitochondrial, synaptosomal, and myelin fractions were obtained for protein determination using bovine serum albumin as a standard (27).

Drugs were obtained from commercial sources with the exception of (+)-nicotine, which was a generous gift from Dr. Leo Abood, University of Rochester (Rochester, N. Y.), and mecamylamine, which was a gift from Merck Sharp & Dohme (Rahway, N. J.). Dr. Barbara Morley, Boys Town Institute for Communication Disorders in Children (Omaha, Neb.) kindly supplied the purified 2.2 α-bungarotoxin.

RESULTS

Specific binding of [^{3}H]acetylcholine (in the presence of 1.5 μM atropine) was greatest when the assay mixture was incubated at 0° (Table 1). At 25° the specific binding was decreased by 31%, and at 37° specific binding was decreased by 65% (Table 1). In order to prevent hydrolysis of the [^{3}H]acetylcholine by cholinesterase, DFP was included in the assay mixture. In the absence of DFP, specific [^{3}H]acetylcholine binding was reduced owing to a decrease in total binding and an increase in nonspecific binding. The presence of 100 μM DFP, routinely used, increased total binding while reducing the nonspecific binding, resulting in higher specific binding (Table 1). Concentrations as low as 10 μM were effective in preventing acetylcholine hydrolysis, and concentrations as high as 1 mM did not interfere with [^{3}H]acetylcholine binding (data not shown).

When atropine was omitted from the buffer, total and specific binding were increased (Table 1). This may have been due to specific binding to muscarinic cholinergic receptors. The addition of 1.5 μM atropine to the assay buffer was sufficient to block muscarinic binding sites (28) without interfering with nicotinic cholinergic sites (see Table 3). No specific binding of [^{3}H]acetylcholine was detected in heat-denatured tissue (Table 1).

To reduce total and specific (carbachol-displaceable) binding to the glass-fiber filters, the filters were prewet with a 0.05% PEI solution. This treatment reduced total binding of [^{3}H]acetylcholine to the filters and eliminated specific binding of [^{3}H]acetylcholine to the filters (Table 1). Measurement of specific binding of [^{3}H]acetylcholine to non-muscarinic cholinergic sites was therefore optimal in the presence of 1.5 μM atropine, 100 μM DFP, and PEI-treated filters. Under these conditions, specific binding was linear between 5 and 20 mg of tissue.

TABLE 1

Binding of [^{3}H]acetylcholine under various assay conditions

Binding of 10 nM [^{3}H]acetylcholine was determined by incubating the assay mixture as described in the text, at 0° for 40 min in the presence of 1.5 μM atropine and 100 μM DFP unless otherwise noted below. Nonspecific binding is that occurring in the presence of 100 μM carbachol. Heat-denatured tissue was prepared by heating the cortex in a boiling water bath for 10 min. PEI treatment of Whatman GF/C filters consisted of wetting the filters with a 0.05% solution of PEI in buffer prior to filtering. Untreated filters were prewet with buffer alone. The data are reported as means ± standard error of the mean obtained from at least three animals.

Condition	Binding		
	Total	Nonspecific	Specific
	dpm		
Cortex			
0°	5115 ± 331	2082 ± 31	3033 ± 362
25°	4735 ± 255	2629 ± 117	2106 ± 324
37°	3362 ± 113	2281 ± 150	1081 ± 263
Without DFP	3612 ± 187	2728 ± 355	882 ± 369
Without atropine	10941 ± 1442	1714 ± 150	9228 ± 1551
Heat-denatured	1795 ± 55	1769 ± 52	24 ± 4
Filter			
Untreated	1576 ± 125	1294 ± 18	281 ± 107
PEI-treated (0.05%)	684 ± 108	619 ± 115	65 ± 14

A saturation analysis of [^{3}H]acetylcholine binding to cerebral cortex homogenates indicated a dissociation constant (K_D) of 12.6 nM and a binding capacity (B_{max}) of 4.6 pmoles/g of tissue (Fig. 1a and b). Five separate analyses yielded a value for the K_D of 12.3 ± 0.8 nM and a value for the B_{max} of 4.6 ± 0.1 pmoles/g of cortex (67.2 ± 3.0 fmoles/mg of protein). Specific binding was 40–75% of total binding, depending on the concentration of [^{3}H]acetylcholine. At a concentration of 10 nM, specific binding was approximately 60% of total binding (Fig. 1a). A Hill plot of the saturation data revealed a Hill coefficient (n_H) of 0.99 (Fig. 1c), indicating lack of cooperativity and that [^{3}H]acetylcholine was binding to a single site.

The rates of association and dissociation of the [^{3}H]acetylcholine-receptor complex were studied in cerebral cortex homogenates. Both association and dissociation were rapid. The half-time ($t_{1/2}$) for association was 3.2 min, and the binding reached equilibrium within approximately 30 min (Fig. 2a). The $t_{1/2}$ for dissociation was 2.5 min, and the dissociation curve reached a plateau within 15 min (Fig. 2b). The rate constants for association (k_1) and dissociation (k_{-1}) were determined graphically to be $k_1 = 0.01$ nM^{-1} min^{-1} and $k_{-1} = 0.12$ min^{-1}. The K_D determined by the ratio k_{-1}/k_1 was 12.0 nM, in close agreement with the K_D determined by equilibrium studies (Fig. 1). The dissociation rates were also studied at 25° and at 37° and yielded a $t_{1/2}$ for dissociation of 30 sec and 2 sec, respectively (data not shown). The more rapid dissociation may account for the lower binding observed at these higher temperatures (see Table 1).

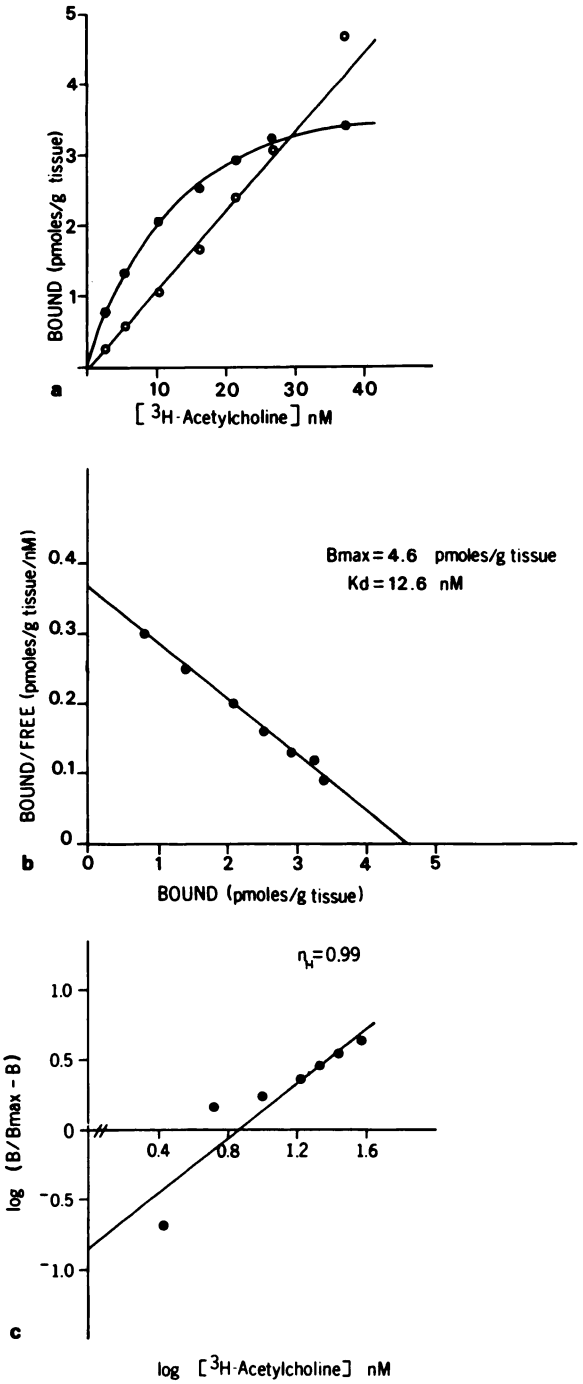

FIG. 1. *[^{3}H]Acetylcholine binding in brain*

a. Saturation analysis of [^{3}H]acetylcholine binding to cerebral cortex. Homogenized cortex in buffer containing 1.5 μM atropine was incubated with varying concentrations of [^{3}H]acetylcholine (2.5–37.0 nM) for 40 min at 0°. Nonspecific binding (○——○) was determined in the presence of 100 μM carbachol. Specific binding (●——●) is the difference between total binding and nonspecific binding.

b. Scatchard analysis of the specific binding of [^{3}H]acetylcholine. This experiment is representative of five separate experiments. The K_D and B_{max} were determined by least-squares linear regression.

c. Hill plot of the binding data in *a*. The Hill coefficient (n_H) was 0.99, determined by least-squares linear regression.

In subcellular distribution studies, binding was measured in the synaptosomal, mitochondrial, and myelin fractions and compared with the binding in the total homogenate. Among the three subcellular fractions, specific binding was highest in the synaptosomal fraction. Very little binding was found in the myelin fraction, and binding was virtually absent in the mitochondrial fraction (Table 2).

The pharmacological characteristics of the [^{3}H]acetylcholine binding site in brain were studied by examining the inhibition of binding by various drugs (Table 3; Fig. 3). In general, cholinergic agonists were more than 1000 times more potent than antagonists in displacing [^{3}H]acetylcholine binding (Table 3; Fig. 3). Acetylcholine and (−)-nicotine were approximately equipotent. The affinity

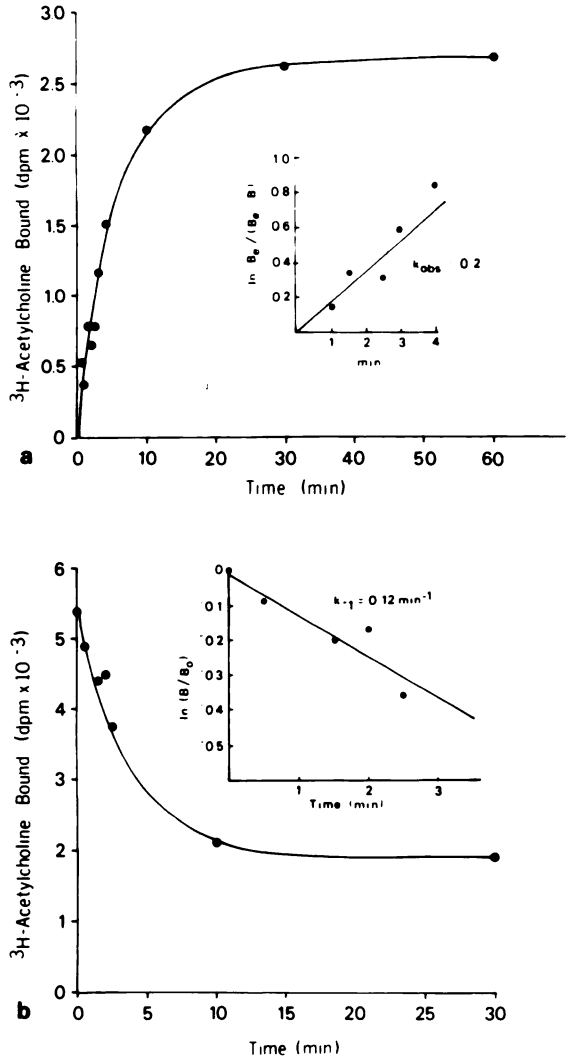

FIG. 2. *Rate of association and dissociation of [^{3}H]acetylcholine binding to cortex*

a. The association rate was determined by incubating the assay mixture as described in the text for various times before filtration. Specific binding is plotted and is representative of four experiments. *Inset*, pseudo-first order kinetic plot of [^{3}H]acetylcholine specific binding. K_1 was determined by the equation $k_1 = (k_{obs} - k_{-1})/[^3\text{H}]$acetylcholine.

b. The rate of dissociation was determined in cortical homogenates by initially incubating the tissue with 10 nM [^{3}H]acetylcholine at 0° for 40 min and subsequently adding 100 μM carbachol. The reaction was then stopped at various times by filtration. The data are representative of four experiments. *Inset*, first-order kinetic plot of [^{3}H]acetylcholine specific binding. k_{-1} is represented by the negative slope, determined by least-squares linear regression.

TABLE 2

Subcellular distribution of [^{3}H]acetylcholine binding in rat cortex

The binding of 10 nM [^{3}H]acetylcholine was determined in the total homogenate (S_1 fraction) and in the synaptosomal, mitochondrial, and myelin fractions which were isolated by sucrose gradient centrifugation as described in the text. Aliquots of each fraction were analyzed for protein content, and specific binding was obtained as femtomoles per milligram of protein. The data are reported as means ± standard error of the mean obtained from three animals.

Fraction	Protein	Specific binding	Specific binding
	mg/fraction	*fmoles/fraction*	*fmoles/mg protein*
S_1 total homogenate	10.5 ± 1.5	489.2 ± 49.2	47.4 ± 3.1
Synaptosome	5.3 ± 0.8	282.8 ± 49.1	53.0 ± 2.9
Myelin	1.7 ± 0.2	17.1 ± 6.6	10.3 ± 4.3
Mitochondria	0.6 ± 0.0	0	0

of carbachol for the binding site was approximately one-half that of acetylcholine. Cytisine, a ganglion-stimulating alkaloid, was the most potent drug examined. Its affinity for the binding site was 5–6 times higher than

TABLE 3

Displacement of [^{3}H]acetylcholine binding in rat cortex by various drugs

Cortical homogenates were incubated with 10 nM [^{3}H]acetylcholine and four to seven concentrations of competing drugs for 40 min at 0° as described in the text. The concentration of drug which inhibited the specific binding of [^{3}H]acetylcholine by 50% (IC_{50}) was determined graphically. The IC_{50} was converted to an inhibitory constant (K_I) by using the equation $K_I = IC_{50}/(1 + ([^3H]\text{acetylcholine}/K_D))$. The K_I reported is the mean ± standard error of the mean obtained from at least three animals.

Drug	K_I
	nM
Cholinergic agonists	
Cytisine	1.3 ± 0.5
(−)-Nicotine	6.4 ± 0.5
Acetylcholine	7.6 ± 1.4
Carbachol	13.4 ± 2.5
(+)-Nicotine	146 ± 28.5
Succinylcholine	6,700 ± 1,900
Piperidine	8,200 ± 2,600
Decamethonium	9,700 ± 2,300
Methacholine	84,300 ± 4,400
Choline	134,000 ± 63,400
Cotinine	175,000 ± 16,400
Cholinergic antagonists	
d-Tubocurarine	28,500 ± 4,400
Gallamine	157,000 ± 1,600
Hexamethonium	182,000 ± 37,000
Atropine	476,000 ± 189,000
Pancuronium	556,000 ± 144,000
Trimethaphan	610,000 ± 166,000
Chlorisondamine	686,000 ± 187,000
Mecamylamine	822,000 ± 273,000
α-Bungarotoxin (Sigma)	Displaced <20% at 40 μM
α-Bungarotoxin (2.2 fraction)	Displaced 50% at 40 μM
Other[a]	
Hemicholinium-3	80,200 ± 9,900
Spiperone	260,000 ± 42,500
4-Aminopyridine	284,000 ± 12,500

[a] The following drugs displaced less than 30% of binding at 100 μM: thioridazine, piperazine, amitriptyline, and diazepam.

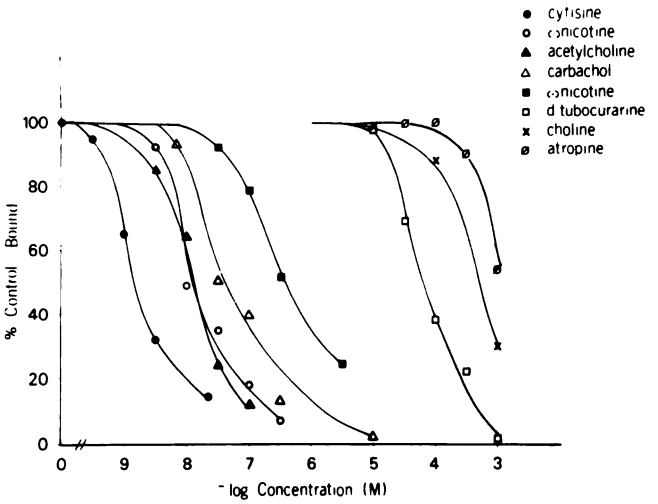

FIG. 3. *Displacement of [^{3}H]acetylcholine binding in cortex by various drugs*

Cortical homogenates were added to tubes containing 10 nM [^{3}H] acetylcholine and the competing drug at various concentrations. The mixture was incubated for 40 min at 0° and filtered as described in the text. Specific binding is expressed as percentage of control (no competing drug present). The curves are representative of at least three separate experiments per competing drug, each assayed in duplicate or triplicate.

that of (−)-nicotine or acetylcholine. Decamethonium and succinylcholine, which produce depolarization blockade at the neuromuscular junction, were among the weakest agonists in displacing [^{3}H]acetylcholine binding. Choline and cotinine, metabolites of acetylcholine and nicotine, respectively, were much less potent than the parent compounds. The much lower affinity of (+)-nicotine as compared with (−)-nicotine (Table 3; Fig. 3) indicated that the binding site displayed a high degree of stereospecificity. Since the (+)-nicotine used was only approximately 96% optically pure, the actual affinity of (+)-nicotine was probably even lower.

Among the antagonists, *d*-tubocurarine was the most

TABLE 4

Relative distribution of [^{3}H]acetylcholine binding in several areas of rat brain

The specific binding of 10 nM [^{3}H]acetylcholine to various areas of rat brain was determined as described in the text. Values are means ± standard error of the mean for the number of animals indicated in parentheses.

Brain region	Specific binding
	pmoles/g tissue
Thalamus (5)	2.66 ± 0.13
Posterior cortex (3)	2.05 ± 0.01
Frontal cortex (3)	1.79 ± 0.02
Whole cortex (7)	1.61 ± 0.15
Superior colliculus (3)	1.44 ± 0.36
Striatum (5)	1.19 ± 0.11
Hypothalamus (5)	1.05 ± 0.24
Cerebellum (3)	1.03 ± 0.16
Brain stem (6)	0.79 ± 0.14
Spinal cord (5)	0.61 ± 0.09
Septum (3)	0.54 ± 0.15
Inferior colliculus (3)	0.51 ± 0.08
Hippocampus (5)	0.44 ± 0.07
Pyriform cortex (3)	0.29 ± 0.08

TABLE 5

Binding constants of [3H]acetylcholine in various areas of rat brain

Tissue homogenates (10 mg) were incubated with varying concentrations of [3H]acetylcholine at 0° for 40 min as described in the text. The number of determinations is designated in parentheses. Brain areas were pooled from two to eight rats to provide enough tissue for Scatchard analyses.

Brain region	B_{max}	K_D
	pmoles/g tissue	*nM*
Thalamus (2)	9.0	14.4
Striatum (2)	5.6	12.2
Cortex (6)	4.7	12.3
Inferior colliculus (1)	2.4	14.0
Hippocampus (2)	2.0	12.6
Cerebellum (2)	1.9	8.4
Hypothalamus (3)	1.8	14.9

potent drug in displacing [3H]acetylcholine binding, but it was approximately 4000 times less potent than nicotine or acetylcholine (Table 3; Fig. 3). The ganglion-blocking drugs were very weak inhibitors of [3H]acetylcholine binding, as were atropine and methacholine (Table 3). At concentrations as high as 40 μM, α-bungarotoxin obtained from a commercial source (Sigma Chemical Company) failed to displace [3H]acetylcholine binding. However, in the presence of 0.2% bovine serum albumin a preparation of α-bungarotoxin (2.2 fraction) purified by Dr. Barbara Morley displaced [3H]acetylcholine binding by 50% at a concentration of 40 μM (Table 3).

The binding of [3H]acetylcholine in different regions of the brain and in spinal cord varied over a 9-fold range (Table 4). Highest binding was found in the thalamus, cortex, superior colliculus, and striatum. In most areas of the cerebral cortex, binding was relatively high, but in the prepyriform (olfactory) cortex, binding was very low. Scatchard analyses in several regions of brain revealed that the variation in binding in different areas was due primarily to changes in the density of the binding sites, and that the K_D was relatively constant (Table 5).

DISCUSSION

Under the conditions employed here, [3H]acetylcholine binds rapidly, reversibly, and with high affinity to brain homogenates. The binding site is saturable, concentrated in the synaptosomal fraction, and displays pharmacological characteristics of a nicotinic cholinergic receptor binding site. Previous attempts to use [3H]acetylcholine to measure and characterize nicotinic cholinergic receptor binding sites in brain have encountered problems due to the low specific radioactivity of the ligand, binding to muscarinic cholinergic receptors, and long separation times (36 hr) by equilibrium dialysis (21).

The relationship between specific binding sites for radiolabeled α-bungarotoxin and nicotinic cholinergic receptors in brain is unclear, since nicotinic cholinergic drugs are relatively weak in displacing it and the toxin does not block cholinergic function in various neuronal tissues (13–17). [3H]Nicotine binds to a site in brain slices (5) and homogenates (6, 19). Abood *et al.* (5) reported that [3H]nicotine binds to a non-cholinergic receptor in brain. However, others have characterized [3H]nicotine binding in brain and concluded that it binds to two sites, one of which has characteristics of a nicotinic cholinergic receptor (6, 19). [3H]Nicotine binds avidly to glass-fiber filters in a stereospecific and displaceable manner (20). This problem was overcome by Romano and Goldstein (6) by presoaking the filters in a polylysine solution. Under these conditions [3H]nicotine bound to two sites in brain homogenates, and the high-affinity site displayed characteristics expected of a nicotinic cholinergic receptor binding site (6).

There are notable similarities and differences between the [3H]nicotine binding site reported previously (6) and the [3H]acetylcholine binding site reported here. The similarities include relatively low affinities of nicotinic cholinergic antagonists as compared with agonists. The lack of potency of α-bungarotoxin is particularly noteworthy. At concentrations up to 0.6 μM the toxin failed to reduce [3H]-nicotine binding (6), and the most potent α-bungarotoxin which we used displaced [3H]acetylcholine binding by 50% only at a concentration of approximately 40 μM (Table 3). Among the agonists, cytisine and nicotine are the most potent drugs in displacing [3H]nicotine binding (6) and [3H]acetylcholine binding (Table 3). In addition, (−)-nicotine is much more potent than (+)-nicotine in displacing the binding of the two 3H-labeled ligands, indicating that the binding sites are stereospecific. The major differences between the [3H]acetylcholine binding reported here and [3H]nicotine binding reported previously (6) are that [3H]nicotine labels two sites whereas [3H]acetylcholine, under the conditions employed here, appears to label only one site. In addition, the apparent affinities of both nicotinic cholinergic agonists and antagonists are consistently higher (5–90 times) when estimated by displacement of [3H]acetylcholine than by displacement of [3H]nicotine. The difference in drug affinities may be explained in part by the fact that [3H]nicotine binds to two sites in brain (6), one of which may be non-cholinergic.

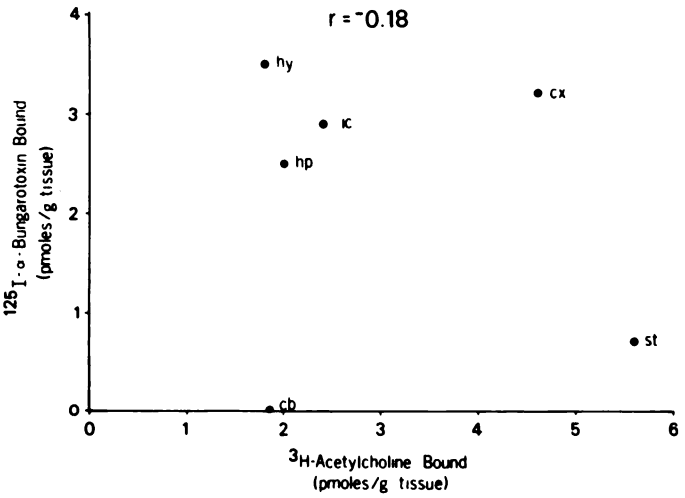

FIG. 4. *Correlation of regional binding of [3H]acetylcholine and 125I-labeled α-bungarotoxin in rat brain*

Maximal specific binding of [3H]acetylcholine was determined in several regions of rat brain according to the procedure described in Fig. 1. 125I-Labeled α-bungarotoxin binding data were taken from Morley *et al.* (7) and are plotted as maximal binding in each area. The lack of correlation is evident from the correlation coefficient (r) of −0.18, determined by a least-squares linear regression. *cb*, Cerebellum; *cx*, cortex; *hp*, hippocampus; *hy*, hypothalamus; *ic*, inferior colliculus; *st*, striatum.

The low potency of α-bugarotoxin in displacing [^{3}H] acetylcholine in brain is particularly interesting. If this low potency were due to an agonist-induced shift of the binding site to an agonist-selective state, the shift would have to be nearly absolute, since the affinity of α-bungarotoxin for the [^{3}H]acetylcholine binding site is at least 10,000 times lower than the affinity indicated by the dissociation constant of ^{125}I-labeled α-bungarotoxin binding in brain (7, 8). Another, more probable, explanation is that α-bungarotoxin and acetylcholine bind to separate sites in brain. If this is the case, the question arises of whether the two binding sites are on the same or different molecules. The distribution of [^{3}H]acetylcholine binding sites varies by approximately 9-fold among the 12 areas examined, with the highest binding occurring in the thalamus and the lowest occurring in the pyriform cortex and hippocampus (Table 4). A comparison of the nominal density of ^{125}I-labeled α-bungarotoxin (7) and [^{3}H]acetylcholine binding sites in several areas of rat brain (Fig. 4) reveals a very low correlation between the two sites and suggests that the two binding sites are not on the same molecule.

The low potencies of neuromuscular and ganglionic nicotinic cholinergic antagonists in displacing [^{3}H]acetylcholine binding in brain is interesting and potentially important. It is possible that the low affinities of antagonists as compared with agonists for the [^{3}H]acetylcholine binding site are due to an agonist-induced shift of the binding site to a high-affinity, agonist-selective state as suggested by Romano and Goldstein (6) for [^{3}H]nicotine binding. Alternatively, the [^{3}H]acetylcholine binding site described here may represent the recognition site on the receptor, which may be distinct from the site at which antagonists act. However, it is also possible that the low affinities of the ganglionic and skeletal muscle antagonists for the brain nicotinic cholinergic binding site indicate that the brain nicotinic cholinergic receptor is substantially different from either of the two peripheral nicotinic receptors.

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