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# PHARMACOLOGICAL SIGNIFICANCE OF ACETYLCHOLINESTERASE INHIBITION BY TETRAHYDROAMINOACRIDINE

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**Abstract**—Tetrahydroaminoacridine (THA; Tacrine) is a potent, non-competitive inhibitor of the neuronal enzyme acetylcholinesterase (AChE) and, consequently, a potent modulator of central cholinergic function. The compound reportedly improves the memory deficits of Alzheimer's dementia. Experiments were run with purified bovine caudate AChE to examine the kinetic properties of THA–AChE interaction within the scheme of multiple binding sites on the enzyme and a proposed "map" of the enzyme surface. The kinetic analyses were also designed to determine whether chemical modification of peripheral anionic sites on AChE may provide insight into mechanisms for selective pharmacological alteration of cholinergic function in the brain. The studies demonstrated that THA is a reversible, non-competitive inhibitor with an $I_{50}$ of 160 ± 10 nM. THA bound primarily at a hydrophobic area outside of the catalytic sites, and binding of THA enhanced the effect of $Ca^{2+}$ binding to a separate group of "accelerator" sites. Experiments with $Al^{3+}$ demonstrated non-competitive inhibitor effects that were additive with THA inhibition and consistent with a model suggesting interaction of THA and $Al^{3+}$ at the enzyme surface. *In vitro* enzyme inhibition studies also provide evidence for THA "protection" of the catalytic site against inhibition by the high-affinity phosphorylating agent, DFP (isoflurophate).

Summers *et al.* [1] reported a significant improvement in Alzheimer Disease (AD) patients treated with oral tetrahydroaminoacridine (THA; Tacrine). THA was first synthesized in 1945 by Albert and Gledhill [2] as a possible antibacterial agent. The compound was found to be a poor antibiotic, but it was later shown by Heilbronn [3] to be a potent inhibitor of cholinesterases (ChE), with apparently greater affinity for serum- or butyrylChE than for neuronal- or acetylcholinesterase (AChE). In 1976, Patočka *et al.* [4] reported a detailed kinetic study of ChE inhibition by THA, and demonstrated that the compound is indeed a non-competitive ChE inhibitor, but it is selective for the neuronal enzyme (AChE).

A number of recent studies have examined the topology of regulatory and catalytic sites on the surface of AChE. Using site-selective fluorescence probes, for example, the enzyme has been shown to consist of an active center or catalytic site and one or more peripheral anionic sites. The structure and function of the active center, including both the esteratic site and the catalytic anionic site, have been well described, but the peripheral anionic sites—their number, arrangement, structure, and function—are still under investigation. In fact, AChE represents a good model enzyme for studying non-competitive, "allosteric" mechanisms, as a great deal of information is available regarding the molecular biology of the enzyme. However, the details of the regulatory role of peripheral anionic sites in tertiary and quaternary protein structure, and their corresponding functions, remain unclear. Thus, while many drugs may be described as non-competitive inhibitors of AChE, in nearly every instance the binding of such ligands to AChE is not elucidated sufficiently, and little is understood of the implications of peripheral site interactions for enzyme function *in vivo*. This problem is applicable to a large number of pharmacologically important ligands, including local anesthetics, muscle relaxants, and opiates [5–7].

Many investigators, including ourselves, have described the multiple binding of a number of different compounds to AChE and have mapped these binding sites on the enzyme surface. Some of this work is summarized in Table 1. However, very few of these studies have related binding to cholinergic function. Steinberg *et al.* [14] defined a conformationally flexible, hydrophobic area on eel AChE which may bind many non-competitive ligands, including THA. By examining the interaction of THA with calcium ion activation of erythrocyte AChE, Patočka *et al.* [4] found that the two ligands are independently associated with the enzyme, and also proposed that THA is a selective ligand for a hydrophobic region, or "$\gamma$" anionic site on AChE. The recent clinical studies of Summers *et al.* [1] provide a functional corollary to these binding data, and may provide some insight into the physiological significance of peripheral sites. Their work may also suggest alternative mechanisms for pharmacological modification of enzyme activity. Their clinical studies further suggest that it is possible to achieve functional effects with therapeutic rather than toxic doses of a drug that is effective via peripheral sites on the enzyme.

We have also been studying the reactivity of peripheral anionic sites on caudate nucleus AChE by

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Table 1. Anionic sites of acetylcholinesterase

| Anionic sites | | Symbols | | Ligands (Ref.) |
|---|---|---|---|---|
| 1. | Catalytic | $\alpha$ ↑ ~14Å ↓ | C | Edrophonium [8]<br>Phenyltrimethylammonium and pyridine methiodide [9]<br>Decamethonium [10] |
| 2. | Allosteric "accelerator" | $\beta$ | $P_1$ | Decamethonium [11]<br>Calcium [12]<br>Tetraethylammonium [13]<br>Propidium [9] |
| 3. | Hydrophobic | $\gamma$ | $P_2$–$P_4$ | Gallamine [14–16]<br>Tetrahydroaminoacridine [4]<br>Tetrapropylammonium [17]<br>Aluminium [18] |

The representative data summarized in this table are the results of experiments with AChE from bovine erythrocytes or eel electric organ.

attempting to chemically modify the enzyme in such a way as to preserve catalytic integrity while altering susceptibility to inhibition by several different types of compounds. We demonstrated that water-soluble carbodiimides, which purportedly react selectively with protein carboxyl groups, significantly modify the reactivity of the $Ca^{2+}$-binding ("$\beta$", or $P_1$) peripheral anionic sites on purified AChE [19]. These and other so-called "group-specific" protein modifying reagents may, therefore, provide useful tools for identifying the THA bindings sites on AChE.

As aluminum ions have been implicated in the etiology of AD, and $Al^{3+}$ has been shown to bind to at least one group of hydrophobic peripheral anionic sites on AChE [18, 19], we also investigated the possibility that THA and $Al^{3+}$ may compete for alterations of AChE function, and that THA may even protect the enzyme against $Al^{3+}$ toxicity.

## METHODS

*Bovine caudate nucleus AChE purification.* Whole calf brains were obtained from a nearby slaughterhouse and transported on ice to the laboratory. The caudate nuclei were dissected and frozen at −70° until used. Membrane-bound AChE was purified by affinity chromatography, as described by Sørensen *et al.* [20]. Briefly, the caudate nuclei were homogenized in a high salt buffer (1 g wet wt/10 mL buffer) of 10 mM Tris–HCl, pH 7.4, 1 M NaCl, 50 mM $MgCl_2$. The homogenate was then centrifuged at 100,000 *g* for 1 hr. The pellet was resuspended in 10 vol of 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100 and stirred for 3 hr at 5°. The suspension was rehomogenized and centrifuged at 100,000 *g* for 1 hr. The supernatant fraction was then applied to an affinity column of trimethylammonium aniline attached to Sepharose 4B and washed extensively. The enzyme was eluted with 20 mM edrophonium and then dialyzed. The specific activity of the final preparation was greater than 2560 I.U./mg protein (the final protein concentration was less than 0.001 mg/mL), representing a purification of greater than 7000-fold. Protein concentrations were determined using the method of Wang and Smith [21], a modification of the Lowry method which excludes interference by Triton X-100.

*Measurements of enzyme inhibition.* Enzyme activity was determined by the colorimetric method of Ellman *et al.* [22] in 2 mM Tris buffer, pH 7.2, or 2 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) buffer, pH 6.8, chosen to optimize solubility. For enzyme inhibition studies, the effects of THA were assayed in the presence of 0.05 to 1.2 mM acetylthiocholine (ASCh) as substrate. $K_{m,app}$ and $V_{max}$ were determined from double-reciprocal plots of reaction rate as a function of substrate concentration. The effects of cations such as $Ca^{2+}$ and $Al^{3+}$, and quaternary ammonium compounds such as gallamine and decamethonium, were assayed by adding them directly to the reaction buffer. Inhibition of AChE by organophosphates was measured using a 5-min preincubation of the enzyme and organophosphate, followed by addition of the substrate and secondary ligand, unless pretreatment with the ligand is indicated.

*Chemical modification of bovine caudate AChE.* EDAC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide], a carboxyl-group reagent previously shown to specifically block the AChE-activating effect of multivalent cations, was used to chemically modify reactive sites on the purified enzyme preparation described above. EDAC was prepared in 2 mM Pipes buffer, pH 6.8, as the lower pH should favor the reaction of EDAC with carboxyl groups [23].

Aluminum chlorohydrate was purchased from Pfaltz & Bauer, Stamford, CT. Paraoxon was purchased from Chem Services, Inc., West Chester, PA. All other compounds were purchased from the Sigma Chemical Co., St. Louis, MO.

## RESULTS

The concentration of THA required to inhibit 50% of AChE activity under the assay conditions described above was measured as the $I_{50}$ (by extrapolation from a dose–inhibition curve); the data are summarized in Table 2. Pretreatment of the enzyme with THA did not alter the $I_{50}$, indicating that the THA inhibition of caudate nucleus AChE, like the

Table 2. $I_{50}$ for THA inhibition of purified bovine caudate acetylcholinesterase

| Condition | $I_{50}$ ($\mu$M) |
|---|---|
| No pretreatment | 0.16 ± 0.01 (4) |
| 5-min THA pretreatment | 0.16 ± 0.20 (4) |
| 30-min THA pretreatment | 0.15 ± 0.10 (4) |
| EDAC treatment | 0.17 ± 0.02 (3) |

Data are the means ± SD for (N) experiments.

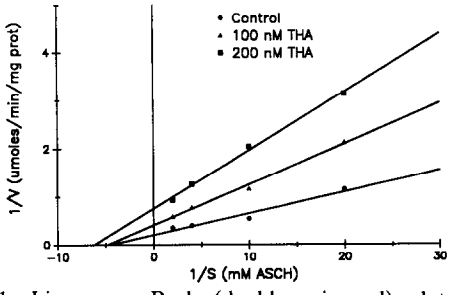


Fig. 1. Lineweaver–Burk (double-reciprocal) plots of AChE activity over a range of substrate (ASCh) concentrations in the absence and presence of 100 or 200 nM THA. $K_m$ values (mM): control = 0.12 ± 0.4; 100 nM THA = 0.18 ± 0.2; and 200 nM THA = 0.21 ± 0.03. $V_{max}$ values ($\mu$mol/min/mg protein): control = 3.62 ± 0.4; 100 nM THA = 2.29 ± 0.12; and 200 nM THA = 1.5 ± 0.1. Data are given as the means ± SE of four experiments. The standard errors fall within the area of the symbols used to denote a point.

inhibition of eel AChE, was fully reversible. This is in contrast to the inhibition of erythrocyte AChE by THA, which was reported to be very stable and reversed only after lengthy dialysis [4]. Pretreatment of the enzyme with a carbodiimide (EDAC) also did not alter the $I_{50}$ for THA. As EDAC is a covalent cross-linking reagent, and has been shown to be an irreversible enzyme ligand [19], it may be concluded that EDAC and THA bind to separate sites on the enzyme surface.

Double-reciprocal analysis of inhibition data for THA is shown in Fig. 1 and demonstrates non-competitive inhibition of purified bovine caudate AChE.

Table 3 describes the results of experiments designed to determine whether THA alters inhibition of AChE by ligands that are known to interact with a serine residue in the catalytic site, namely, the carbamate physostigmine, and the organophosphates paraoxon and diisopropyl fluorophosphate (DFP). It has been suggested that chemical modification of AChE at peripheral sites may alter active site reactivity toward these inhibitors and, consequently, provide protection against these compounds [19]. The data in Table 3 indicate that there were no significant effects of THA on the potency of enzyme inhibition by physostigmine or paraoxon, but there was a significant increase in the $I_{50}$ for DFP (i.e. decreased inhibitory potency) in THA-treated enzyme, either with or without prior enzyme modification with a carbodiimide.

In an effort to pharmacologically "dissect" the binding sites for THA on the caudate nucleus AChE, the enzyme was incubated with several different peripheral site ligands. Double-reciprocal plots demonstrated that none of the ligands studied changed the substrate affinity of the catalytic site.

$Al^{3+}$ and gallamine are both hydrophobic ligands that bind to a group of peripheral sites collectively identified as the "$\gamma$" sites (see Table 1). Based on their observation that THA has effects on enzyme activity similar to those seen with gallamine, Patočka *et al.* [4] concluded that THA binds to this subset of sites on bovine erythrocyte enzyme. The binding of THA to erythrocyte enzyme, however, was irreversible, and may reflect the observation by numerous investigators that AChE from different tissues has identical catalytic sites and overall similar catalytic activity, but these various enzymes may also have quite different peripheral sites, particularly since they undergo considerably different post-translational modifications and, for example, are attached to the membrane by different structural mechanisms [24].

In the present studies, the interactions of $Ca^{2+}$ and THA, and gallamine and THA, were examined in purified caudate nucleus AChE chemically modified with the carbodiimide EDAC. This compound was shown previously to covalently block carboxyl groups at the $Ca^{2+}$-binding, or "$\beta$" peripheral anionic, sites. As expected, $Ca^{2+}$ activation of AChE was blocked (Fig. 2A). However, while inhibition by 100 nM THA was evident in the $Ca^{2+}$-free controls, $Ca^{2+}$ actually reversed THA inhibition in this modified enzyme preparation, presumably by binding to EDAC-insensitive "accelerator" sites.

Patočka *et al.* [4] studied $Ca^{2+}$-THA interactions on erythrocyte AChE, and reported an increase in the relatively small activating effect of $Ca^{2+}$ in the presence of THA. As shown in Table 1 and discussed above, experimental evidence suggests that THA binds to a "$\gamma$" inhibitory site, and $Ca^{2+}$ binds to a "$\beta$" accelerator site. It is known that the "$\gamma$" anionic site can affect the activity of both the catalytic and "$\beta$" sites. The activating effect of $Ca^{2+}$ is thus enhanced by THA.

Roufogalis and Quist [11] have shown that gallamine has an effect similar to that of THA on $Ca^{2+}$ activation of erythrocyte enzyme. As seen in Fig. 2B, gallamine inhibition of EDAC-modified caudate AChE was evident in the absence of THA, and 100 nM THA retained its inhibitory activity in the gallamine-free controls with modified enzyme. However, exposure to increasing gallamine concentrations in the presence of 100 nM THA produced a significant increase of enzyme activity relative to the controls. Thus, if THA blocks gallamine binding to inhibitory, hydrophobic "$\gamma$" sites, the quaternary ammonium compound may be shifted to accelerator sites that are EDAC insensitive. Such a shift was also predicted by Steinberg *et al.* [14] in their studies with eel AChE.

To elucidate further the effects of THA binding on the reactivity of peripheral sites, unmodified purified caudate AChE was pretreated with 50, 100 or 200 nM

Table 3. Inhibition of purified bovine caudate AChE with and without THA treatment

| Condition | $I_{50}$ ($\mu$M) | | |
|---|---|---|---|
| | Physostigmine | Paraoxon | DFP |
| Control | 5.9 ± 0.18 | 0.34 ± 0.01 | 2.53 ± 0.65 |
| 100 nM THA | 7.8 ± 0.99 | 0.99 ± 0.40 | 10.02 ± 0.59 ($P < 0.05$) |
| 100 nM THA pretreatment | 4.8 ± 0.37 | 0.53 ± 0.01 | |
| EDAC control | | | 4.38 ± 1.01 |
| EDAC + 100 nM THA | | | 8.77 ± 0.05 ($P < 0.05$) |

Data are the means ± SD of four experiments. DFP = diisopropyl fluorophosphate.

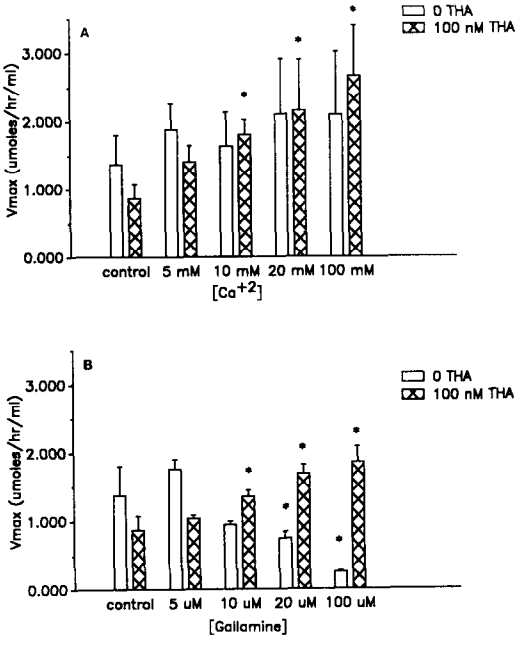


Fig. 2. Effects of various calcium (A) and gallamine (B) concentrations on maximal AChE activity in the absence and presence of 100 nM THA. The error bars indicate the SD for four different kinetic experiments, each run in duplicate. The (*) indicates data significantly different from control ($P < 0.05$) by non-paired $t$-tests.

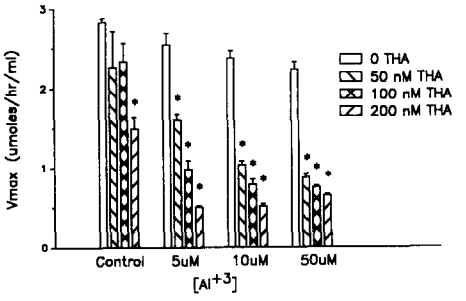


Fig. 3. Effects of various aluminum concentrations on maximal AChE activity in the absence and presence of various THA concentrations. The error bars indicate the SD for four different kinetic experiments. The (*) indicates data significantly different from control ($P < 0.05$) by non-paired $t$-tests.

THA, and then incubated with another "$\gamma$" site ligand, $Al^{3+}$. The double-reciprocal analysis of enzyme assays over a range of 5 to 50 $\mu$M Al chlorohydrate demonstrated additivity, and suggests potentiation of these two non-competitive inhibitors (see Fig. 3). While the data support the possibility of overlapping binding sites, one cannot rule out the additional possibility of interacting but independent sites for THA and $Al^{3+}$.

## DISCUSSION

THA is a reversible, non-competitive inhibitor of caudate nucleus AChE. The biochemical and physiological implications of non-competitive inhibition of an enzyme that appears to function primarily by terminating the action of a neurotransmitter are certainly not well understood. As a xenobiotic, a non-competitive inhibitor may assume the role of an exogenous regulatory signal (or biochemical messenger), and thereby alter the normal sequence of biochemical events at the cholinergic synapse.

Marquis and Lerrick [18] reported that aluminum chlorohydrate is a potent modifier of AChE activity, and acts primarily at binding sites outside of the catalytic or active site. In view of the multiple peripheral anionic sites characteristic of this enzyme and our own experimental observation that $Al^{3+}$ effects are independent of the effects of "$\beta$" ligands, we concluded that $Al^{3+}$ may alter substrate binding at the catalytic site by interaction at the "$\gamma$" peripheral anionic sites. Later, Marquis and Black [19] observed that $Al^{3+}$ potentiates $Ca^{2+}$ effects on AChE activity in a manner similar to that reported for THA [4]. The observation that the catalytic activity of AChE and the effects of peripheral sites on catalytic activity can both be modified by the binding of multivalent inorganic cations and other peripheral site ligands [25] suggested a possible mechanism of interaction between THA and $Al^{3+}$. For example, THA may reverse $Al^{3+}$ inhibition and, consequently, protect the enzyme from the cholinergic, neurotoxic effects of this metal ion [26].

The data from our multi-ligand studies support the suggestion of interaction between these two ligands

on the enzyme surface, but do not provide a definitive mechanism, especially as the total effect of the two ligands was often greater than inhibition by either ligand alone. It also remains to be shown whether these observations in isolated enzyme systems are directly relevant to alterations of neurochemical function *in vivo*.

Sherman and Messamore [27] recently described studies with acute and chronic exposure to THA in rats which suggest presynaptic cholinergic effects of the drug. AChE is a heterogeneous enzyme that can be separated into multiple molecular forms. A tetrameric membrane-bound form ($G_4$) and a monomeric soluble form ($G_1$) are the two predominant enzyme species in mammalian brain, and evidence suggests that a substantial proportion of $G_4$ enzyme is associated with cholinergic nerve terminals, and is thus presynaptic. It has also been shown that the $G_4$ molecular form of AChE is selectively decreased in several areas of Alzheimer brain, and that this loss is markedly associated with both the loss of ChAT and the severity of the neuropathological lesion in that area. No data are available at present to determine whether THA is selective for a particular molecular form of AChE, and especially whether it may be selective for a presynaptic form of the enzyme [28].

The data in these studies also indicate that the effects of THA on AChE activity represent a panorama of actions, including inhibition, both by direct binding and indirect potentiation of "$\gamma$" inhibitory anionic sites, and acceleration by enhanced actions of $Ca^{2+}$, for example, and possibly other cations at the "$\beta$" anionic sites. The independence of these potentiation sites from the effects of carboxyl group reagents such as EDAC further demonstrates that there are multiple subclasses of each of the major peripheral sites outlined in Table 1.

A direct correlation of AChE inhibition and therapeutic improvement in the behavioral parameters important to AD has not been demonstrated, but the theoretical efficacy of hypercholinergic stimulation in the CNS remains as a valid working model for developing pharmacologically active agents. Other mechanisms of THA action should also be investigated. The very early observation of potent analeptic activity of THA, for example, is still undefined. Finally, the potent antiChE activity of THA also suggests that it may exhibit numerous nonspecific side effects on the enzymes of normal metabolism.

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