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ACCELERATED COMMUNICATION

Tetrahydroaminoacridine Block of *N*-Methyl-D-aspartate-Activated Cation Channels in Cultured Hippocampal Neurons

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SUMMARY

The action of tetrahydroaminoacridine (THA), a centrally active cholinesterase inhibitor that may provide symptomatic benefit in Alzheimer's disease, was studied on responses to the excitatory amino acid *N*-methyl-D-aspartate (NMDA) in cultured hippocampal neurons, using whole-cell voltage-clamp and single-channel recording techniques. THA produced a concentration-dependent block of NMDA-evoked inward current responses (IC_{50}, 190 μM at −60 mV), without affecting responses to quisqualate or kainate. THA block of NMDA responses was voltage dependent and was nearly completely relieved at positive holding potentials. Analysis of the voltage dependency indicated that the THA binding site senses 56% of the transmembrane electrostatic field. In single-channel recordings from outside-out membrane patches, THA appeared to reduce the frequency and duration of NMDA-evoked single-channel currents, without affecting the single-channel amplitude. The effects of THA on NMDA responses occur at concentrations 1–2 orders of magnitude greater than the therapeutic serum concentrations and, therefore, blockade of NMDA receptor-mediated responses is unlikely to contribute to the putative therapeutic action of THA. However, because NMDA receptors may play a critical role in cognitive and memory function, THA has the potential to produce undesirable central nervous system side effects at high doses.

THA is a centrally acting cholinesterase inhibitor that may improve cognitive function in Alzheimer's disease (1). Because other centrally active cholinesterase inhibitors have failed to provide as substantial a benefit as THA (2), pharmacological actions other than cholinesterase inhibition, such as blockade of voltage-dependent K^+ channels (3–6), could account for the putative therapeutic activity of THA. Recently, Albin *et al.* (7) demonstrated an interaction between THA and NMDA-type excitatory amino acid receptors in rat brain homogenates, using radioligand binding techniques. In addition, THA has been shown to protect against NMDA receptor-mediated neurotoxicity in cultured cells (8). These observations are particularly intriguing in view of the hypothesis that NMDA receptor-mediated excitotoxicity may contribute to the neuronal injury in Alzheimer's disease (9, 10).

In the present study, we used electrophysiological techniques to provide insight into the nature of the interaction between THA and NMDA receptors. Our results indicate that THA can block NMDA responses by binding to an acceptor site that is located within the transmembrane electrostatic field, perhaps in the ionophore portion of the NMDA receptor-channel complex. The effect of THA on NMDA responses was selective, in that THA failed to affect responses produced by non-NMDA excitatory amino acid agonists.

Materials and Methods

Cell culture. Neurons from the hippocampi of 19-day-old Sprague-Dawley rat embryos were grown in primary cell culture, according to methods described by Segal (11). In brief, the hippocampal tissue was triturated by passage through a Pasteur pipette, and the resulting cell suspension was plated onto polyornithine-coated 35-mm culture dishes, at a density of approximately 3×10^5 cells/dish, in Eagle's modified minimal essential medium supplemented with 10% fetal bovine serum, 10% horse serum, and 1% glutamine. The cultures were incubated at 37° in a humidified atmosphere containing 10% CO_2. One-half of the culture medium was replaced every 4 days with medium similar to that used for the initial plating, except that it lacked fetal bovine serum. Culture media were obtained from GIBCO (Grand Island, NY); all other chemicals and drugs were from Sigma Chemical Co. (St. Louis, MO), except THA, which was from Aldrich Chemical Co. (Milwaukee, WI).

Whole-cell voltage-clamp recording. Electrophysiological recordings were performed at room temperature (23–25°) on the stage of an inverted phase-contrast microscope. Before each experiment, the

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ABBREVIATIONS: THA, 1,2,3,4-tetrahydro-9-aminoacridine (tacrine); NMDA, *N*-methyl-D-aspartate; QA, quisqualic acid; KA, kainic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

culture medium was removed, the cells were rinsed twice, and the culture dish was partially filled with 1.5 ml of recording solution containing 140 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, and 10 mM HEPES. The bathing medium also contained 1 μM tetrodotoxin to block voltage-dependent Na^+ channels, 1 μM glycine to maintain a defined glycine concentration (12), and 1 μM strychnine to block glycine-evoked Cl^- current. Mg^{2+} was omitted from the solution to avoid depression of NMDA-evoked current responses (13, 14). The solution was adjusted to a pH of 7.40 with NaOH and an osmolality of 307 mOsm/kg of H_2O with glucose.

Patch electrodes were prepared from 1.5-mm o.d. filament fused, borosilicate glass capillaries (TW150F-4; WPI instruments, New Haven, CT), by using a Flaming-Brown micropipette puller (model P80; Sutter Instruments Co., San Rafael, CA). The electrodes were filled with a solution containing 145 mM potassium gluconate, 2 mM $MgCl_2$, 1.1 mM EGTA, 0.1 mM $CaCl_2$, and 5 mM HEPES, except in experiments where current-voltage curves were constructed, in which case 145 mM CsCl was used instead of potassium gluconate. The electrode solutions were adjusted to an osmolality of 307 mOsm/kg of H_2O with glucose and to a pH of 7.40 with KOH (CsOH in the CsCl solution). The solutions were filtered through a 0.22-μm membrane filter (Millipore Corp., Bedford, MA) immediately before use. Pipette-to-bath resistances were typically 4–10 MΩ.

Whole-cell currents were recorded with an Axopatch 1B patch-clamp amplifier (Axon Instruments, Burlingame, CA). After seal formation and before establishment of a whole-cell recording, electrode capacitance was neutralized. Voltages corresponding to the membrane currents were displayed on a high-speed ink pen recorder with a direct current input stage (Gould Electronics, Cleveland, OH).

Drug application. Drugs were dissolved in bathing medium and were applied to the cell surface with blunt micropipettes (tip diameter, 1 μm), using a pneumatic pressure ejection system (Medical Systems, Greenvale, NY). The tip of the pressure pipette was situated <4 μm from the cell membrane, and the drug solution was expelled as needed during the experiment by application of a back pressure of 0.8–2.5 psi. In most experiments, we used two pressure pipettes, one containing an excitatory amino acid and the second containing THA. A third pressure pipette, containing a second excitatory amino acid, was used in some experiments. To assay for block, excitatory amino acids were applied with brief (300-msec) pressure pulses before, during, and after the sustained ejection of THA. To avoid desensitization, excitatory amino acid ejections were not made more frequently than every 30 sec. In a previous study (15), we found that the hydrodynamic effect of a second superfusion solution produced no significant decrement in the excitatory amino acid response ("blow-away" artifact). The pressure pipettes were tested for patency before and after each experiment, by verification that there was sufficient fluid flow from the tip to displace small particles of debris (1–2 μm in size) on the bottom of the culture dish. We used 100 μM NMDA and KA and 10 μM QA.

Single-channel recording. Single-channel recordings were carried out in the outside-out configuration, using the 50-GΩ headstage feedback resistor of the Axopatch amplifier system. The patch electrodes were coated with Sylgard, fire polished, and filled with a cesium gluconate solution whose composition was similar to that used to fill the electrodes for whole-cell recording. Tip resistances were typically 4–10 MΩ. After an outside-out patch was obtained, the pipette tip was situated near the mouth of a superfusion pipette carrying a continuous stream of bathing medium containing the drug solutions. Membrane currents were filtered at 2 kHz (−3 dB, four-pole low-pass Bessel filter), digitally sampled at 10 kHz, and stored on magnetic media in digital form for later analysis. Data were recorded in 10-sec epochs. For display in Fig. 5, the single-channel current records were digitally filtered at 300 Hz.

Data analysis. We calculated the fractional block (B) produced by THA in whole-cell recording experiments according to the formula $B = 1 - I'/I$, where I is the peak evoked current amplitude immediately before the onset of the drug superfusion and I' is the current evoked 5 sec after the onset of the superfusion. Differences between two means were compared by Student's t test, as performed by the Pharmacologic Calculation System computer programs accompanying the work of Tallarida and Murray (16). The criterion for statistical significance was $p < 0.05$. Multiple comparisons were made with a one-way analysis of variance and the Newman-Keuls test, using the same program. Concentration-effect data were fit with a nonlinear least squares program [ALLFIT, as described by De Lean *et al.* (17)], according to the logistical equation

$$B = \frac{1}{1 + (K_D/[\mathrm{THA}])^n} \quad (1)$$

where [THA] is the THA concentration, K_D is the concentration of THA resulting in 50% block, and n is an empirical parameter that describes the steepness of the curve and has the same meaning as the Hill coefficient. Final plotting and linear regression analysis were performed on a microcomputer with the GRAPHER software package (Golden Software, Golden, CO). All quantitative data are expressed as mean ± standard error.

Amplitude determinations were made from the single-channel current records using the pCLAMP software package (FETCHAN and pSTAT programs; Axon Instruments). Estimates of the mean unitary current amplitudes were made by fitting of the amplitude histograms to Gaussian curves, using a least-squares algorithm.

Results

Response to NMDA. In all experiments except those carried out explicitly to test the voltage sensitivity of the THA block, the holding potential was set at −60 mV. At this potential, 100 μM NMDA invariably elicited a robust inward current response that rose rapidly during the 300-msec superfusion and decayed in the 1–2 sec period after the drug application. Peak NMDA responses ranged in amplitude from 150 to 1800 pA (mean ± standard error, 510 ± 62 pA, 31 cells).

THA block. As illustrated in Fig. 1A, THA caused a concentration-dependent block of NMDA-evoked inward current responses that was completely reversible 30 sec after termination of the superfusion. Maximal blockade was achieved in <2 sec after the initiation of the THA superfusion; we routinely quantitated the fractional block approximately 5 sec after the onset of the superfusion, so that the values reported represent steady state (equilibrium) determinations. No use-dependent increase in block was observed with repeated NMDA applications during the continuous application of THA, suggesting that, if the block occurs by a use-dependent mechanism, the apparent rate of the blocking reaction is sufficiently fast that equilibrium is achieved during the initial NMDA application. There were no consistent changes in the resting current at THA concentrations of 1–100 μM. However, THA concentrations of 1 and 10 mM resulted in a small outward shift in resting current, of 16 ± 10 pA (10 cells) and 65 ± 9 pA (five cells), respectively (data not shown).

Concentration dependence. Concentration-effect curves were constructed from experiments with 31 cells exposed to varying concentrations of THA (Fig. 1B). THA concentrations as low as 10 μM produced a small depression of the NMDA response, and there was near-complete block at 10 mM. The data were fitted with a sigmoidal curve, according to the logistical equation (eq. 1), with an apparent K_D of 193 ± 33 μM.

Voltage dependence and reversal potential. We evaluated the voltage dependence of the THA (1 mM) block in three cells. The current recordings from one cell are shown in Fig. 2A. Although there was substantial blockade of the NMDA

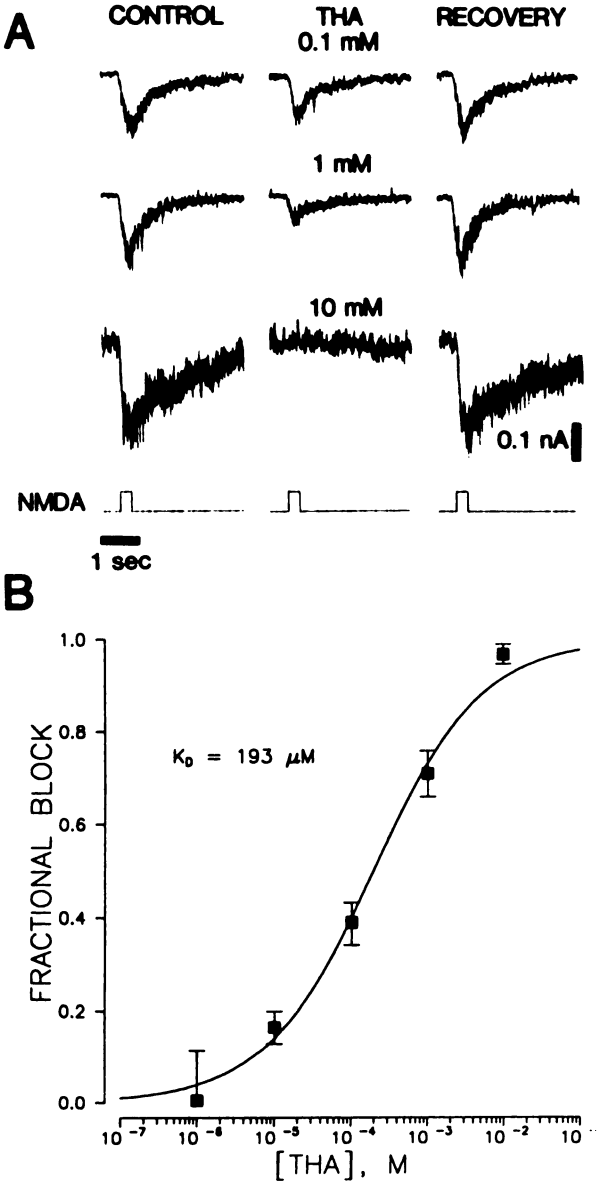

Fig. 1. Concentration-dependent suppression of NMDA-evoked inward current responses by THA. A, NMDA responses before (*CONTROL*), 5 sec after the onset of (*THA*), and 30 sec after the termination of (*RECOVERY*) THA superfusion. Each *row* represents a different cell. The duration of the NMDA (100 μM) pressure pulse is indicated below the current records. B, Concentration-effect curve for THA inhibition of NMDA-evoked current responses. Each *point* represents the mean ± standard error of data from 6–17 cells. The smooth curve was fit to the logistical equation (Eq. 1), with apparent K_D = 193 ± 33 μM and n = 0.61 ± 0.06.

response at a holding potential of −60 mV, less depression was apparent as the holding potential was brought to more positive levels, and almost no depression was observed at +40 to +60 mV. The mean current amplitude data (normalized to the control current at −60 mV) for the three cells are plotted in Fig. 2B. Under control conditions, this plot appeared roughly linear. However, in the presence of 1 mM THA, NMDA evoked a substantially smaller inward current at negative holding potentials than outward current at positive holding potentials. Statistical comparisons of the control NMDA responses with those during THA superfusion at the different holding potentials revealed significant differences between treatment and control groups only at −20, −40, and −60 mV. The NMDA reversal potential was approximately +5 mV and was not affected by THA superfusion in the three cells examined. We calculated the mean fractional block at the various holding potentials, and these values are plotted in Fig. 2C. There was a statistically significant decrement in the fractional block values over the potential range −60 to +60 mV (comparing values at −60 and −40 mV with values at +40 and +60 mV; analysis of variance and Newman-Keuls test). The increase in the standard errors at a holding potential of +20 mV resulted from the increased variability in measuring very small currents near the reversal potential.

The voltage dependence of the block was further analyzed according to the method of Woodhull (18), which provides a means of calculating the fraction of the transmembrane electrostatic field sensed by a charged blocking ligand at its acceptor site. THA contains two nitrogen atoms, one within the acridine ring and a second as a primary amino group. The pK_a of the ring nitrogen is 9.912, so that it is protonated at physiological pH. In contrast, the amino nitrogen has a very low pK_a and is only protonated in concentrated acid (19). Therefore, the molecule would be expected to carry a single positive charge at physiological pH. In the Woodhull model, the ratio of NMDA current in the absence and presence of THA (I_o/I_{THA}) can be related to the transmembrane voltage (V) by the relationship:

$$I_o/I_{THA} = 1 + \{[THA]/K_D(0)\}\exp(-zFV\delta/RT) \quad (2)$$

where $K_D(0)$ represents the dissociation constant of the THA-acceptor site complex at 0 transmembrane potential, δ is the fractional voltage drop experienced at the acceptor site, z is the charge of THA (= 1), and F, R, and T have their usual meanings. Eq. 2 can be linearized by rearranging and taking the natural logarithms of both sides, resulting in the following equation for a straight line:

$$\ln(I_o/I_{THA} - 1) = \ln\{[THA]/K_D(0)\} - (z\delta F/RT) \times V \quad (3)$$

in which $K_D(0)$ and δ can be determined from a plot of $\ln(I_o/I_{THA} - 1)$ against V (20–22). Data from Fig. 2 expressed in this fashion are plotted in Fig. 3. Linear least-squares fits give a $K_D(0)$ of 814 μM and δ of 0.56. The K_D at an arbitrary voltage [$K_D(V)$] is related to the $K_D(0)$ by the following equation (18):

$$K_D(V) = K_D(0)\exp(\delta FV/RT) \quad (4)$$

Substituting −60 mV for V, we obtained a $K_D(-60)$ of 218 μM. This independent measure of $K_D(-60)$ is close to the K_D determined from the concentration-effect data shown in Fig. 1, which were obtained at a holding potential of −60 mV (193 μM).

Specificity of block. To assess the specificity of THA, we compared its effects on NMDA-evoked inward current responses with its action on responses elicited by the selective excitatory amino acid agonists QA and KA. THA blocked the NMDA-induced current but produced only a minimal reduction in the KA-induced current in the same cells (Fig. 4A). Similar results were obtained in comparisons of the effects of THA on NMDA- and QA-induced currents (Fig. 4B). The fractional block produced by 1 mM THA on the KA (four cells) and QA (five cells) responses was 0.13 ± 0.10 and 0.04 ± 0.05, respectively.

Block of NMDA-induced single-channel currents. The blocking action of THA was further examined in single-channel recordings from outside-out patches. Channel activity was

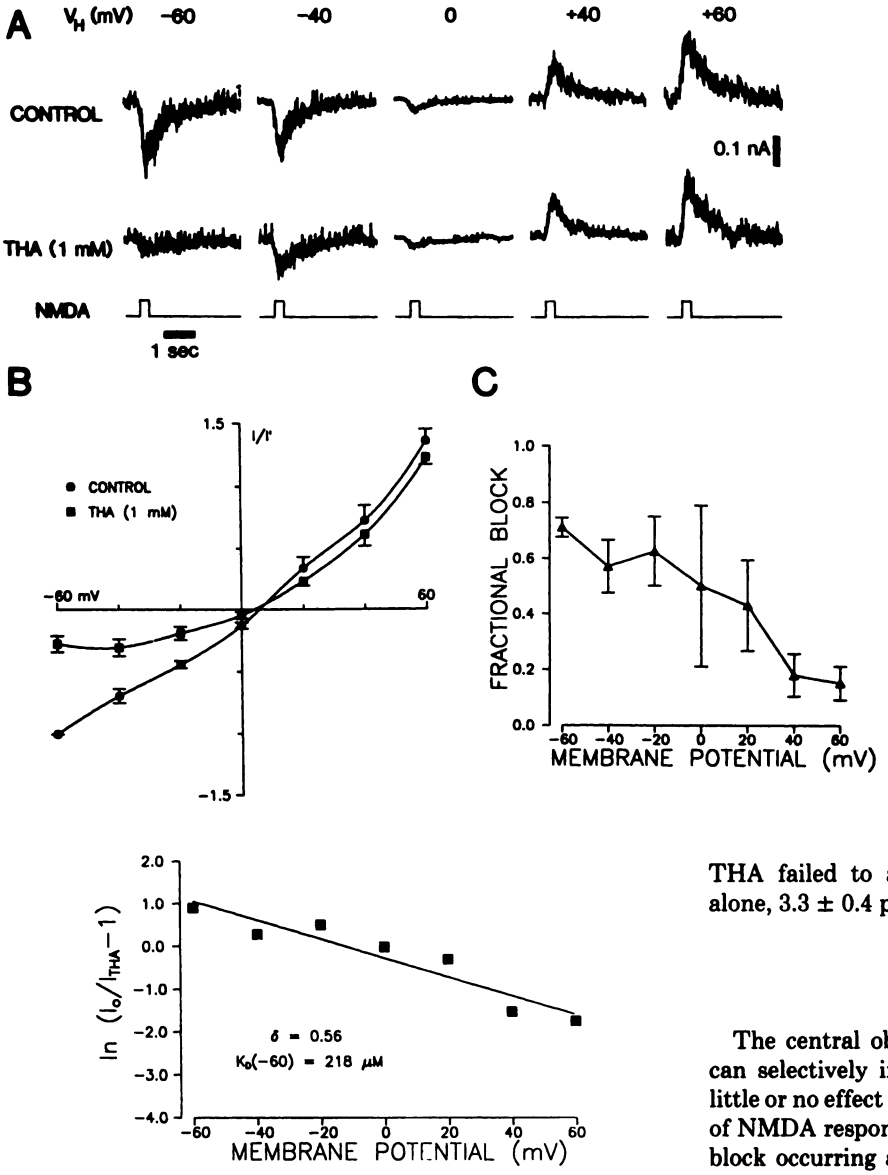

Fig. 2. Voltage dependence of the THA block. A, NMDA (100 μM)-induced currents evoked at various holding potentials (V_H) before (*CONTROL*) and during (*THA*) superfusion with 1 mM THA. B, Current-voltage curves for NMDA-evoked current before (●) and during (■) superfusion with 1 mM THA. The data were derived from three separate cells (including the cell in A) and were normalized to the control current at −60 mV. C, Fractional block as a function of membrane potential, calculated from data presented in B.

Fig. 3. Analysis of the voltage dependence of THA block, according to the method of Woodhull (18). The raw data (same as in Fig. 2C) were transformed according to Eq. 3. The *straight line* is the best linear least-squares fit to the data. The derived parameters are as shown.

evoked by superfusion of the patches with 100 μM NMDA, which elicited frequent inward single-channel currents at the holding potential of −60 mV (Fig. 5, *top*). Such openings were never observed in the absence of NMDA. The estimated single-channel conductance was approximately 50 pS (assuming a reversal potential of 0 mV), which is typical of NMDA-activated single-channel currents (23–25). A relatively high concentration of NMDA was intentionally used, so as to allow block of the channel to be easily demonstrated. In two of the patches studied, only a single channel appeared to be active (no superimposed channel openings). However, in the third patch, two channels may have been active in the patch (Fig. 5). During the simultaneous application of 100 μM NMDA and 1 mM THA, the frequency of channel openings decreased and there was a suggestion that the channel openings were briefer, as is evident in the sample recordings shown in Fig. 5. However, THA failed to alter the single-channel amplitude (NMDA alone, 3.3 ± 0.4 pA; NMDA + THA, 3.4 ± 0.3 pA).

Discussion

The central observation in the present study is that THA can selectively inhibit NMDA-evoked currents while having little or no effect on QA- or KA-evoked currents. The inhibition of NMDA responses was voltage dependent, so that the strong block occurring at negative potentials was almost completely reversed as the cell was brought to depolarized potentials. The high specificity and strong voltage dependency of the THA-induced block are reminiscent of the block produced by magnesium (13, 14, 26, 27) and high concentrations of zinc (24), by the dissociative anesthetics phencyclidine, ketamine, and MK-801 (28–30), and by tricyclic antidepressants (25). These agents are thought to block NMDA responses by binding to a site within the ionophore portion of the NMDA receptor-channel complex. They enter NMDA channels predominantly when the channels are in the open state and, because they are charged, the affinity with which they bind to their acceptor sites is modified by the transmembrane electrostatic field. Our results suggest that THA may also antagonize NMDA responses by a similar mechanism. Furthermore, our calculations using the Woodhull approach indicate that the THA binding site senses approximately 56% of the transmembrane potential (measured from the outside). This suggests that the THA binding site may be located near the middle of the membrane-spanning region of the ionophore portion of the NMDA receptor-channel complex. The results from single-channel recording, in particular the apparent decrease in channel open time, are not inconsistent with the concept of open channel block, because the entry

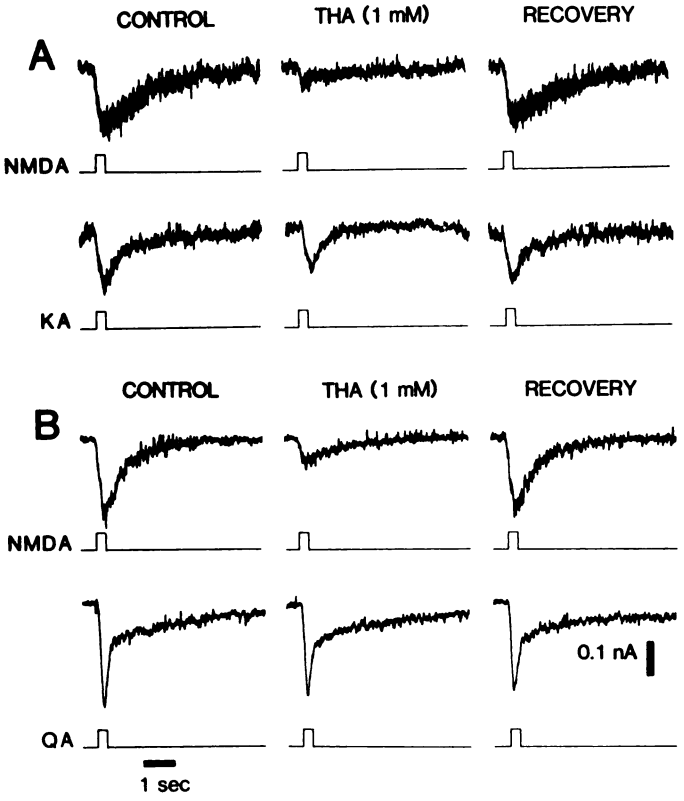

Fig. 4. Specificity of THA block. A, Comparison of the effects of THA (1 mM) on the responses evoked by NMDA (100 μM) and KA (100 μM) in the same cell. B, Comparison of the effect of THA (1 mM) on responses evoked by NMDA and QA (10 μM) in the same cell. The excitatory amino acid responses were obtained before (*CONTROL*), 5 sec after the onset of (*THA*), and 30 sec after the termination of (*RECOVERY*) the THA superfusion.

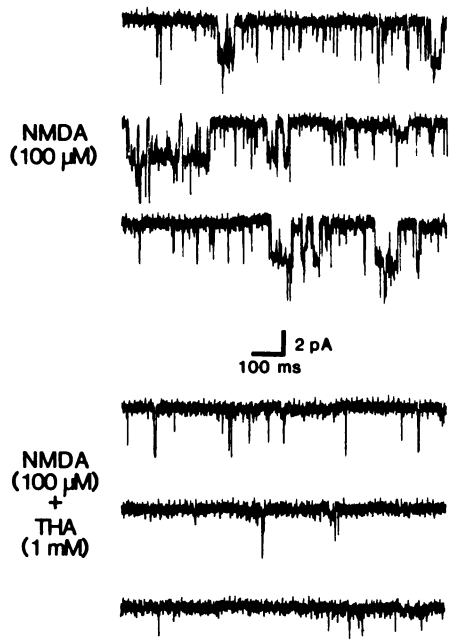

Fig. 5. Outside-out patch recordings showing the effect of THA on NMDA-evoked single-channel currents. The current tracings are from a single patch. The holding potential was −60 mV.

of a THA molecule into the open channel would abort current flow through the channel before its normal closing.

Although an open-channel blocking mechanism is perhaps the simplest model compatible with our voltage-dependency data and seems especially reasonable in view of the results obtained with single-channel recording, we cannot exclude the possibility that THA binds to an allosteric site that is not strictly within the ionophore of the channel but that, nevertheless, senses the transmembrane electrostatic field. Instead of sterically blocking ion transit through the channel, in this alternative mechanism the drug would modify channel gating, influence its desensitization, or otherwise alter the activity of the channel. On the other hand, it seems unlikely that THA antagonizes NMDA responses by blocking the recognition sites for either NMDA or the coagonist glycine (12), because the block produced by antagonists acting at these sites is not voltage dependent (15, 31, 32).

We also note that apparent voltage dependency need not invariably be due to electrostatic effects on the blocking drug *per se*. In fact, MacDonald *et al.* (29) have recently suggested that the reduction in the dissociative anesthetic block of NMDA responses that occurs at positive membrane potentials is in fact due to displacement of the anesthetic molecules from their acceptor site by intracellular cations flowing outward through the conduction pathway. This mechanism is similar to that proposed by MacKinnon and Miller (33) for the current-dependent relief of charybdotoxin block of Ca^{2+}-activated K^+ channels. However, the close agreement between the dissociation constant value determined by titration and that derived independently using the Woodhull approach argues against this interpretation, because it appears that the bulk of the voltage dependence can be accounted for by an action of the membrane potential on the THA molecule. In the case of charybdotoxin, the blocking site is near the external mouth of the channel. Similarly, MacDonald *et al.* (29) have proposed that the dissociative anesthetic blocking site is in the vicinity of the outer mouth or vestibule of the channel. In contrast, our results indicate that THA penetrates more deeply into the channel, as does the tricyclic antidepressant desipramine ($\delta = 0.72$; see Ref. 25). Consequently, the dissociative anesthetic acceptor site may be distinct from the binding site for low affinity ligands such as THA and tricyclic antidepressants. In fact, if these ligands were to bind to the dissociative anesthetic site, they should theoretically be more easily displaced by outward cationic current and show a greater apparent voltage dependency than do high affinity ligands (such as ketamine), yet this is not what has been observed experimentally (31, 32). Nevertheless, in radioligand binding studies, Albin *et al.* (7) have demonstrated that THA competitively displaces [^{3}H]*N*-(1-[2-thienyl]cyclohexyl)-3,4-piperidine from binding to the PCP acceptor site, indicating that the THA and PCP sites may be identical or are at least situated near one another. Clarification of the relationship between the two sites awaits a better definition of the precise locus of dissociative anesthetic binding.

THA has been reported to be protective against NMDA- and glutamate- but not QA-induced excitotoxicity in neuronal cell cultures (8). The IC_{50} value for THA in the latter study was 500 μM, which is compatible with the K_D of 193 μM observed in the present work. In one report, it was claimed that lower doses of THA (25–50 μM) actually enhanced *in vivo* quinolinic acid (an NMDA agonist) neurotoxicity (34). These investiga-

tors suggested that THA may act to augment NMDA responses, and it has been proposed that THA might have NMDA agonist activity (7). We found no evidence that THA could potentiate NMDA-evoked currents or elicit an inward current by itself. In fact, at high concentrations, THA often caused a small outward current response that could be due to blockade of low background levels of excitatory amino acids in the culture dish or to inhibition of the effects of presynaptically released glutamate (35).

The role of NMDA receptor-mediated excitotoxicity in the etiology of Alzheimer's disease is uncertain. Areas that are most sensitive to cellular degeneration in Alzheimer's dementia (i.e., entorhinal cortex, hippocampus, and subiculum) have a glutamatergic input (36). Because the number of uptake sites for glutamate may be reduced in the Alzheimer's brain (37, 38), the resulting excess glutamate could lead to cell death by excitotoxicity. If this were true, there should be a reduction in NMDA receptors in the brains of patients with Alzheimer's disease, a finding that has sometimes, but not always, been observed (9, 10, 36, 38).

Although it is tempting to speculate that a reduction in excitotoxicity by NMDA antagonism may play a role in the possible therapeutic efficacy of THA in Alzheimer's disease, there are reasons to doubt this. The concentrations required to antagonize NMDA responses are approximately 1–2 orders of magnitude greater than the serum THA levels observed in clinical studies (0.1–1.0 μM) (1, 39). Of all the known effects of THA, only its anticholinesterase activity (ED_{50}, 0.1–1.3 μM) (5, 40, 41) occurs at therapeutic serum concentrations. Furthermore, the usefulness of THA in Alzheimer's dementia has not yet been conclusively proven, and recent results from more complete clinical trials have not been as encouraging as the original study (42, 43).

Although antagonism of NMDA receptor-mediated responses probably does not contribute to the putative therapeutic efficacy of THA in Alzheimer's disease, the effect could potentially limit the usefulness of such an agent in the treatment of dementia. NMDA receptors appear to be critically important in cognitive and memory function. Thus, NMDA receptors are required for long term potentiation (44, 45), a possible cellular model of memory, and NMDA antagonists interfere with learning in animals (46). At high doses, THA could have significant side effects, particularly in patients with underlying defects in cognitive and memory function.

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