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Interaction of Selected Cholinergic Effector Molecules with Acetylcholinesterase in Physiological Eel Ringer's Solution

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SUMMARY

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Interaction of cholinergic effectors with eel acetylcholinesterase was investigated in physiological eel Ringer's solution, pH 7. The acceleration of methanesulfonation of the soluble enzyme by the cholinergic agonists decamethonium and trimethylbutylammonium ions was indistinguishable from that observed with the membrane-bound enzyme. Maximum acceleration of the methanesulfonation reaction by decamethonium and succinylcholine with the particulate acetylcholinesterase was essentially the same. A limited acceleration caused by trimethylbutylammonium ion is attributed to its partial overlap with the esteratic site, in contrast to the postulated "exo" mode of binding for the bisquaternary accelerator, decamethonium. Kinetic studies with trimethylbutylammonium and decamethonium ions using acetylcholine and phenyl acetate as substrates, as well as equilibrium binding studies to the enzyme, support the "exo" mode of binding for decamethonium, the "endo" mode of binding for 3-hydroxyphenyltrimethylammonium ion, and an intermediate mode of binding for trimethylbutylammonium ion. The effects produced by the quaternary nitrogen agonists on acetylcholinesterase in physiological eel Ringer's solution can be explained on the basis of binding of the effectors to the anionic subsite of the active center.

INTRODUCTION

Acetylcholinesterase is involved in chemical synaptic transmission (1). A possible regulatory function of this enzyme in cholinergic synapses has stimulated a number of kinetic studies relating to the possible existence of an allosteric modifier site or sites on the acetylcholinesterase molecule.

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Changeux (2) has shown that both the structure and catalytic properties of soluble acetylcholinesterase from *Torpedo* depend on the ionic strength of the environment. He suggested that the enzyme can exist in at least two conformational states which differ in their affinities toward quaternary nitrogen ligands, and that the response of acetylcholinesterase to the binding of various cholinergic effector molecules at a site distinct from the active site is mediated through conformational alterations of the enzyme. Kitz *et al.* (3) studied the effects of certain neuromuscular blocking compounds on rates of inhibition of acetylcholinesterase by carbamates and on rates of spontaneous recovery

of the enzyme activity. These workers reported that under conditions in which modifiers had no effect on substrate hydrolysis, the neuromuscular blocking drugs accelerated rather than inhibited carbamylation and decarbamylation of the active site. The results were interpreted as evidence for an allosteric site mechanism.

Roufogalis and Quist (4) investigated the interaction of bovine acetylcholinesterase with calcium, tetramethylammonium, tetraethylammonium, decamethonium, gallamine, and *d*-tubocurarine. The authors postulated three different anionic sites on the enzyme surface to account for the observed kinetics of interaction. Interaction of quaternary nitrogen ligands with membrane-bound acetylcholinesterase from erythrocytes was studied by Wombacher and Wolf (5), who also postulated the presence of a regulatory site in order to explain certain kinetic phenomena, and suggested that the regulatory site could have a possible receptor function. However, most experiments were performed under conditions of low ionic strength, where physiologically unimportant phenomena can be observed (6).

An interesting approach to the correlation of pharmacodynamic properties of drug-receptor and drug-enzyme interactions was taken by Belleau *et al.* (7), who showed that the relative efficiency of a drug as an accelerator of methanesulfonation of the erythrocyte acetylcholinesterase correlates well with the potency of the drug. Wilson (8) and Belleau and DiTullio (9) suggested that acceleration of the methanesulfonation reaction by cholinergic drugs reflects conformational changes which the enzyme undergoes upon binding of quaternary nitrogen ligands. Kitz and Kremzner (10) demonstrated directly that conformational changes accompany binding of substrates or inhibitors to acetylcholinesterase. However, conformational changes that occur upon interaction of effector molecules with acetylcholinesterase do not necessarily signify the presence of an allosteric site.

During studies of the relationship of various cholinergic effector binding sites in excitable membrane fragments from the electric organ of *Electrophorus electricus*, the question of the existence of a functional peripheral anionic site which is distinct from the anionic subsite of the active center was reinvestigated. Of particular interest was whether (a) the membrane-bound acetylcholinesterase and the soluble eel enzyme differ with respect to their interaction with cholinergic effectors, as tested by the effect on the rate of the methanesulfonation reaction, and (b), in the case of identity of the response of both the membrane-bound and free enzyme, whether a peripheral regulatory site is involved in the response of acetylcholinesterase to the depolarizing drugs decamethonium and trimethylbutylammonium ion.

MATERIALS AND METHODS

Enzyme. Either commercial eel acetylcholinesterase (Sigma, grade V; Worthington, grade ECHP; specific activity, approximately 70 mmoles of acetylcholine hydrolyzed per hour per milligram of protein) or enzyme extracted from the electric organ of *E. electricus* and partially purified by ammonium sulfate precipitation (specific activity, 1–19 mmoles/hr/mg) was used.

All experiments were performed in physiological eel Ringer's solution, pH 7.0 and ionic strength 0.186 (11) containing NaCl, 169 mM; KCl, 5 mM; $CaCl_2$, 3 mM; $MgCl_2$, 1.5 mM; Na_2HPO_4, 1.2 mM; and NaH_2PO_4, 0.3 mM.

In the acceleration of the methanesulfonation reaction, 1-ml aliquots of the enzyme in physiological eel Ringer's solution, pH 7.0, were initially incubated with the desired concentrations of effector compounds for 30 min at 4°. The tubes were then placed in a temperature-controlled water bath at 26°, and 1–5 μl of 0.1 M methanesulfonyl fluoride prepared in 50% ethanol (v/v) were added at zero time to the enzyme solution. Final concentrations of MSF[2] ranged from 100 to 500 μM. Progress of the methanesulfonation of acetylcholinesterase was followed by assaying aliquots of the reaction mixture for en-

[2] The abbreviations used are: MSF, methanesulfonyl fluoride; 3-HOPTA, 3-hydroxyphenyltrimethylammonium iodide; TMBA, trimethylbutylammonium iodide.

zyme activity according to Ellman *et al.* (12), with 500 μM acetylthiocholine and 500 μM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.05 M sodium phosphate buffer, pH 8. Plots of percentage inhibition gave a straight line on $\log \frac{E}{Eo}$ vs. time graphs, indicating a pseudo-first-order reaction between MSF and acetylcholinesterase (13).

Phenyl acetate hydrolysis in the absence and presence of inhibitors was followed at 270 nm on a Cary 14 spectrophotometer at 26°. The reaction medium consisted of 3 ml of the desired concentration of phenyl acetate prepared in eel Ringer's solution, pH 7, and containing 2% (v/v) methanol, plus the desired concentration of effector compound. The reaction was initiated by adding 5–20 μl of enzyme solution.

The kinetics of acetylcholine hydrolysis in the presence of decamethonium was followed titrimetrically on a Radiometer pH-stat with 0.01 N NaOH at 26°. The reaction volume was 10 ml.

For equilibrium binding of radioactive ligands to the native or methanesulfonated acetylcholinesterase, specially constructed microcells were used. The two 80-μl compartments of each microcell were separated by cellulose dialysis tubing. Binding assays were carried out at 26° for 9 hr or longer. After equilibration had been achieved, duplicate 20-μl aliquots were withdrawn from both the protein-containing and the protein-free compartment, and were counted for radioactivity on a Packard scintillation counter. The counting efficiency for tritium was 35–40%. Aliquots of 20 μl were also assayed for protein according to Lowry *et al.* (14). The amount of ligand bound per milligram of protein was calculated from the known specific radioactivity of a ligand. From the knowledge of the concentration of acetylcholinesterase active sites per milligram of protein, the number of moles of ligand bound per mole of active sites could be calculated. In the experiments with methanesulfonated enzyme the concentration of active sites was estimated from the knowledge of cholinesterase equivalents per milligram of total protein in the uninhibited preparation and the protein content of the inhibited preparation. The active site concentration was determined by titration of the stock enzyme solution with Tetram (15).

Methanesulfonation was done by dialyzing aliquots of free or membrane-bound acetylcholinesterase against five changes of a 1 mM solution of *N*-methylpyridinium 3-*O*-methanesulfonate iodide in Ringer's solution, pH 7, until the enzyme was inhibited 99.9%, and was followed by dialysis against two changes of Ringer's solution to remove the methanesulfonating reagent.

Membrane-bound enzyme. Excitable membrane fragments from electric eel were prepared according to Changeux *et al.* (16). The membrane preparation was washed once with cold Ringer's solution and was centrifuged at 100,000 × *g* for 1 hr to remove completely any contaminating soluble proteins. The preparation used in kinetic experiments had a specific activity of 1.1 mmoles of acetylthiocholine hydrolyzed per hour per milligram of membrane proteins.

All chemicals used other than those listed below were reagent grade. Acetylcholine bromide and phenyl acetate (Fisher Scientific Company) were recrystallized and redistilled, respectively. Decamethonium dibromide, *d*-tubocurarine, and gallamine triiodide (K & K Laboratories) were used without further purification. Succinylcholine dichloride (Mann Biochemicals) was also used without further purification. Methanesulfonyl fluoride (Eastman Organic Chemicals) was used directly. Trimethylbutylammonium iodide was prepared by quaternizing *N*,*N*-dimethylbutylamine (K & K Laboratories) with methyl iodide. *N*-Methylpyridinium 3-*O*-methanesulfonate iodide was prepared according to a published procedure (17). [^{3}H]3-Hydroxyphenyltrimethylammonium iodide was prepared by methylation of *N*,*N*-dimethyl-3-hydroxyaniline (Eastman Organic Chemicals) followed by catalytic tritiation at New England Nuclear Corporation as described previously (18). The specific radioactivity of the [^{3}H]3-HOPTA was 229 mCi/mmole. [^{3}H]Decamethonium dichloride had a specific radioactivity of 178 mCi/mmole (Amersham/Searle).

All data were treated statistically by least-squares regression analysis.

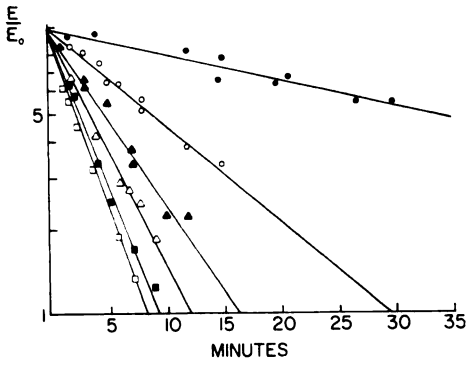

FIG. 1. *Acceleration of methanesulfonation of acetylcholinesterase by decamethonium*

Soluble enzyme preparation (specific activity, 1.5 mmoles of acetylthiocholine hydrolyzed per hour per milligram of protein) was used. Other experimental details are given inthe legend to Table 1. ●, control, decamethonium absent; ○, 0.5 μM decamethonium; ▲, 1 μM decamethonium; △, 5 decamethonium; ■, 10 μM decamethonium; □, 50 μM decamethonium.

RESULTS AND DISCUSSION

Figure 1 illustrates a typical acceleratory effect of decamethonium on the methanesulfonation of acetylcholinesterase. The solid lines were computed by the least-squares method from duplicate experimental points. Figure 2 represents the plot of α vs. $(\alpha\text{-}1)/A$, where α is the acceleration factor at any given concentration of modifier A. The intercept of the line with the y axis is the maximal acceleration obtained when the enzyme was completely saturated with the modifier A, and the slope is equal to the dissociation constant for the enzyme-modifier complex. The equation used in the plot is a rearranged form of the relationship derived by Kitz and Wilson (13):

$$k_{obs} = \frac{k_3 \cdot (1 + k_a/k_3 \cdot A/K_A)}{1 + A/K_A} \qquad (1)$$

where the constants are defined by the following set of relations:

$$E + I \xrightarrow{k_3} E'$$

$$E + A \rightleftharpoons EA \quad (k_A)$$

$$EA + I \rightleftharpoons E' \quad (k_a)$$

with E = free enzyme, I = MSF, A = accelerator, e.g., decamethonium, succinylcholine, etc., EA = accelerator-enzyme complex, and E' = methanesulfonated acetylcholinesterase.

Rearranging Eq. 1, one obtains

$$\alpha = \frac{k_a}{k_3} - K_A\left(\frac{\alpha - 1}{A}\right) \qquad (2)$$

where $k_a/k_3 = \alpha_{max}$ and $k_{obs}/k_3 = \alpha$.

Table 1 summarizes the values for α_{max} and the K_A for selected effectors obtained with both the membrane-bound and the soluble acetylcholinesterase. Contrary to the results obtained with erythrocyte enzyme by Belleau *et al.* (7), the maximal effect on the rate of the MSF reaction with acetylcholinesterase from the electric eel is, within experimental error, the same with both decamethonium and succinylcholine. Their potency is reflected in K_A values, which agree with K_i values for these compounds as inhibitors of the active site of the enzyme. It therefore appears that, at least for these two bisquaternary modifiers, the existence of a correlation between their kinetic and pharmacological properties is not substantiated.

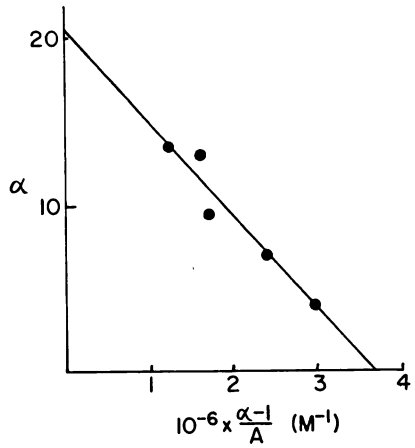

FIG. 2. *Acceleration of methanesulfonation of acetylcholinesterase by decamethonium*

α is acceleration at any given concentration of modifier. A is molar concentration of modifier. Each point represents the ratio of velocity of the MSF reaction in the presence of decamethonium to the rate of the control reaction, obtained from the slopes of the lines shown in Fig. 1. For further details, refer to the text.

TABLE 1

Interaction parameters for cholinergic effector molecules with soluble and membrane-bound acetylcholinesterase as tested by their effect on methanesulfonation of the enzyme

α_{max} and K_A values were obtained as described in the text. The following concentrations of methanesulfonyl fluoride and modifiers were used: decamethonium, 5×10^{-7}, 1×10^{-6}, 5×10^{-6}, 1×10^{-5}, and 5×10^{-5} M (MSF, 1.3×10^{-4} and 2×10^{-4} M with membrane-bound and free enzyme, respectively); succinylcholine, 1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 5×10^{-5}, and 1×10^{-4} M (MSF, 3.25×10^{-4} M); trimethylbutylammonium ion, 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M (MSF, 3.25×10^{-4} M); curare and gallamine, 1×10^{-4}, 5×10^{-4}, and 1×10^{-3} M (MSF, 5×10^{-4} M). Membrane-bound enzyme had a specific activity of 1.1 mmoles of acetylthiocholine hydrolyzed per hour per milligram of protein. The specific activity of soluble enzyme ranged from 1 to 70 mmoles of acetylthiocholine hydrolyzed per hour per milligram of protein. The methanesulfonation reaction mixture contained an amount of enzyme that hydrolyzed 0.63 mmole of acetylthiocholine per hour, or about 0.9 μg of acetylcholinesterase.

Modifier	α_{max}	K_A
Membrane-bound enzyme		M
Decamethonium	22.9 ± 1.7	$4.6 \pm 0.6 \times 10^{-6}$
Succinylcholine	17.6 ± 0.7	$3.1 \pm 0.2 \times 10^{-5}$
Trimethylbutylammonium ion	4.5 ± 0.2	$1.7 \pm 0.1 \times 10^{-4}$
d-Tubocurarine	0.67[a]	
Gallamine	0.59	
Soluble enzyme		
Decamethonium	20.4 ± 1.7	$5.5 \pm 0.8 \times 10^{-6}$
Trimethylbutylammonium ion	4.1 ± 0.2	$1.3 \pm 0.2 \times 10^{-4}$

[a] Inhibition of methanesulfonation by a 1 mM concentration of modifier.

It is also evident, however, that trimethylbutylammonium ion has a distinctly smaller effect on both the free and particulate enzyme. Since the responses of soluble and membrane-bound acetylcholinesterase to cholinergic agonists as tested by the methanesulfonation reaction are similar, further experiments were conducted with only the soluble enzyme preparation.

In order to clarify the observed difference in the reactivity of the esteratic site toward methanesulfonation in the presence of decamethonium and trimethylbutylammonium ion, the effects of these compounds on the kinetics of hydrolysis of a good substrate were studied.

Reinvestigation of some elementary kinetics was necessitated by the existence of conflicting reports on the type of inhibition of acetylcholinesterase by decamethonium, as well as by the absence of data on the inhibition of acetylcholinesterase by decamethonium and trimethylbutylammonium in physiological eel Ringer's solution.

Figures 3 and 4 show the results of the inhibition of phenyl acetate hydrolysis by decamethonium and TMBA, respectively. It can be seen that decamethonium produces purely noncompetitive inhibition whereas TMBA behaves as a purely competitive inhibitor. These results indicate that while decamethonium does not prevent accessibility of the nonpolar substrate to the active site, the TMBA molecule must do so.

Apparently the hydrophobic alkyl chain of TMBA interacts at least partially with the hydrophobic region of the esteratic site (20), preventing phenyl acetate from forming the *ES* complex. One corollary of the above is that the acceleration can be produced by purely competitive inhibitors of acetylcholinesterase, and hence is a result of binding of quaternary nitrogen modifiers to the anionic subsite of the active center, and that conformational changes that may accompany this interaction (7, 9) are not transmitted allosterically. Direct proof that TMBA interacts only with the anionic subsite of the active center would require demonstration of 1:1 stoichiometry of TMBA binding and complete displacement of TMBA by the active site-specific ligand 3-HOPTA, as was subsequently demonstrated for decamethonium. Because of the

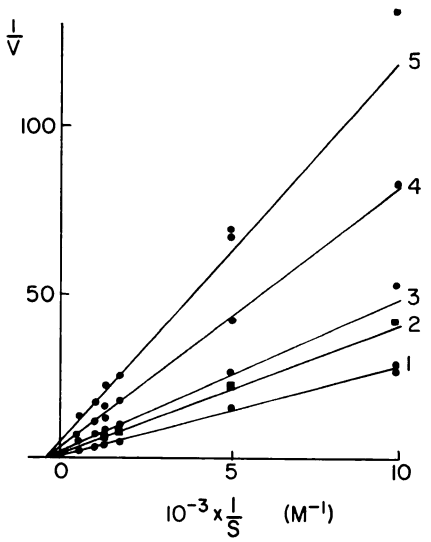

FIG. 3. *Reciprocal plot of results of decamethonium inhibition of acetylcholinesterase-catalyzed hydrolysis of phenyl acetate*

Reaction conditions are described in the text. Phenyl acetate was used at concentrations from 0.1 to 2 mM. The enzyme was the Sigma grade preparation (specific activity, 69.6 mmoles of acetylcholine hydrolyzed per hour per milligram of protein). Enzyme purified by ammonium sulfate precipitation (specific activity, 17 mmoles/hr/mg of protein) gave the same results. The following concentrations of decamethonium were used: *1*, none; *2*, 5 μM; *3*, 10 μM; *4*, 50 μM; *5*, 100 μM. The noncompetitive inhibition constant, K_i, was obtained from the plot of $(V_{max}/v) - 1$ vs. I and was 9.5 ± 0.4 μM. The coordinates of the lines were obtained using the weighted least-squares program of Wilkinson (19).

relatively high K_d (K_i) for the enzyme-TMBA complex, a direct binding experiment would be prohibitively expensive and technically difficult.

Podleski (21) showed that trimethylbutylammonium ion dipolarizes electroplax at concentrations of 2–10 μM. If the acetylcholinesterase molecule indeed bears a kinetically unrecognizable, receptor-like binding site in addition to the active center anionic subsite, TMBA would be expected to bind to the enzyme in the micromolar concentration region. Attempts to observe such binding to the enzyme at 5 μM trimethylbutylammonium ion were negative. Table 2 shows that TMBA displaces decamethonium from the enzyme only at concentrations above 50 μM. The 40% displacement seen at 100 μM TMBA is qualitatively consistent with the K_A for TMBA from the methanesulfonation acceleration experiment (Table 1).

Figure 5 (inset) shows the result of equilibrium binding of a specific "endo" inhibitor, 3-hydroxyphenyltrimethylammonium ion, to the active site of acetylcholinesterase. One mole of 3-HOPTA is bound per mole of Tetram-titratable sites. The binding is completely abolished by irreversible methanesulfonation of the enzyme esteratic site. In contrast, methanesulfonated acetylcholinesterase still can bind 1 molecule of decamethonium per active site. Considerable uncertainty is involved in the estimation of the stoichiometry and the K_d values of the in-

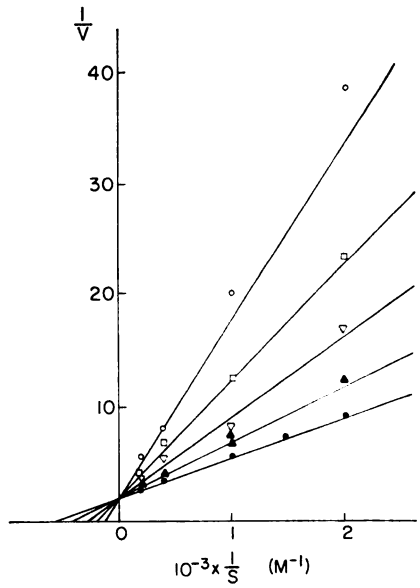

FIG. 4. *Reciprocal plot of results of trimethylbutylammonium inhibition of acetylcholinesterase-catalyzed hydrolysis of phenyl acetate*

Reaction conditions are described in the text. Phenyl acetate was varied from 0.1 to 2 mM. Eel enzyme purified by ammonium sulfate precipitation (specific activity, 19 mmoles of acetylcholine hydrolyzed per hour per milligram of protein) was used. The following concentrations of trimethylbutylammonium iodide were used: ●, none; ▲, 0.1 mM; ▽, 0.25 mM; □, 0.5 mM; ○, 1 mM. The coordinates of the lines were obtained using the weighted least-squares program of Wilkinson (19). The competitive inhibition constant, K_i, is 4.5 ± 0.7 × 10^{-4} M.

TABLE 2

Displacement of decamethonium from acetylcholinesterase by trimethylbutylammonium ion

Equilibrium binding of [^{3}H]decamethonium to soluble acetylcholinesterase (Worthington grade ECHP; specific activity, 64.7 mmoles of acetylcholine hydrolyzed per hour per milligram of protein) was assayed in the presence of increasing concentrations of trimethylbutylammonium iodide. The equilibrium concentration of decamethonium was 3 μM. Approximately 1.39 mg of protein per milliliter were used in the binding assay. Each value represents the average of two experiments.

TMBA	Decamethonium bound
M	%
0	100
1×10^{-6}	109
5×10^{-6}	94.8
1×10^{-5}	90.0
5×10^{-5}	93.0
1×10^{-4}	60.6

hibited enzyme, since no direct measure of the degree of denaturation that could occur during the inhibition of the enzyme can be made. Nevertheless, from the number of moles of decamethonium bound per milligram of protein (7.8×10^{-10}), as obtained from the Scatchard plot in Fig. 5, and from the knowledge of the concentration of active sites per milligram of uninhibited enzyme preparation (9.1×10^{-10}), as determined by active site titration prior to methanesulfonation, it is calculated that 0.86 mole of the ligand is bound per mole of methanesulfonated enzyme.

These results substantiate the "exo" mode of decamethonium binding, as first proposed by Belleau and DiTullio (7), and suggest the existence of a peripheral anionic site with which the second cationic head of decamethonium presumably interacts.

The partially competitive character of inhibition of acetylcholine hydrolysis by decamethonium (Fig. 6), and the complete displacement of this drug from enzyme by 3-HOPTA (Table 3), indicate that decamethonium does not bind independently to the peripheral site, but rather bridges it with the anionic subsite of the active center as depicted by Belleau and DiTullio (7).

As has been noted, whereas TMBA is a competitive inhibitor, decamethonium is noncompetitive with respect to phenyl acetate but is mixed with respect to acetylcholine. The competitive component with charged substrates is easily understood, since both the substrate and the inhibitor compete for the same anionic binding site; the noncompetitive component is presumably

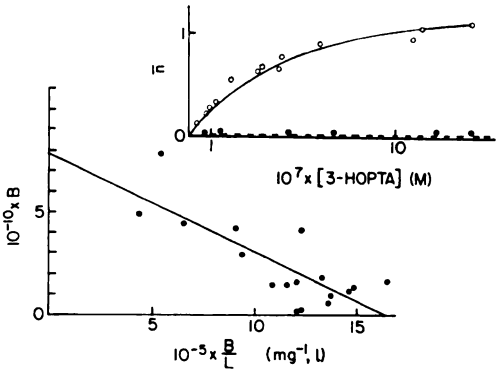

FIG. 5. *Scatchard plot of binding of decamethonium to methanesulfonated acetylcholinesterase*

The inset shows binding of 3-HOPTA to the active and methanesulfonated enzyme. In binding assays with 3-HOPTA, acetylcholinesterase (Worthington grade ECHP; specific activity, 70 mmoles of acetylcholine hydrolyzed per hour per milligram of protein) was used. The concentration of the enzyme active sites varied from 0.1 to 5 μN. Acetylcholinesterase (Sigma grade V; specific activity, 70 mmoles of acetylcholine hydrolyzed per hour per milligram of protein) was used in the decamethonium binding experiments. The concentration of active sites in the uninhibited preparation was 0.91 nmole/mg of protein. The protein concentration of the methanesulfonated preparation used in the binding assay was 2-3 mg/ml. ○, binding to active preparation; ●, binding to methanesulfonated enzyme. $\bar{n}$ is the number of moles of 3-HOPTA bound per mole of active sites of acetylcholinesterase; B is moles of decamethonium bound per milligram of protein; and L is the equilibrium concentration of free decamethonium. The solid lines were computed by the least-squares method from the experimental points. The dissociation constant, K_d, for the 3-HOPTA–acetylcholinesterase complex is $2.5 \pm 0.2 \times 10^{-7}$ M, and n is 1.1 ± 0.03. The K_d for the decamethonium–methanesulfonated enzyme complex is 4.7 ± 0.9 μM, and $B = 7.8 \pm 1.1 \times 10^{-10}$ mole/mg of protein, or approximately 0.86 mole of ligand bound per mole of active sites.

mechanistically of the same nature for both charged and neutral substrates. In general, noncompetitive inhibition can be explained as due either to irreversible inactivation of enzyme or to an effect on the rate-limiting step. Since no progressive inactivation of the enzyme under the experimental conditions used was observed, the second explanation must be considered. It has been demonstrated by Krupka (23) that certain quaternary nitrogen ions, such as tetraethylammonium ion, can form a ternary complex with the acyl-acetylcholinesterase, resulting in a decreased rate of deacylation, k_3. Two modes of binding of the effectors which have an effect on the velocity of the acetylcholinesterase-catalyzed reactions can be considered: (a) interaction of a quaternary nitrogen group with the peripheral anionic site allosterically affects the active site in such a way that the rate-limiting step is either altered or less efficient; (b) the rate-limiting deacylation is affected simply by binding of the effector molecule to the anionic subsite of the active center of the acyl-enzyme.

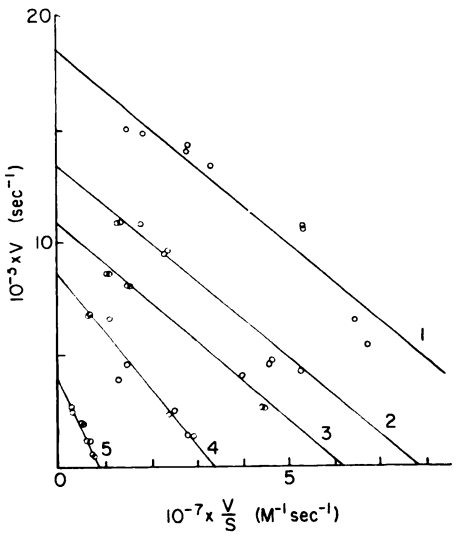

FIG. 6. *Decamethonium inhibition of acetylcholinesterase-catalyzed hydrolysis of acetylcholine*

The experiments were done at 26° in eel Ringer's solution, pH 7.0. The data were plotted according to the method of Eadie (22). The acetylcholine concentrations were varied from 0.05 to 2 mM. Acetylcholinesterase (Sigma Grade V; specific activity, 70 mmoles of acetylcholine hydrolyzed per hour per milligram of protein) was used. The following concentrations of decamethonium were used: curve *1*, none; *2*, 2.5 μM; *3*, 5 μM; *4*, 10 μM; *5*, 50 μM. The coordinates of the lines were obtained using the weighted least-squares program of Wilkinson (19). The noncompetitive inhibition constant was calculated from the y intercepts for values where the slope does not change. The apparent K_i value thus obtained is 6 μM. The competitive apparent K_i value is 10 μM, as calculated from the change in slopes.

TABLE 3

Displacement of decamethonium from acetylcholinesterase by 3-hydroxyphenyltrimethylammonium ion

Equilibrium binding of [^{3}H]decamethonium to soluble acetylcholinesterase (Sigma grade V; specific activity, 69.9 mmoles of acetylcholine hydrolyzed per hour per milligram of protein) was assayed in the presence of increasing concentrations of 3-hydroxyphenyltrimethylammonium iodide. The equilibrium concentration of decamethonium was 1.2 μM. The concentration of protein used in the binding assay was 1.35 mg/ml.

3-HOPTA	Decamethonium bound
μM	%
0	100
0.5	35.6
1.0	16.6
2.5	16.6
5.0	0.0

Although in both cases the formation of a ternary complex is implicit, the important difference is in the mode of binding of a modifier to the enzyme. In case (a), allosteric properties are implied; in case (b), a localized conformational change of the active site region, whether or not large enough to be transmitted over the whole protein, is sufficient to explain the kinetically observable modifications of acetylcholinesterase activity. These requirements are also met by decamethonium, presumably by the virtue of its binding "away" from the esteratic site. By contrast, trimethylbutylammonium ion is a purely competitive inhibitor, even with the uncharged phenyl acetate, indicating that it must sterically hinder the former from forming an *ES* complex. It appears that this partially "endo" binding may be also responsible for reducing the rate of methanesulfonation relative to that observed

with decamethonium. It could be argued that competitive inhibition against phenyl acetate may be related to the bulk of the substrate rather than to the partially "endo" binding of TMBA. However, were this the case, there should be no reason why TMBA should not form a ternary complex with acetyl-acetylcholinesterase and thus exhibit noncompetitive inhibition. The same argument might also indicate that noncompetitive inhibition (decrease in the rate-limiting deacetylation step, k_3) is unlikely to result from interaction of quaternary nitrogen compounds with the peripheral anionic site. It is difficult to envision why this site should be made inaccessible to the charged TMBA by a neutral substrate, such as phenyl acetate, or by an acetyl group in acetyl-acetylcholinesterase.

CONCLUSION

The monoquaternary trimethylbutylammonium ion and the bisquaternary decamethonium are rather typical cholinergic effectors which activate the acetylcholine receptor and also interact with acetylcholinesterase. The results presented in this report indicate that in physiological eel Ringer's solution, pH 7, the effects of these drugs on kinetic parameters of acetylcholinesterase result from their interaction with the anionic subsite of the catalytic center. The observed differences between decamethonium and TMBA in acceleration of the methanesulfonation reaction are consistent with the different effects of these drugs on the catalytic activity of acetylcholinesterase with acetylcholine and phenyl acetate as substrates. These differences are explained as resulting in part (but see also ref. 9) from the orientation of ligands on the enzyme surface. Decamethonium, which binds "away" from the esteratic cleft, is a good accelerator of the methanesulfonation reaction and is capable of binding to acetyl-acetylcholinesterase, as evidenced by its effect on the rate-limiting deacetylation. Trimethylbutylammonium ion seems to bind in partially "endo" fashion. It is thus less efficient in accelerating the methanesulfonation reaction and is incapable of interacting with acetylated enzyme, as evidenced by the purely competitive inhibition it produces.

The function of the peripheral anionic site, apart from its contribution to binding energy and orientation of bisquaternary decamethonium on the enzyme surface, is not apparent.

The above interpretation is not in agreement with the recent report by Jung and Belleau (24) that erythrocyte acetylcholinesterase can be fractionated into subspecies, one of which is inhibited only noncompetitively by decamethonium. This suggests that in that particular enzyme subspecies decamethonium and acetylcholine do not compete for the common binding site in the catalytic center, and consequently the noncompetitive inhibition is probably due to interaction of decamethonium with a peripheral site.

Two cholinergic antagonists, gallamine and curare, affect the methanesulfonation of membrane-bound acetylcholinesterase at concentrations at least two orders of magnitude higher than those at which physiological response is observed. Preliminary binding studies of di[*methyl*-^{14}C]*d*-tubocurarine, 0.1–5 μM, to soluble acetylcholinesterase in physiological eel Ringer's solution showed negligible interaction of this drug with acetylcholinesterase.[3] Mooser and Sigman (25) have demonstrated that in 5 mM phosphate buffer curare interacts strongly with eel acetylcholinesterase, and Belleau and DiTullio (26) reported affinity labeling of curare-specific site in erythrocyte enzyme which had been dialyzed against water. These results are in agreement with the previous findings of Changeux (2) and Kitz *et al.* (3); however, they may not be relevant to conditions of physiological ionic strength and pH. Crone (27) has recently confirmed that, as with the eel enzyme, the interaction of gallamine and curare with mammalian acetylcholinesterase is strongly dependent on ionic strength. An increase in ionic strength to 0.15, which is comparable to the ionic strength of physiological saline solution, "obliterated all effects of gallamine." This author suggested that the effects of inorganic ions and of gallamine-like compounds may be brought about through a common mechanism.

In summary, in physiological eel Ringer's

[3] Unpublished observations.

solution, pH 7, interaction of the cholinergic agonists decamethonium and trimethylbutylammonium ion with eel acetylcholinesterase does not reveal properties suggestive of allosterism. In view of the demonstrated dependence of the quaternary structure of acetylcholinesterase on ionic strength and pH (28, 29), the physiological significance of results obtained in Ringer's solution, as compared to those obtained at low ionic strength, cannot be assessed fully without knowledge of the ionic microenvironment of the membrane-bound enzyme.

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