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Ambenonium Is a Rapidly Reversible Noncovalent Inhibitor of Acetylcholinesterase, with One of the Highest Known Affinities

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SUMMARY

Steady state patterns of inhibition of purified human erythrocyte acetylcholinesterase by three inhibitors were analyzed. Edrophonium acted essentially as a competitive inhibitor, whereas tacrine and ambenonium gave mixed competitive and uncompetitive inhibition with acetylthiocholine as substrate. Inhibition constants for the competitive components were 470 μM for edrophonium, 65 μM for tacrine, and 0.12 nM for ambenonium. The extremely high affinity of ambenonium permitted analysis of the rates of approach to steady state inhibition. These rates were characterized by a single exponential time course with rate constants, k_{exp}, that showed a linear dependence when plotted against ambenonium concentration, at fixed substrate concentration. The intercepts of these plots were independent of the substrate concentration and indicated an ambenonium dissociation rate constant of 0.013 ± 0.002 sec^{-1}. The slope of the plot at the lowest substrate concentration approximated the ambenonium bimolecular or association rate constant and gave a value of 5.2 ± 0.6 × 10^7 M^{-1} sec^{-1}. Three models were examined to account for the nearly linear dependence of the slopes of these plots on the substrate concentration. These models indicated that ambenonium and acetylthiocholine competed for a peripheral anionic site in the acetyl-enzyme intermediate formed during substrate hydrolysis. The apparent equilibrium dissociation constant of acetylthiocholine for this peripheral site (1.2–1.4 mM) was significantly different from that calculated from substrate inhibition data (20.1 ± 2.8 mM). We propose that acetylthiocholine can interact with the acetyl-enzyme both at the peripheral site and at the active site but that only the latter interaction inhibits substrate hydrolysis.

AChE (EC. 3.1.1.7) hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses in the central and peripheral nervous systems. Inhibitors of AChE activity promote increases in the concentration and duration of action of synaptic acetycholine and result in increased activation of synaptic nicotinic and muscarinic acetylcholine receptors. The extent of AChE inhibition determines whether the inhibitors are toxic or pharmacologic. The complete and poorly reversible inhibition of AChE produced by toxic agents like organophosphate nerve gases can lead to first overactivation and then inactivation of nicotinic receptors at skeletal neuromuscular junctions, with subsequent skeletal muscle paralysis. However, partial inhibition of AChE, with consequent moderate elevation of synaptic acetylcholine, is beneficial in certain diseases. In myasthenia gravis, AChE inhibitors like edrophonium (Fig. 1A) and ambenonium (Fig. 1C) have been shown to provide enhanced neuromuscular transmission and increased muscular strength. In certain types of glaucoma, partial AChE inhibition leads to contraction of the ciliary muscle and the iris and produces a fall in intraocular pressure. More recently, administration of the AChE inhibitor tacrine (Fig. 1B) has been reported to give significant improvement in patients with Alzheimer's disease (1). In this disease there is a degeneration of the cholinergic projections originating in the nucleus basalis of Meynert, and AChE inhibitors may compensate for the reduced amount of acetylcholine released by synaptic transmission from the remaining neurons.

Although the AChE inhibitors shown in Fig. 1 share a general structure that involves tertiary or quaternary amine groups linked to aromatic hydrocarbon groups, an unusually high affinity of ambenonium for mammalian AChE was reported by Lands *et al.* (2). Webb (3) determined an inhibition constant of 0.5 nM for ambenonium with eel electric organ AChE. Most reversible AChE inhibitors that approach this level of affinity involve some type of covalent bond formation at the AChE active site. Thus, carbamate inhibitors form a carbamoylated AChE that is hydrolyzed relatively slowly (see Ref. 4), and quaternary ammonium alkylborinic acid inhibitors appear to form a tetrahedral adduct with an active site group (5). In this report, we confirm the high affinity of ambenonium with human erythrocyte AChE, and we show association and dissociation rate constant data consistent with a rapidly reversible, noncov-

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ABBREVIATION: AChE, acetylcholinesterase.

alent, ambenonium-AChE complex. Analysis of the inhibition by acetylthiocholine of the ambenonium association rate indicates an acetylthiocholine interaction with a peripheral anionic site on AChE.

Experimental Procedures

Materials. Human erythrocyte AChE was purified to homogeneity as outlined previously (6). Ambenonium chloride (a generous gift of Lyle Norton, Sterling Drug Inc.), edrophonium chloride (Sigma), and tacrine (Aldrich) were used without further purification.

Assay. Acetylthiocholine was used as the substrate in the spectrophotometric assay of Ellman *et al.* (7), at 25°, as modified to pH 7.0 (6). The 3-ml assay solvent contained 1.0% Triton X-100, 0.1 M sodium phosphate, 0.33 mM 5,5′-dithiobis-(2-nitrobenzoic acid), and substrate and inhibitors at the indicated concentrations. When ambenonium was introduced as an inhibitor, a continuous record of the absorbance at 412 nm was used to monitor the approach to a linear steady state hydrolysis rate.

Model of enzyme catalysis. Rate measurements were analyzed according to the model of AChE catalysis in eq. 1, as originally proposed by Krupka and Laidler (8) (see Ref. 4).

$$\begin{array}{ccccccc} & & & & & bk_3\ \mathrm{ES} + \mathrm{P} & \\ & & & & \mathrm{EAS} & \rightarrow & \\ & & & & k_{s2} \downuparrows k_{-s2} & & \\ & & & & \mathrm{S} & & \\ & & k_1 & k_2\ \mathrm{X} & + & k_3\ \ \mathrm{E} + \mathrm{P} & \\ \mathrm{S} + & \mathrm{E} & \rightleftharpoons\ \mathrm{ES} & & \mathrm{EA} & \rightarrow & \\ & + & k_{-1} & & + & & \\ & \mathrm{I} & & & \mathrm{I} & & \\ & k_{i1} \downuparrows & k_{-i1} & & k_{i21} \downuparrows & k_{-i2}\ ak_3 & \end{array} \qquad (1)$$

$$\mathrm{EI} \qquad\qquad \mathrm{EAI} \rightarrow \mathrm{EI} + \mathrm{P}$$

In eq. 1, S is the acetylthiocholine substrate, X is the thiocholine leaving group, and P is the acetate product. The initial enzyme-substrate complex ES proceeds to an acetyl-enzyme, EA, before regeneration of free enzyme E. The inhibitory ligand I may block activity by combining with the free enzyme E (with association and dissociation rate constants of k_{i1} and k_{-i1}, respectively) and/or with the acetyl-enzyme complex EA (with corresponding rate constants k_{i2} and k_{-i2}). Acetylthiocholine also can form an inhibitory complex by binding to the acetyl-enzyme (with corresponding rate constants k_{s2} and k_{-s2}).

Steady state inhibition constants. Equilibrium formation of the inhibitory complexes EI and/or EAI and EAS can occur when deacylation of EAI and EAS is completely inhibited ($a = b = 0$). This condition results in the expression for the steady state enzyme activity, v_{ss}, shown in eq. 2.

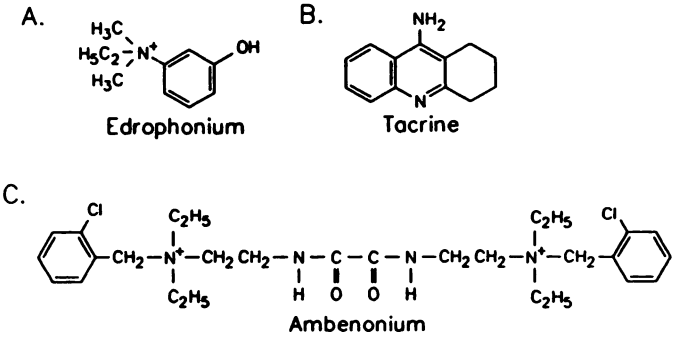

Fig. 1. Structures of AChE inhibitors examined in this report. A, Edrophonium (ethyl(3-hydroxyphenyl)dimethylammonium); B, tacrine (1,2,3,4-tetrahydro-9-aminoacridine); C, ambenonium ([oxalylbis(iminoethylene)] bis[(*o*-chlorobenzyl)diethylammonium]).

$$v_{ss} = \frac{d[\mathrm{X}]}{dt} = \frac{V_{max}[\mathrm{S}]}{[\mathrm{S}](1 + [\mathrm{I}]/K_{iu} + [\mathrm{S}]/K_{su}) + K_{app}(1 + [\mathrm{I}]/K_{i1})} \qquad (2)$$

In eq. 2, V_{max} is given by $k_{cat}[\mathrm{E}]_{tot}$, where $k_{cat} = k_2k_3/(k_2 + k_3)$; $[\mathrm{E}]_{tot}$ is the total concentration of all enzyme species; K_{app} corresponds to $(k_{-1} + k_2)k_{cat}/k_1k_2$; $K_{i1} = k_{-i1}/k_{i1}$; $K_{iu} = k_3k_{-i2}/k_{cat}k_{i2}$; and $K_{su} = k_3k_{-s2}/k_{cat}k_{s2}$. To obtain estimates of the competitive inhibition constant K_{i1}, reciprocal plots of $1/v$ versus $1/[\mathrm{S}]$ were constructed at relatively low concentrations of substrate, where EAS was negligible ($[\mathrm{S}]/K_{su} = 0$). The plots were assessed by a weighted least squares analysis that assumed the variance of v to be a constant percentage of v for the entire data set. Slopes of these reciprocal plots were then plotted against [I] in a similar weighted analysis, and K_{i1} was determined as the ratio of the replot intercept to the replot slope (9). In similar fashion, values of K_{iu} were estimated from weighted least squares analysis of reciprocal plot intercepts versus [I] as the ratio of the replot intercept to the replot slope.

Rate of approach to the steady state. An inhibitor with high enzyme affinity exhibits a low rate constant for enzyme dissociation, and equilibrium binding may not be achieved for seconds or minutes after mixing of the reaction partners. The approach to equilibrium inhibitor binding may be monitored by the enzyme activity toward substrate, in the model in eq. 1. Because the reaction pathway involving acetylthiocholine and AChE achieves a steady state in <1 msec, concentrations of E, ES, EA, and EAS will remain at their steady state ratios during the approach to equilibrium inhibitor binding. Incorporating this assumption into the eq. 1 model, a general solution to the rate of approach to the steady state will involve two exponential rate constants, because the model allows inhibitor binding to both E and EA. In this report, however, three special cases of this model that result in rates characterized by a single exponential rate constant will be considered. In the first special case, I is assumed to bind only to E and no ligands bind to EA (i.e., $1/K_{i2} = 1/K_{s2} = 0$). In this case, the enzyme activity v approaches v_{ss} with an exponential time course corresponding to the rate constant k_{exp_1} given in eq. 3.

$$k_{exp_1} = \frac{k_{i1}[\mathrm{I}]}{(1 + [\mathrm{S}]/K_{app})} + k_{-i1} \qquad (3)$$

In the second special case, I binds only to EA and forms a dead-end complex (i.e., $1/K_{i1} = 0$ and $a = 0$). Then v approaches v_{ss} exponentially, with the rate constant k_{exp_2} given in eq. 4.

$$k_{exp_2} = \frac{k_{i2}[\mathrm{I}]k_{cat}/K_3}{\frac{K_{app}}{[\mathrm{S}]}\left[1 + \frac{[\mathrm{S}]}{K_{app}}\left(1 + \frac{[\mathrm{S}]}{K_{su}}\right)\right]} + k_{-i2} \qquad (4)$$

The third special case assumes that EAI is not a dead-end complex but rapidly deacetylates to EI (i.e., $ak_3 >> k_{-i2}$). In this case, EAI will remain in a low constant steady state ratio to E throughout the approach to equilibrium of EI, and the exponential approach to v_{ss} occurs with the rate constant k_{exp_3} given in eq. 5.

$$k_{exp_3} = \frac{[\mathrm{I}]\left(k_{i1} + \frac{k_{i2}k_{cat}[\mathrm{S}]}{k_3K_{app}}\right)}{1 + \frac{[\mathrm{S}]}{K_{app}}\left(1 + \frac{[\mathrm{S}]}{K_{su}}\right)} + k_{-i1} \qquad (5)$$

Results

The graphical analysis of steady state inhibition data for the inhibitors in Fig. 1 is shown in Fig. 2. Reciprocal plots involving edrophonium inhibition (Fig. 2A) intersect near the $1/v$ axis, consistent with previous reports that this inhibitor acts competitively by binding to the free enzyme E, with little binding to the acetylated enzyme EA, in the model in eq. 1 (10). In contrast, reciprocal plots involving both tacrine and amben-

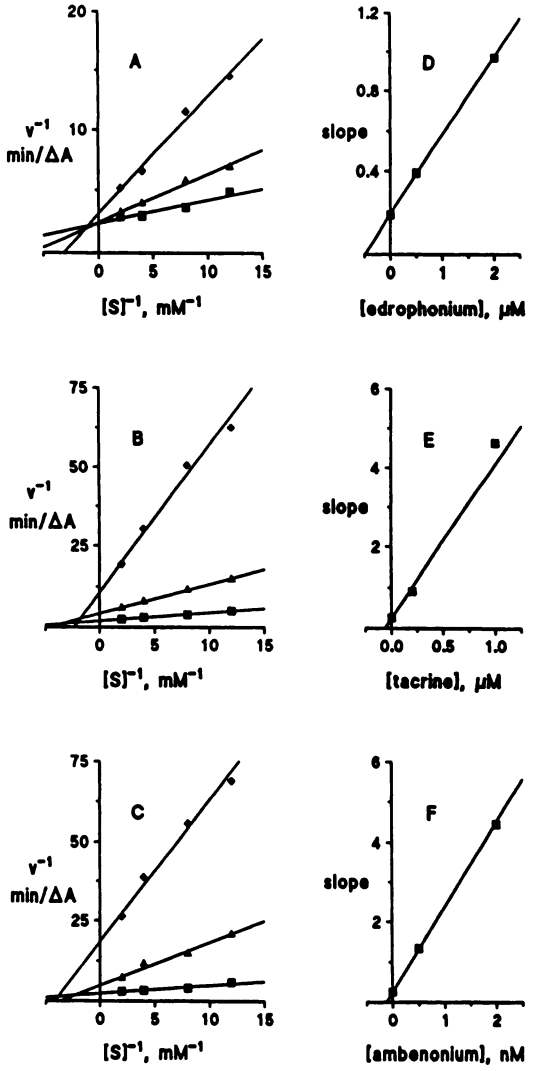

Fig. 2. Steady state inhibition by three inhibitors of AChE hydrolysis of acetylthiocholine. Reciprocal plots of initial velocities and substrate concentrations (A–C) and replots of the slopes of the reciprocal plots versus inhibitor concentration (D–F) were obtained as outlined in Experimental Procedures. Acetylthiocholine concentrations did not exceed 0.5 mM, and no EAS species (see eq. 1) was apparent. Reciprocal plots of initial velocities in the absence of inhibitors gave an estimate of K_{app} for acetylthiocholine of 115 ± 13 μM (four experiments). *Lines* were calculated from a weighted least-squares analysis of the data points.

TABLE 1

Competitive inhibition constants for three inhibitors of human erythrocyte AChE

Values of K_{ii} were estimated, as outlined in Experimental Procedures, from the data in Fig. 2.

Inhibitor	K_{ii}
	nM
Edrophonium chloride	470 ± 130
Tacrine	65 ± 30
Ambenonium chloride	0.12 ± 0.03

onium (Fig. 2, B and C) show both increasing slopes and increasing intercepts with higher inhibitor concentration. This pattern indicates mixed inhibition, arising from significant inhibitor interaction with both E and EA. Replots of the slopes versus the inhibitor concentration give the estimates of competitive inhibition constants K_{i1} in Table 1. Values are in reasonable agreement with those reported previously for tacrine (0.2 μM) (11) and ambenonium (0.5 nM) (3) but are slightly higher than those reported for edrophonium with eel electric organ AChE (100–150 nM) (see Ref. 12).

The K_{i1} value of 0.12 nM for ambenonium in Table 1 is one of the highest reported for any AChE inhibitor. To determine whether the binding of ambenonium to the enzyme is reversible on a time scale consistent with a simple noncovalent interaction, the rate of approach to steady state inhibition was measured under two conditions. In the first, ambenonium was added to an assay in progress, and the decrease in substrate hydrolysis rate to a new steady state level was recorded (Fig. 3A). In the second, ambenonium was premixed with the stock AChE before dilution of an aliquot of the mixture into the assay, and the increase in rate to the final steady state value was monitored (Fig. 3B). In both cases the pre-steady state rates approached the final steady state values with a single exponential time course, within experimental error.

The dependence of the pre-steady state rate constants (k_{exp}) on the concentrations of ambenonium and substrate was examined in Fig. 4, where observed values of k_{exp} are plotted against ambenonium concentration at three fixed levels of substrate. The plots are linear, as predicted by all three special cases in eqs. 3–5. The slopes of the plots reveal a substrate concentration dependence that is addressed in the Discussion, but the intercepts of the plots do not depend on substrate concentration, within experimental error. The intercept average corresponds to an inhibitor dissociation rate constant of 0.013 ± 0.002 sec^{-1} in eqs. 3–5, and its invariance with substrate concentration indicates either that $k_{-i1} = k_{-i2}$ (a combination of eqs. 3 and 4) or that k_{-i2} is kinetically insignificant (eq. 5). At the lowest substrate concentration employed (50 μM), the slope approximated a bimolecular or association rate constant (k_{i1} in eq. 3) of 5.2 ± 0.6 × 10^7 M^{-1} sec^{-1}. The equilibrium dissociation constant K_{i1} should be given by the ratio of the dissociation to the association rate constants. This ratio is 0.22 ± 0.07 nM, in reasonable agreement with the 0.12 ± 0.03 nM value for K_{i1} from steady state inhibition data in Table 1. Part of the discrepency is accounted for by the fact that the association rate constant should be slightly higher than the value estimated in the presence of 50 μM substrate.

Under steady state hydrolysis conditions, the model in eqs. 1 and 2 predicts that substrate inhibition will occur at high concentrations of substrate, because substrate will combine with acetyl-enzyme in an EAS complex in which deacetylation is blocked. The inhibition constant for substrate binding to the acetyl-enzyme, K_{su}, was estimated to be 20.1 ± 2.8 mM from a series of measurements at high substrate concentration, as shown in Fig. 5. This compares with a value of 44 ± 19 mM measured previously for eel electric organ AChE (13).

Discussion

The competitive inhibition constant of 0.12 nM observed in this study for ambenonium inhibition of human erythrocyte AChE is consistent with previous reports of a very high ambenonium affinity for AChE. To investigate whether the inhibition involves a rapidly reversible noncovalent complex, association and dissociation rate constants were estimated from the exponential rate of approach to steady state inhibition. The estimated association constant of 5.2 × 10^7 M^{-1} sec^{-1} determined here can be compared with reported association rate constants

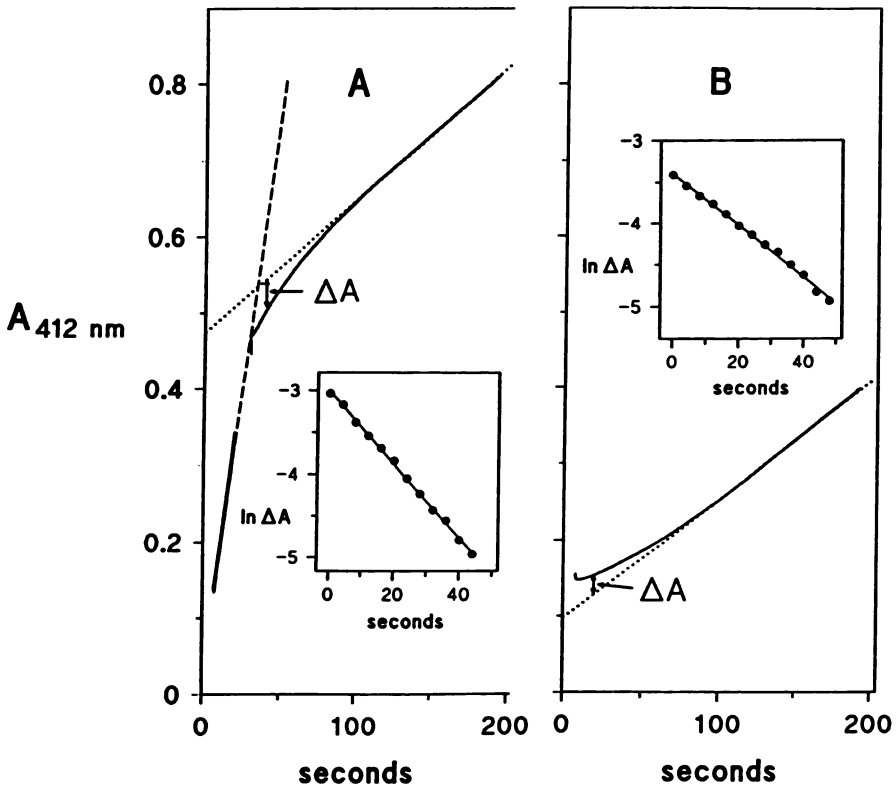

Fig. 3. Measurements of the rate of approach to steady state hydrolysis of 2.5 mM acetylthiocholine in the presence of ambenonium. A, AChE (0.1 unit) was added to substrate alone and an immediate steady state rate was observed (– – –). Addition of 50 μl of ambenonium chloride stock, to a final concentration of 1.64 nM, resulted in a progressive decrease in substrate hydrolysis rate to a new steady state (···). The rate of approach to the new steady state was calculated by plotting the $\log_e$ of ΔA (the difference between the absorbance extrapolated from the *dotted line* and the observed absorbance) versus time (*inset*). The linearity of the plot indicated a single exponential rate, and its slope gave a rate constant, k_{exp}, of 0.044 sec^{-1}. B, AChE (0.1 unit in 10 μl) was preincubated with 60 μl of 100 nM stock ambenonium chloride for 2 min. Addition of 60 μl of the mixture to the assay (final concentration of ambenonium, 1.68 nM) resulted in a progressive increase in substrate hydrolysis rate until a steady state was obtained. A value of k_{exp} of 0.031 sec^{-1} was calculated from the *inset* plot constructed as outlined in A. Estimates of k_{exp} by this method require that the approach to steady state occurs over a small range of substrate concentration, where the substrate hydrolysis rate in the absence of inhibitor is effectively constant. To ensure this condition, an absorbance scale of 0–1.0 was used for measurements with 2.5 mM and 0.5 mM acetylthiocholine and of 0–0.1 for 50 μM acetylthiocholine.

for other cationic inhibitors of AChE determined by fluorescence quenching. Although less than one-tenth that obtained for the monoquaternary *N*-methylacridinium cation (14), the value here is comparable to the estimate of 9.7×10^7 M^{-1} sec^{-1} reported for a bisquaternary benzoquinone dication by Bolger and Taylor (15). These authors suggest that, in comparison with a monoquaternary cation like *N*-methylacridinium, bisquaternary dications like benzoquinones and ambenonium require more critical alignment with complementary anionic sites in the enzyme for productive binding, with a consequent reduction in the relative association rate constant. Of particular interest here, however, the close correspondence of the value of the association rate constant for ambenonium to those of a series of benzoquinones (15) is compelling evidence that ambenonium shows rapid reversibility typical of other bisquaternary noncovalent inhibitors of AChE. The unusually high affinity of ambenonium for AChE, thus, is a result of its dissociation rate constant of 0.013 ± 0.002 sec^{-1}, a value that is at least 2 orders of magnitude lower than those of the benzoquinone bisquaternary inhibitors.

The structure of ambenonium includes a chlorine atom in the *ortho* position of the phenyl groups, and a halide group in this position appears important for the high affinity of ambenonium for AChE. Replacement of chlorine with bromine has little effect on AChE affinity, but replacement of chlorine with hydrogen decreases AChE affinity by about 3 orders of magnitude (3). The high affinity of ambenonium for AChE should make it a more selective AChE inhibitor than tacrine for potential use in the treatment of Alzheimer's disease. However, the bisquaternary structure of ambenonium will prevent its passage through the blood-brain barrier after conventional oral or intravenous dosing. It may be feasible to design an analog of ambenonium in which one ethyl group is deleted from each quaternary ammonium center to give a bistertiary amine compound that could penetrate the blood-brain barrier more readily.

In addition to demonstrating the practical point that ambenonium binding to AChE is rapidly reversible, this report also introduces ambenonium as an important tool for exploring AChE catalytic mechanism. It is the first reported analysis of the rate of approach to equilibrium binding of any rapidly reversible noncovalent inhibitor with AChE during steady state hydrolysis of a substrate. This type of analysis has the potential to add new insights into the interactions of inhibitors with AChE, beyond those derived strictly from steady state rate data. Although the data here are limited to the interaction of ambenonium and acetylthiocholine at AChE sites that regulate AChE activity, useful information about these interactions can be obtained by examining the dependence of the rate of ambenonium binding on the substrate concentration. In the model in eq. 1, ambenonium and acetylthiocholine compete for binding sites in both the free enzyme and the acetyl-enzyme. A ternary complex of free enzyme with substrate and inhibitor (ESI) is not included. Although an ESI complex is important when the substrate and inhibitor involve one neutral and one cationic ligand (12, 13, 16), it appears to be kinetically insignificant when both the substrate and the inhibitor are cationic and when assays are conducted near physiologic ionic strength (4, 8, 9, 12, 13, 16, 17).

The dependence of both the slopes and the intercepts of reciprocal plots on ambenonium concentration in the steady state data shown in Fig. 2 indicate that this inhibitor binds to both the free enzyme and the acetylenzyme. If inhibitor bound to only one of these species, rate constant expressions for the approach to the steady state could be formulated explicitly, as indicated in the special cases in eqs. 3 and 4. In the general

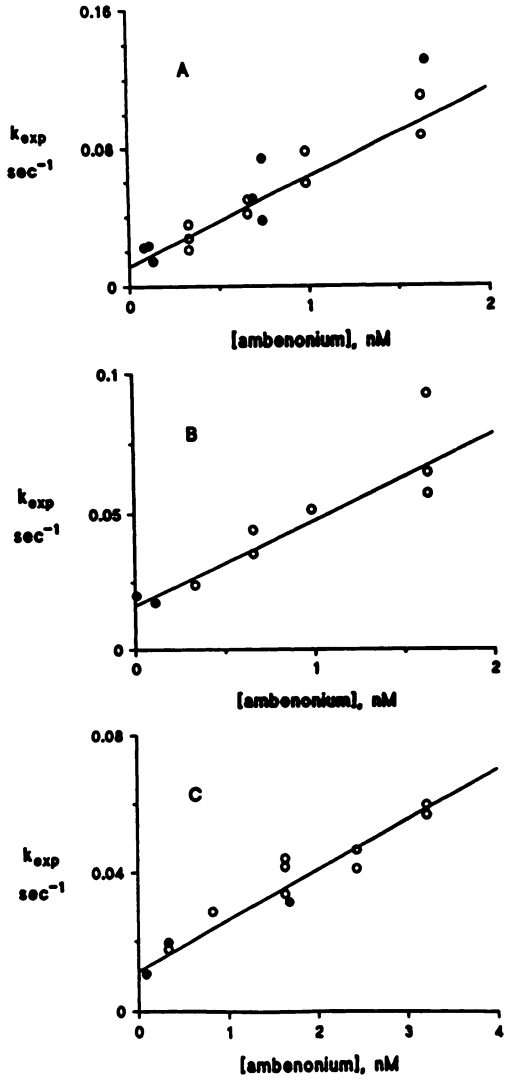

Fig. 4. Dependence of the rates of approach to steady state acetylthiocholine hydrolysis on the concentration of ambenonium. Pre-steady state rates were estimated either from decreasing substrate hydrolysis velocities (○), as in Fig. 3A, or from increasing velocities (●), as in Fig. 3B, and were fit to a single exponential time course as in Fig. 3. Acetylthiocholine concentrations corresponded to 50 μM (A), 500 μM (B), or 2500 μM (C). The *y*-axis intercepts corresponded to 0.0115 ± 0.0027 sec^{-1} (A), 0.0164 ± 0.0022 sec^{-1} (B), and 0.0113 ± 0.0013 sec^{-1} (C) and the slopes to 0.052 ± 0.006 nM^{-1} sec^{-1} (A), 0.031 ± 0.004 nM^{-1} sec^{-1} (B), and 0.0148 ± 0.0012 nM^{-1} sec^{-1} (C).

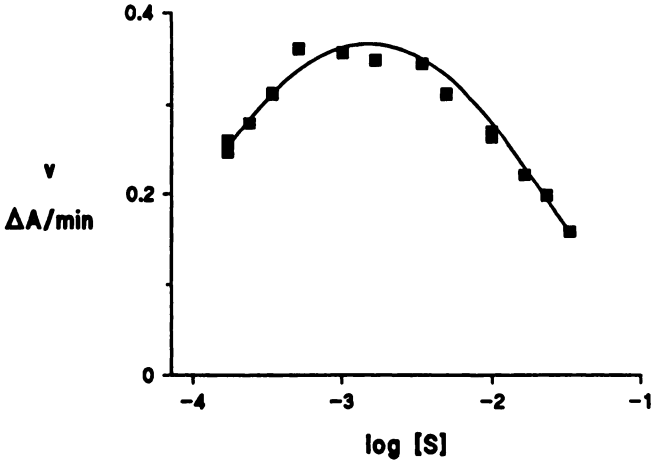

Fig. 5. Inhibition of enzyme-catalyzed acetylthiocholine hydrolysis at high substrate concentrations. Substrate concentrations are shown as log_{10} of substrate, in M units. The line is calculated from eq. 2 (where [I] = 0), with fixed parameters K_{app} = 115 ± 13 μM (from Fig. 2), V_{max} = 0.422 ± 0.016 δA_{412}/min (observed), and K_{su} = 20.1 ± 2.8 mM (from linear least squares analysis of eq. 2, assuming the former two parameters). The error in K_{su} was estimated from the variation in K_{su} fitted at the error limits of K_{app} and V_{max}.

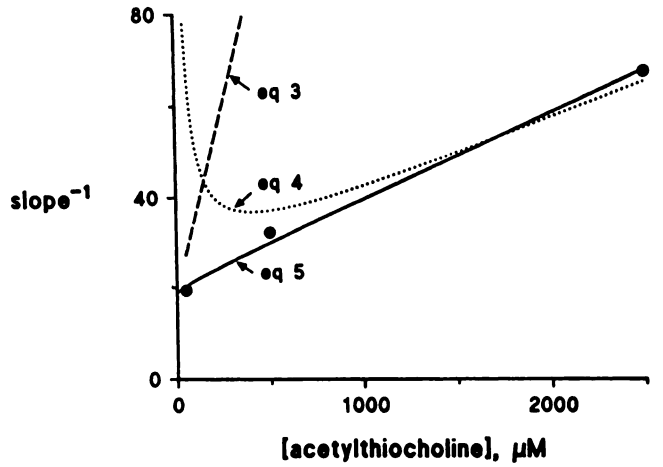

Fig. 6. Dependence of the apparent association rate constants for ambenonium on the concentration of acetylthiocholine. The apparent constants are defined as the slopes of the plots in Fig. 4 and correspond to the coefficients of [I] in eqs. 3–5. Reciprocals of the slopes were generated to emphasize their near-linear dependence on [S]. For comparison with eqs. 3–5, the following parameters were fixed: K_{app} = 115 ± 13 μM, K_{ii} = 0.31 ± 0.09 nM (as determined from the steady state data in Fig. 2), and $k_{i1} = k_{i2}$ = 0.013 ± 0.002 sec^{-1} (as determined from the intercepts in Fig. 4). Calculated lines were produced by a program (Fig. P; Biosoft) that generated an unweighted nonlinear least squares fit to the reciprocal of the [I] coefficient in eqs. 3–5. The following estimates were obtained from the calculated lines: eq. 5 (——), k_{i1} = 0.053 nM^{-1} sec^{-1} and K_{su} = 1.2 mM (this value of k_{i1} was used in fitting eqs. 3 and 4); eq. 4 (· · · ·), K_{su} = 1.4 mM; eq. 3 (– – –), no free parameters.

case, however, these two binding equilibria are coupled, and the approach to the steady state is characterized by two exponential rate constants. The relationship of coupled relaxation rate constants to rate constants for the isolated equilibria has been given by Eigen and DeMaeyer (18) (see examples in Ref. 14), and evaluation of individual kinetic parameters from the kinetic data is complex. However, if experimental conditions can be adjusted so that the amplitude of one of the coupled equilibria is much larger than the other, then its exponential rate constant predominates and can be approximated by the theoretical rate constant for the isolated equilibrium. In the model in eq. 1, the substrate concentration can be manipulated to vary the ratio of [EA] to [E] over a wide range. At high concentrations of substrate, this ratio is large and the observed k_{exp} can be approximated by eq. 4. At the lowest substrate concentration practical in the current experiments (50 μM), [E] is 2–3 times [EA] and k_{exp} approaches the expression in eq. 3.

To illustrate these points, the observed dependence of ambenonium association rates on acetylthiocholine concentration are compared with those predicted from eqs. 3 and 4 in Fig. 6. It is evident that the theoretical lines corresponding to eqs. 3 and 4 approach the experimental points only at the expected extremes of substrate concentration. At intermediate substrate concentrations, the general case of coupled equilibria holds and the rate of approach to the steady state should involve two

exponential rate constants. However, data analysis resolves only a single exponential term. Two possible explanations may be considered that are consistent with the experimental data. In the first, inhibitor binding is little influenced by the state of enzyme acetylation ($k_{i1} = k_{i2}$ and $k_{-i1} = k_{-i2}$). This approximation is in agreement with the observation that the dissociation rate constants indicated by the intercepts in Fig. 4 show no substrate dependence. In this situation, at intermediate substrate concentrations the exponential rate constants predicted by eqs. 3 and 4 approach common values and would be difficult to distinguish from a single exponential. The second explanation invokes the special case proposed in eq. 5, where the acetyl-enzyme EAI is allowed to deacetylate ($a > 0$) and the rate of approach to the steady state collapses to a single exponential. The key assumption in eq. 5 ($ak_3 >> k_{-i2}$) is easily justified by noting that k_3 is estimated to be 10^4 sec^{-1} (4), whereas k_{-i2} is likely to be on the order of 10^{-2} sec^{-1}, from the intercepts in Fig. 4. The explanation involving eq. 5 is attractive because a calculated line for the data in Fig. 6 is readily obtained. This line, determined in part from parameters fixed by the steady state data in Fig. 2, is in reasonable agreement with the experimental points.

The analysis in Fig. 6 reveals important information about one kinetic parameter in eq. 1, the constant, K_{su}, that reflects the competition between substrate and inhibitor for a common site in the acetyl-enzyme EA. K_{su} in Fig. 6 was not fixed by the steady state data but, instead, was determined from a best fit of the three experimental points. Both eqs. 4 and 5 indicate a K_{su} value of 1.2–1.4 mM. In classical steady state kinetics, K_{su} is determined from a substrate inhibition curve like that in Fig. 5, where a value of 20.1 ± 2.8 mM was observed. The difference between these two K_{su} estimates is far greater than the experimental error, and the most direct explanation for the discrepency is that they reflect substrate binding at different acetyl-enzyme sites. The concept of peripheral anionic sites outside the active site is well established for AChE (17, 19) (see Ref. 4). Propidium, which binds primarily to a peripheral site, and edrophonium, which binds primarily to the active site, do not compete with each other. In contrast, bisquaternary ligands, including ambenonium, compete with both propidium and edrophonium and, thus, appear to bridge both sites. The most straightforward interpretation of the divergent estimates of K_{su} noted above, therefore, is that the 1.2–1.4 mM value represents acetylthiocholine binding to the peripheral site occupied by one end of the ambenonium molecule. This interaction of acetylthiocholine with a peripheral site has not been reported previously because it has no detectable effect effect on k_{cat} or K_m for acetylthiocholine at the active site ($b = 1$ in the eq. 1 model) and is revealed only by its competition with ambenonium for this site on the acetyl-enzyme. We expect that this site also would be revealed by competition experiments with other bisquaternary or peripheral site ligands, but it is unlikely to be detected by direct titration of higher concentrations of the enzyme with acetylthiocholine, because this substrate is hydrolyzed too rapidly. It remains reasonable to assume that the K_{su} value of 20.1 mM represents the classical substrate inhibition site involving acetylthiocholine interaction at the active site of the acetylated enzyme, as has long been proposed (see Ref. 4). This interaction does block deacetylation ($b < 1$ in the eq. 1 model). The indication that acetylthiocholine can bind to a peripheral anionic site without blocking AChE deacetylation raises the interesting possibility that acetylcholine binding to this peripheral site may be significant or even obligatory in the catalytic pathway for acetylcholine hydrolysis. In view of the low affinities reflected in the K_{su} value for acetylthiocholine and the high catalytic efficiency of AChE, this possibility will be difficult to address without the development of additional reporter probes for this enzyme.

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