High-Affinity [^{3}H]THA (Tetrahydroaminoacridine) Binding Sites in Rat Brain

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Tetrahydroaminoacridine (THA), an acetylcholinesterase inhibitor that is reported to have significant effects on cognition and memory in Alzheimer's disease patients, binds to rat brain membranes in a saturable and reversible manner. Computer analysis of the binding data revealed high- and low-affinity sites with K_d values of 97.8 n*M* and 4.65 μ*M* and B_{max} values of 4.13 and 114 pmol/mg protein. Autoradiographic studies show that these binding sites are not colocalized with acetylcholinesterase activity. The binding of [^{3}H]THA to membranes does not appear to be related to receptors for several neurotransmitters/neuromodulators, including acetylcholine and other acetylcholinesterase inhibitors. Amiridin, a closely related acetylcholinesterase inhibitor, was able to block specific [^{3}H]THA binding (IC_{50} = 1.05 μ*M*). While the function of THA mediated by these sites is unknown, they may be responsible in part for the distinct clinical effects of tetrahydroaminoacridine compared to other acetylcholinesterase inhibitors.

KEY WORDS: tetrahydroaminoacridine (THA); THA binding sites; Alzheimer's disease; acetylcholinesterase inhibitors.

INTRODUCTION

Alzheimer's disease (AD) is a neurological disorder characterized by progressive decline in cognitive function (1). A large body of evidence indicates that this deterioration of the cholinergic system is responsible for these deficits in cognitive function and memory (2). Thus, therapeutic interventions in AD have focused largely on enhancing acetylcholine (ACh) function in the central nervous system (CNS). However, clinical trials with the ACh precursors choline and lecithin have not demonstrated any consistent cognitive improvements (3–5). Similarly, trials with the AChE inhibitor physostigmine have been disappointing, largely because its peripheral side effects severely limit its dose (6–8).

Recently, oral administration of THA (9-amino-1,2,3,4-tetrahydroacridine) was reported to result in a significant improvement in memory, cognitive function and quality of life in patients with AD (9). THA is a potent inhibitor of AChE (10), leading to the speculation that this property accounted for its clinical effects. However, THA does possess other pharmacological properties which may contribute to its putative efficacy in AD. For example, it is known that THA modulates neuronal K^+ currents in several species (11–13) and also blocks neuronal uptake of monoamines (14). However, the relevance of these actions to the clinical effects of THA in AD patients is difficult to judge since THA is much less potent at producing these noncholinergic activities than as an AChE inhibitor.

In an attempt to identify the site of action through which THA exerts it pharmacological effects in the CNS, we examined rat brain membranes for specific binding sites for [^{3}H]THA. Our results show that THA has both high- and low-affinity binding sites in brain membranes which do not appear to be related to the AChE enzyme or receptors for a large number of other neurotransmitters/neuromodulators. This study describes the initial characterization of these sites.

MATERIALS AND METHODS

[^{3}H]THA ([7-^{3}H]-9-amino-1,2,3,4-tetrahydroacridine) was custom synthesized from 7-bromo-9-amino-1,2,3,4-tetrahydroacridine by New England Nuclear, Boston, MA (27.2 Ci/mmol). Greater than 97.5% of the radioactivity comigrated with nonradioactive THA as a single peak on silica gel thin-layer chromatography (methylene chloride:ethanol:triethylamine, 5:5:0.1). [^{3}H]THA binding and the AChE enzyme assay were carried with a membrane P2 fraction prepared from whole rat brain with the cerebellum removed, the striatum, or the hippocampus essentially as described by Gray and Whittaker (15). The P2 was resuspended and washed two additional times with 5 m*M* Tris–HCl (pH7.5) and stored at −20°C until assayed. In order to be able to compare the results from the binding assay, autoradiography, and enzyme assay, the conditions developed for the AChE assay by Ellman *et al.* (16) were used in the binding assays as well (50 m*M* sodium phosphate, pH 8.0). AChE histochemistry was carried out according to Keolle and Fridenwald (17) also with 50 m*M* sodium phosphate buffer, pH 8.0, containing 100 μ*M* tetraisopropylpyrophosphoramide. For the binding assay, 200 μg of membrane protein [determined according to Smith *et al.* (18)] was added to 1.5-ml microfuge tubes containing 1 ml of 100 m*M* sodium phosphate buffer, pH 8.0. THA or drugs to be tested were added in 20-μl aliquots before adding [^{3}H]THA. The tubes were incubated for 20 min on ice, followed by centrifugation (2.5 min at 10,000*g*). The supernatant was aspirated, and the radioactivity in the membrane pellet measured in a Beckman LS5800 liquid scintillation counter. Specific binding was determined by subtracting the binding that occurs in the presence of 100 μ*M* nonradioactive THA and constituted 60 to 70% of the total radioactivity associated with the pellet. The binding results in the competition assays were analyzed by the LUNDON2 computer modeling program (LUNDON Software, Cleveland, Ohio), which uses simultaneous equilibrium concentration equations as described by Feldman *et al.* (19). The program implements an algorithm of rigorous weighted nonlinear least-squares regressions. Autoradiography was carried out by a modification of standard procedures (20). Twenty-micrometer brain sections were freeze-thaw mounted on top gelatin-coated glass slides and incubated with 100 m*M* sodium phosphate buffer, pH 8.0, containing 20 n*M* [^{3}H]THA for 30 min at 0°C. The sections were washed two times for 5 sec each in ice-cold buffer and immediately dried with a stream of cold air. LKB ultrafilm was developed following exposure for 8 to 10 days.

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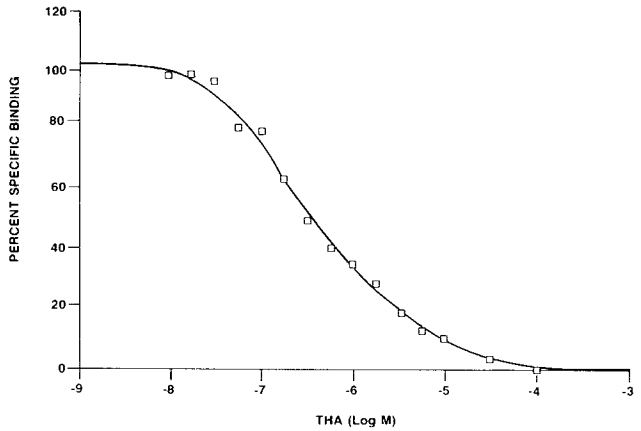

Fig. 1. Inhibition of [^{3}H]THA binding by THA. The binding assays are performed as described above. Each point is the average of triplicate determinations from a representative experiment. The data were analyzed using the LUNDON2 computer modeling program described under Materials and Methods. The two-site model was determined by the program to be a better fit to the data and was significantly different from the one-site fit ($P < 0.001$, F test). The line is the theoretical curve calculated by the LUNDON2 computer modeling program for a two-site model. The experiemnt was repeated five times with similar results.

7-Bromo-9-amino-1,2,3,-tetrahydroacridine, 3-(+)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (CPP), nitrendipine, 3-quinuclidinyl benzilate (QNB), and phencyclidine (phencyclidine) were synthesized by the Department of Medicinal Chemistry, Pfizer Inc. THA was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were from Sigma Chemical Co (St. Louis, MO).

RESULTS

[^{3}H]THA binding to membranes from rat brain (final concentration of 5 nM) was rapid and reached equilibrium after 2 min at 0°C. The binding was reversible, with the bound ligand being completely displaced 2 min after the addition of 100 μM nonradioactive ligand (data not shown). Because of this rapid on-and-off rate a filter assay was impractical and all the results presented here were obtained using a microfuge assay. The kinetic parameters of [^{3}H]THA binding were investigated by incubating membranes with 5 nM [^{3}H]THA in the presence of various concentrations of nonradioactive THA (Fig. 1). Curve-fitting analysis (19) with the LUNDON2 computer modeling program indicated that the data were best fit by a two-site model. The equlibrium binding parameters averaged from five separate experiments are summarized in Table I. The high-affinity site represents the most potent activity of THA yet reported. Since the putative clinical effects of THA are attributed to its effects on AChE, we examined the ability of THA to inhibit AChE in the same membrane preparations that were used for the binding experiments. Additionally, the conditions used for the AChE assay and binding assay were identical, i.e., 50 mM sodium phosphate buffer, pH 8.0 (16). THA exhibited an IC_{50} of 320 ± 97 nM (N = 4). This value does not correspond to either of the K_d values of the binding sites, suggesting that the binding sites may not be associated with the ability of THA to inhibit AChE.

Additional experiments showed that the distributions of the THA receptors and AChE in the rat brain are dissimilar. We examined the relative levels of THA binding and AChE activity in membranes prepared from the striatum and hippocampus. Previous histochemical studies have demonstrated the striatum to be enriched in AChE relative to the hippocampus. Our results confirm this heterogeneity of AChE activity, with the striatum exhibiting over two-fold higher activity than the hippocampus and nearly a four-fold increase over membranes from forebrain (Table I). We found that the K_d values of the [^{3}H]THA binding sites and the B_{max} value of the low-affinity site in these tissues were indistinguishable from each other or from forebrain (Table I). However, the density of the high-affinity site was reduced in the striatum compared to the forebrain or hippocampus, although the reduction did not reach statistical significance ($P > 0.05$) (Table I). We also determined the localization of both [^{3}H]THA binding sites and AChE activity in the rat hippocampus and dentate gyrus by using autoradiographic (20) and histochemical (17) methods. Calculations based of the kinetic parameters in Table I indicate that at 20 mM [^{3}H]THA, 56% of the binding is to the high-affinity site and 44% is to the low-affinity site in the hippocampus. Specific binding is high in the striatum oriens and radiatum in the hippocampus (Fig. 2). In the dentate gyrus, binding is highest near the granular cell bodies and decreased in the outer portions of the molecular later. Binding is lowest in myelinated areas (e.g., the corpus callosum) (Fig. 2). In contrast, AChE activity is higher in the molecular layer of the dentate

Table I. Distribution of AChE Activity and [^{3}H]THA Binding Sites in Membranes from Rat Forebrain, Striatum, and Hippocampus[a]

	Forebrain	Hippocampus	Striatum
High affinity			
B_{max} (pmol/mg protein)	4.13 ± 1.19	3.65 ± 0.43	2.63 ± 0.81
K_d (nM)	97.8 ± 23.3	94.2 ± 23.9	78.1 ± 14.4
Low affinity			
B_{max} (pmol/mg protein)	114 ± 21.7	102 ± 41.2	112 ± 12.8
K_d (μM)	4.65 ± 1.14	3.09 ± 1.02	4.49 ± 0.81
AChE (ΔOD/min/mg)	1.05 ± 0.23	1.59 ± 0.36	3.75 ± 0.84

[a] Membrane fractions were prepared and assays carried out as described in the text. The results are the means ± SE from four or five separate experiments.

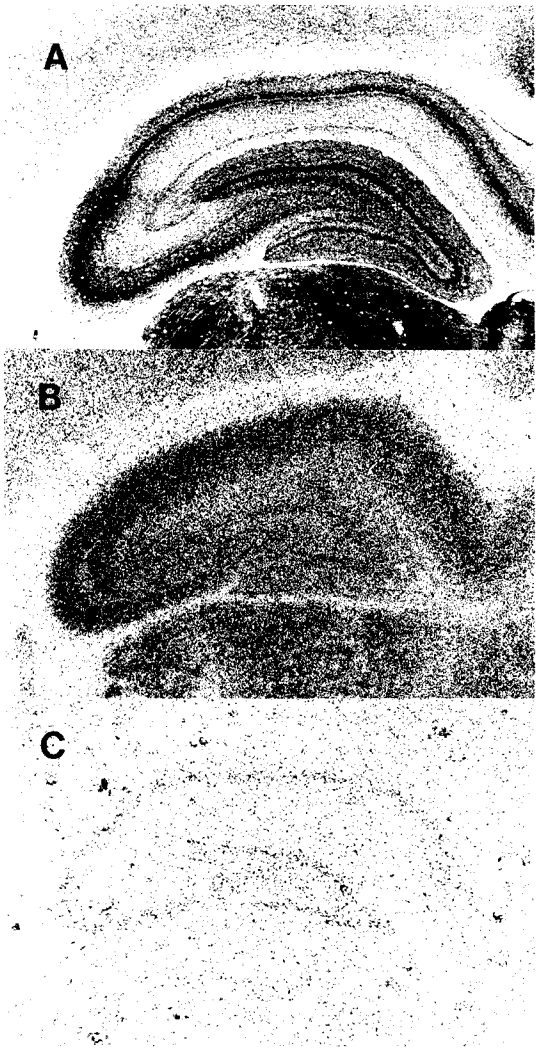

Fig. 2. Comparison of the distribution of AChE activity (A) and [^{3}H]THA binding sites (B) in the rat hippocampus. A small amount of nonsaturable binding on the pyramidal and granule cell layer occurs even in the presence of 100 μM nonradioactive THA (C). AChE histochemistry and autoradiography were performed as described under Materials and Methods.

gyrus and lower in the striatum oriens and radiatum (Fig. 2). If AChE was associated with one of the binding sites, approximately one-half of the binding should be in the dentate gyrus since this structure contains the majority of the AChE activity. Instead the major portion of the binding is in areas of the hippocampus that contain the least amount of AChE activity. Thus, this distribution is inconsistent with either binding site being associated with the AChE enzyme. It is possible that THA is binding to a subpopulation of AChE. However, since THA inhibits all AChE activity in the rat CNS, this binding site is not related to THA's ability to inhibit this enzyme.

We also examined the ability of compounds representative of various neurotransmitter receptors and ion channel ligands to inhibit [^{3}H]THA binding (20 mM). Compounds which interact with receptors for monoamine systems (serotonin, ketanserin, norepinephrine, clonidine, pindolol, propranolol, epinephrine, clozapine, phentolamine, reserpine), other receptors [glutamate (glutamate, CPP), GABA, substance P, diazepam, glycine, phencyclidine], or binding sites for Ca^{2+} channels (nitrendipine) and K^+ channels (4-aminopyridine, apamine) were unable to alter [^{3}H]THA binding (IC_{50}'s > 100 μM). K^+ions (10 mM) also had no effect on the binding. More importantly acetylcholine and physostigmine, either alone or in combination, as well as atropine, scopolamine, hemicholinium, gallamine, QNB, and decamethonium, did not block [^{3}H]THA binding at concentrations of up to 100 μM. Only the close chemical analogue of THA, the acetylcholinesterase inhibitor amiridin, blocked [^{3}H]THA binding (IC_{50} = 1.05 μM; N = 4). We have not determined if amiridin displays any selectivity for either the high- or the low-affinity sites. These data, therefore, indicate that the binding sites for THA are distinct from the binding sites of several other neurotransmitters/neuromodulators.

DISCUSSION

This study is the first to demonstrate the existence of a saturable high-affinity binding site for THA in rat CNS membranes. The binding of [^{3}H]THA is not blocked by several neurotransmitters/neuromodulators and is thus distinct from the sites at which these compounds act. Furthermore, the [^{3}H]THA binding site is not related to the ability of THA to inhibit the AChE enzyme or ACh receptors. Several studies have suggested that the putative clinical effects of THA may be due to its activity on noncholinergic neuronal systems (11–14). The THA molecule has an obvious structural resemblance to the K^+-channel blocker 4-aminopyridine (4-AP) and THA blocks neuronal K^+ currents in several species at concentrations of 6 to 1000 μM (11–13). [^{3}H]THA binding, however, was not affected by 4-AP or K^+ ions (Table I) and THA has a significantly higher affinity for its binding site than any of these effects on K^+ channels. Although THA also inhibits monoamine uptake (14), THA binding is not affected by monoamine agonists or antagonists at pharmacologically relevant concentrations. At the estimated clinically active levels of THA in the CNS (0.1 to 1 μM) (11), THA would be expected to affect only AChE activity, the [^{3}H]THA binding site identified here, and perhaps norepinephrine uptake.

THA enters into the CNS rapidly following systemic administration (21). Additional studies suggest that the THA that enters into the CNS occupies the binding sites described here. Thus, McNally *et al.* (22) found that the distribution of [^{14}C]THA in the CNS 30 min after either an oral (20 mg/kg) or an i.v. (5 mg/kg) dose did not correspond to the distribution of AChE activity in the brain. Furthermore, the distribution of ^{14}C radioactivity that they observed in the hippocampus, dentate gyrus, and corpus callosum was similar to the distribution of [^{3}H]THA binding sites reported here. Additionally, our preliminary autoradiographic results on the distribution of [^{3}H]THA binding sites in the CNS is also in agreement with the *in vivo* distribution reported by McNally *et al.* Thus, these results suggest that the THA binding sites are occupied *in vivo* and are an important mechanism for influencing relative levels of THA in various structures of the CNS. As a result, areas with high levels of THA re-

ceptors will have high concentrations of THA *in vivo*. This may have important consequences for determining the principal sites of action of this compound.

Binding studies have been used to help identify and characterize unknown *in vivo* activities of many compounds. Thus, binding sites for benzodiazepines (23), excitatory amino acids (24), dextromethorphan (25), phencyclidine (26), and others were first identified before their functional significance was recognized. The experiments described here reveal that [^{3}H]THA has nanomolar and micromolar affinities for unique binding sites in brain distinct from the AChE enzyme. While the precise function of the THA binding site is unknown at present, it is likely that its elucidation will aid in understanding the *in vivo* effects of this drug and facilitate the design of improved therapies for AD.

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