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Acetylcholinesterase: Specificity of the Peripheral Anionic Site for Cholinergic Ligands

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SUMMARY

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The activation of erythrocyte acetylcholinesterase by various ligands, including $CaCl_2$, was almost completely and irreversibly blocked by a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), at a peripheral anionic site. $CaCl_2$ either enhanced, antagonized, or had little effect on the inhibition of the unmodified enzyme by cholinergic ligands, depending on the affinity of these ligands for the peripheral anionic site. These effects of $CaCl_2$ were abolished after modification of the peripheral site by EDAC. Whereas both cholinergic agonists and antagonists had affinity for the peripheral site, antagonists (gallamine, pentamethonium, hexamethonium, tetraethylammonium, and atropine) accelerated acetylcholine hydrolysis, while agonists [tetramethylammonium, nicotine, and EDAC (reversible component)] had little effect. Acetylcholine and butyrylcholine inhibited substrate hydrolysis when bound to the peripheral site, but this inhibition accounted for only part of the total substrate inhibition observed, since substrate inhibition of the EDAC-modified enzyme, though delayed, still occurred at high substrate concentrations. Hydrophobic molecules (chlorpromazine and tetracaine) and ligands with structural complementarity to the active site (edrophonium) had little or no affinity for the peripheral anionic site. The peripheral anionic site may be located in a more polar environment than that of the catalytic anionic site. Binding of cholinergic ligands to the peripheral anionic site of acetylcholinesterase appeared to elicit responses parallel to those of cholinoreceptors.

INTRODUCTION

In the past few years acetylcholine catalytic activity has been separated from high-affinity acetylcholine binding activity by both chemical (1) and physical (2-4) methods, and it appears that catalytic and receptor activities may be located in different membrane fractions (5). However, other evidence indicates that occupation of cholinergic receptors alters allosteric properties of acetylcholinesterase, in a manner consistent with the pharmacological differentiation of the tissue toward cholinergic ligands (6, 7). Recent work indicates that acetylcholinesterase from a number of sources has at least one peripheral anionic site (8-11), but the physiological significance of this site in regulation of acetylcholinesterase or cholinergic receptor activity

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remains obscure. Changes in catalytic activity of acetylcholinesterase may reflect a conformational response relevant to the receptor mechanism (12).

In this study we undertook an investigation of the specificity of peripheral sites on bovine erythrocyte acetylcholinesterase for a variety of cholinergic ligands. A peripheral anionic site involved in activation of acetylcholine hydrolysis has been shown in our laboratory to be selectively blocked by a water-soluble carbodiimide (13) and to be sensitive to low concentrations of $CaCl_2$. Modification of the peripheral site, both reversibly and irreversibly, allowed determination of the affinity and consequence of the interaction of cholinergic ligands with this site. Elucidation of molecular aspects of the excitation and inhibition of cholinoreceptive proteins by such agents are of interest in view of the possible regulatory roles of anionic sites in these proteins (14).

MATERIALS AND METHODS

Enzyme Assay

Acetylcholinesterase was a partially purified bovine erythrocyte preparation from Sigma, with a specific activity of 150 μmoles of acetylcholine per hour per milligram of protein. A pH-stat titrimetric assay procedure was used as described previously (11). Control assays in a final volume of 40 ml were performed in the absence of added inorganic salts, since the salts commonly employed in the assay of this enzyme have specific effects at the peripheral site on the enzyme (14, 15) and interfere with identification of binding of cholinergic ligands to this site. The ionic composition of the medium was less than 10 μM with respect to Na^+ and was not appreciably changed during the titrimetric assay (16).

EDAC[2] Modification

Modification of acetylcholinesterase by EDAC, a water-soluble carbodiimide, was achieved essentially as previously described (13). Specifically, a solution of the enzyme in distilled water (1 mg/ml) was allowed to react with 2 mM EDAC for 7.5 hr at pH 7.4 and 25°, until $CaCl_2$ activation of the hydrolysis of 0.4 mM acetylcholine was abolished. After 18–24 hr of dialysis against 1 mM sodium phosphate buffer, pH 7.4, the enzyme was frozen in aliquots and used as required.

[2]The abbreviations used are: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; TEA, tetraethylammonium iodide; TMA, tetramethylammonium iodide.

Chemicals

The following compounds were used as received: acetylcholine perchlorate, atropine sulfate, and procaine hydrochloride (British Drug Houses); tetramethylammonium iodide and tetraethylammonium iodide (Baker Chemical Company); hexamethonium chloride (Matheson, Coleman, and Bell); decamethonium bromide and *d*-tubocurarine chloride (K & K Laboratories, Inc.); and butyrylcholine iodide, EDAC, and CMC (Sigma Chemical Company). Inorganic salts were analytical reagent grade. The following gifts are gratefully acknowledged: gallamine triethiodide (Poulenc, Ltd.); edrophonium chloride (Hoffmann-La Roche), and pentamethonium bromide (Dr. B. Belleau, University of Ottawa). Nicotine base (British Drug Houses) was redistilled. Tetracaine hydrochloride (K & K Laboratories, Inc.) was recrystallized from ethanol–ether, m.p. 147–148°.

RESULTS

Specificity of EDAC Modification

Table 1 shows that activation of the maximum velocity of acetylcholine hydrolysis by $CaCl_2$, gallamine, TEA, and hexamethonium was decreased 85–95% after EDAC modification. Thus the ligands probably activate by interaction at the same peripheral anionic site, previously shown to be a carboxyl group(s) (13). Under these conditions only around 7% of the basal catalytic activity toward acetylcholine was inhibited by EDAC.

Substrate Hydrolysis by EDAC-Modified Enzyme

Figure 1A shows the hydrolysis of acetylcholine by free and EDAC-modified acetylcholinesterase in the absence of added

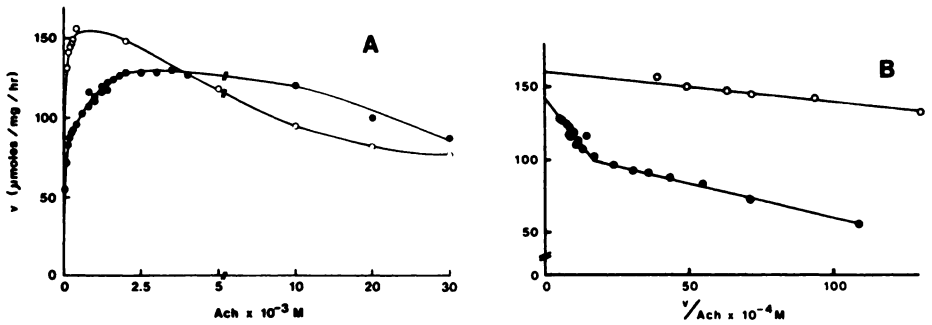

FIG. 1. *Hydrolysis of acetylcholine (Ach) at low ionic strength (A) and Eadie plots at suboptimum substrate concentrations (B)*

O——O, free enzyme; ●——●, EDAC-modified enzyme.

salts. Biphasic dependence at suboptimal substrate concentrations was obtained after EDAC modification. Eadie plots (Fig. 1B) exhibited two slopes, corresponding to high-affinity [K_m(app) = 48.3 μM, V_{max} = 102 μmoles/mg/hr] and low-affinity [K_m(app) = 225 μM, V_{max} = 140 μmoles/mg/hr] catalysis. In contrast, a linear plot was obtained for the free enzyme in this concentration range, but lower substrate concentrations, which showed a deviation from linearity (16), were not investigated. A single band of activity of the EDAC-modified enzyme on 5% polyacrylamide gel in 5 mM Tris-glycine buffer (pH 8.6) and a single elution peak on Sepharose 6B[3] suggested nearly complete modification of the enzyme by EDAC to a single molecular species. Substrate inhibition was delayed from 0.4 mM in the free enzyme to 1.5–3.5 mM after EDAC modification.

Influence of $CaCl_2$ on Ligand Binding

Depending on their affinity for the peripheral anionic site, inhibition of the enzyme by ligands in the presence of $CaCl_2$ was either enhanced, antagonized, or largely unchanged.

Enhancement of ligand inhibition. Inhibition of acetylcholine hydrolysis by some ligands was enhanced on addition of $CaCl_2$ to the reaction medium. Figure 2 shows typical results obtained with pentamethonium (Fig. 2A) and atropine (Fig. 2B), where the curves for ligand inhibition were shifted toward lower concentrations in the presence of 0.2 and 1 mM $CaCl_2$. Similar shifts were found for ligands listed in groups 1 and 2 in Table 2. The increase in inhibitory potency in the presence of $CaCl_2$ resulted from the high affinity of the ligands for the peripheral anionic site and their consequent inhibition of $CaCl_2$ activation of catalytic activity at lower concentrations than required to inhibit acetylcholine hydrolysis at the catalytic anionic site. At higher $CaCl_2$ concentrations (5 mM)

[3] B. D. Roufogalis and G. Beauregard, unpublished observations.

TABLE 1

Effect of various ligands on maximum velocity of acetylcholine hydrolysis after EDAC modification

EDAC modification was carried out as described in MATERIALS AND METHODS. Maximum velocity was determined from plots of v vs. $v/[S]$ with and without the ligand. The acetylcholine concentration was varied from 0.05 to 0.4 mM for the free and from 0.2 to 2 mM for the EDAC-modified enzyme. The two concentrations of ligand shown gave nearly maximum activation of the free and EDAC-modified enzyme, respectively.

Ligand	Concentration	Increase in maximum velocity	
		Control	EDAC-modified
	mM	%	%
$CaCl_2$	1.0	98	0
	100		6
Gallamine	0.02	72	1.3
	0.2		14
TEA	1.0	51	4
	10.0		9
Hexamethonium	0.2	68	8
	1.0		15

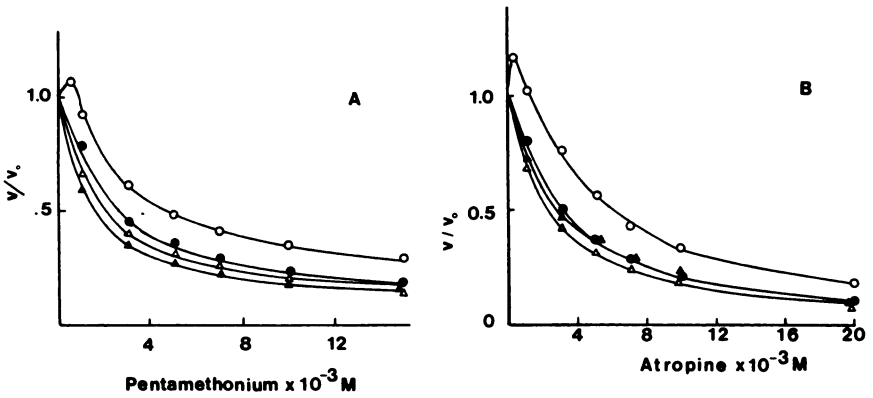

FIG. 2. *Effect of $CaCl_2$ on inhibition of acetylcholinesterase by pentamethonium (A) and atropine (B)*

v/v_0 is the fraction of remaining enzyme activity, where v and v_0 are velocity in the presence and absence of ligand, at 0.4 mM acetylcholine. O——O, no $CaCl_2$; ▲——▲, 0.2 mM $CaCl_2$; Δ——Δ, 1 mM; ●——●, 5 mM.

more ligand was required to displace $CaCl_2$ from the peripheral site, and the shift to lower concentrations was diminished or even reversed (Fig. 2 and Table 2). Compounds listed in group 1 accelerated the maximum velocity of hydrolysis when bound to the peripheral site, while compounds in group 2 had little effect.

As predicted, 0.1 mM TEA, which like $CaCl_2$ activates acetylcholine hydrolysis at the peripheral site (13) (Table 1), also shifted the hexamethonium inhibition curve to lower concentrations (Fig. 3). Results obtained with the EDAC-modified enzyme further supported this interpretation of the data. After irreversible EDAC modification of the peripheral site, $CaCl_2$ no longer enhanced inhibition by ligands in groups 1 and 2 of Table 2. Figure 4 illustrates this with hexamethonium; inhibition of the EDAC-modified enzyme was unaffected by 0.2 and 1 mM $CaCl_2$ and was slightly antagonized by 5 mM $CaCl_2$.

No effect on ligand inhibition. Inhibition by edrophonium [ethyl(3-hydroxyphenyl)-dimethylammonium chloride] was not significantly altered by activator concentrations of $CaCl_2$ (Fig. 5A), indicating that it does not bind to the peripheral anionic site at these concentrations. Linear Dixon plots of edrophonium inhibition (Fig. 5B) are consistent with exclusive inhibition at the catalytic anionic site. The convergence of the plots on the abscissa with increasing $CaCl_2$ concentrations is also consistent

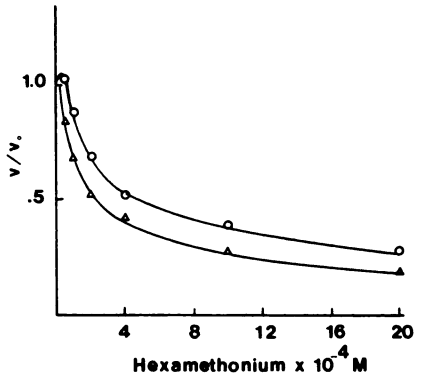

FIG. 3. *Effect of TEA on inhibition of acetylcholinesterase by hexamethonium*

v/v_0 is defined in Fig. 2; v_0 is velocity in the absence of TEA (O——O) or in the presence of 0.1 mM TEA (Δ——Δ).

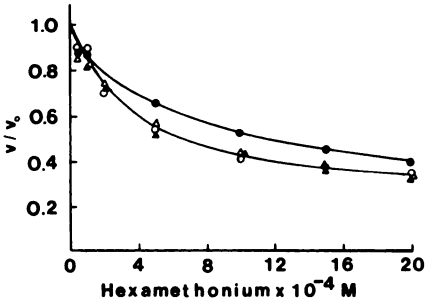

FIG. 4. *Effect of $CaCl_2$ on inhibition of EDAC-modified acetylcholinesterase by hexamethonium*

v/v_0 is defined in Fig. 2. The EDAC-modified enzyme was prepared as described in MATERIALS AND METHODS and assayed with 0.4 mM acetylcholine. O——O, no $CaCl_2$; ▲——▲, 0.2 mM $CaCl_2$; Δ——Δ, 1 mM; ●——●, 5 mM.

TABLE 2

Effect of $CaCl_2$ on I_{50} for ligand inhibition of acetylcholinesterase activity

I_{50} values were determined from plots of v/v_0 vs. ligand concentration, similar to those shown in Figs. 2–5. The acetylcholine concentration was 0.4 mM.

Ligand	I_{50} in presence of $CaCl_2$			
	0 mM	0.2 mM	1.0 mM	5.0 mM
		M × 10^5		
Group 1				
Gallamine	9.5	5.3[a]	5.3	12.6
Pentamethonium	452	200	155	260
Hexamethonium	46.6	25.3	25.3	55.3
TEA	355	270		
Atropine	600	293	227	308
Group 2				
TMA	1060	485		
Nicotine	38.0	29.6	29.6	33.3
CMC	132	65		
EDAC	11	7.7	7.7	
Group 3				
Acetylcholine[b]	650[b]	470[b]		640[b]
Butyrylcholine	17.5	19.0		27.5
Group 4				
Procaine[c]	25.5	25.5	25.5	
Decamethonium[d]	0.0348	0.0469	>0.1	
d-Tubocurarine[e]	1.82	1.5	2.95	
Tetracaine	0.7	1.2		
Chlorpromazine[f]	3.59	4.89	5.10	6.40
Edrophonium	0.152	0.170	0.170	0.155

[a] In the presence of 0.4 mM $CaCl_2$.

[b] Substrate inhibition. Values refer to I_{50} concentrations.

[c] The curves diverge at the I_{50} value. At lower ligand concentrations there is slight potentiation by $CaCl_2$, and at higher values there is slight antagonism.

[d] Substrate concentration was 0.5 mM.

[e] *d*-Tubocurarine has been placed in this group since, like decamethonium and unlike compounds in group 1, it decreases the maximum velocity of acetylcholine hydrolysis and its inhibition is antagonized by 1 mM $CaCl_2$.

[f] Reaction in darkened room to avoid free radical formation.

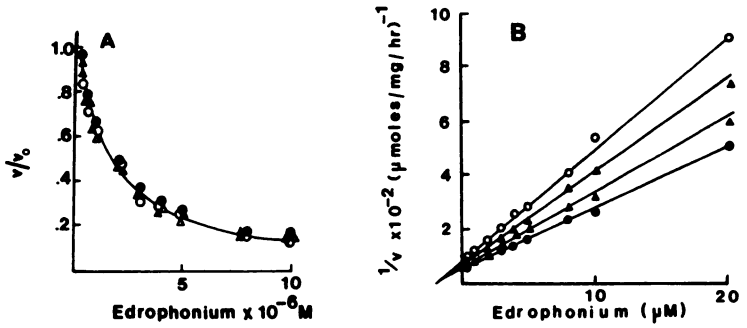

FIG. 5. *Effect of $CaCl_2$ on inhibition of acetylcholinesterase by edrophonium (A) and Dixon plots at various $CaCl_2$ concentrations (B).*

v/v_0 is defined in Fig. 2, at 0.4 mM acetylcholine. O——O, no $CaCl_2$; ▲——▲, 0.04 mM $CaCl_2$; Δ——Δ, 0.2 mM; ●——●, 1 mM.

with independent binding sites of edrophonium inhibition and $CaCl_2$ activation.

Antagonism of ligand inhibition. Our previous study showed antagonism of decamethonium inhibition by low concentrations of $CaCl_2$, most probably as a result of interference with binding of the second quaternary ammonium group of decamethonium to the peripheral anionic site (11), and this was confirmed by Mooser *et al.* (17). As expected, EDAC modification also antagonized decamethonium inhibition (Fig. 6). The K_i for decamethonium inhibition was increased 10-fold, from 0.045 to 0.44 μM, after modification of the peripheral site, and the kinetics of inhibition changed from linear Dixon plots (Fig. 6A) to hyperbolae (Fig. 6B).

The inhibition of acetylcholine hydrolysis by compounds listed in group 4 (Table 2) was also slightly antagonized by $CaCl_2$. The compounds antagonized include chlorpromazine, butyrylcholine, procaine, and tetracaine. Figure 7A shows that the upward-curving Dixon plot for butyrylcholine inhibition became linear in the presence of 5 mM $CaCl_2$ or after EDAC modification indicating that the upward curvature was due to butyrylcholine inhibition at the peripheral site. Antagonism of butyrylcholine inhibition by $CaCl_2$ occurred only at higher butyrylcholine concentrations, where upward curvature was noted (Fig. 7B). Thus butyrylcholine, like acetylcholine, has greater affinity for the catalytic anionic site than for the peripheral site.

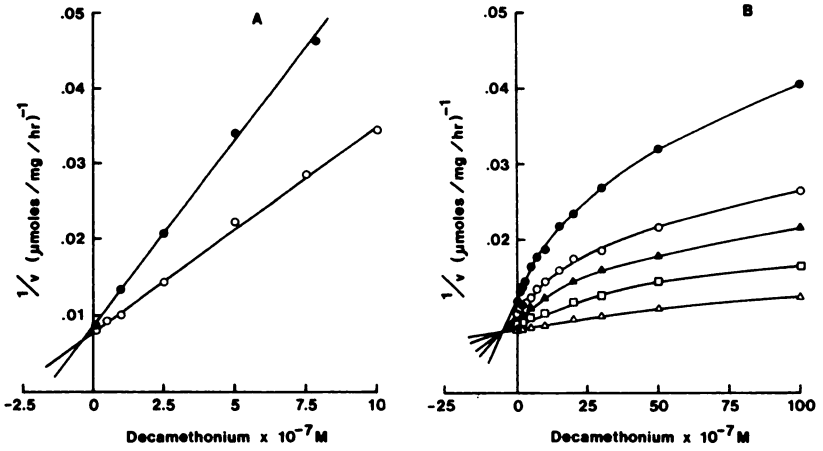

FIG. 6. *Dixon plots for decamethonium inhibition of free (A) and EDAC-modified (B) acetylcholinesterase*
In A the acetylcholine concentration was 0.1 mM (●——●) or 0.5 mM (○——○). In B the acetylcholine concentration was 0.2 mM (●——●), 0.4 mM (○——○), 0.6 mM (▲——▲), 1.0 mM (□——□), or 2.0 mM (△——△).

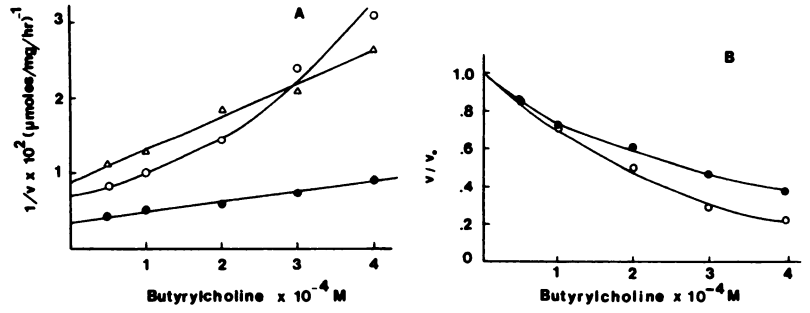

FIG. 7. *Butyrylcholine inhibition of free and EDAC-modified acetylcholinesterase*
No detectable butyrylcholine hydrolysis occurred, and the acetylcholine concentration was 0.4 mM in all experiments. A. ○——○, free enzyme in the absence of $CaCl_2$; ●——●, free enzyme in the presence of 5 mM $CaCl_2$; △——△, EDAC-modified enzyme, no $CaCl_2$. B. Free enzyme, ○——○, in the absence of $CaCl_2$; ●——●, in the presence of 5 mM $CaCl_2$.

Substrate Inhibition

Substrate inhibition of the unmodified enzyme was enhanced in the presence of $CaCl_2$ (0.2–5 mM) or 0.1 mM TEA (Fig. 8A and Table 2). As discussed above, this indicates that at above optimum concentrations, acetylcholine has affinity for the peripheral anionic site. Analysis of substrate inhibition in the absence of $CaCl_2$ according to Brestkin *et al.* (18) indicates two substrate inhibition sites (not shown). After EDAC modification of the peripheral site substrate inhibition was delayed from 0.4 mM in the free enzyme to 1.5–3.5 mM, but still occurred (Fig. 1). Thus loss of the peripheral anionic site abolished only one component of the substrate inhibition, that occurring with higher affinity. The second site of inhibition is probably the catalytic anionic site of the acetylated enzyme intermediate (19), since after EDAC modification of the enzyme the Dixon plot of substrate inhibition was linear, the addition of $CaCl_2$ did not enhance the inhibition, and the inhibition was antagonized by agents known to act at the catalytic anionic site (e.g., edrophonium) (results not shown).

DISCUSSION

The finding that the irreversible interaction between carboxyl groups of acetylcholinesterase and EDAC, a water-soluble carbodiimide, blocks activation of the enzyme by a variety of ligands without blocking acetylcholine hydrolysis to a significant extent has provided a valuable tool for investigation of the specificity of the peripheral site toward various cholinergic ligands. Since the stoichiometry of the reaction with EDAC is unknown, the possibility of nonuniform carbodiimide labeling may complicate the interpretation of the data. Although investigation of the interactions between both $CaCl_2$ and EDAC with the binding of various cholinergic ligands to bovine erythrocyte acetylcholinesterase has allowed the determination of the specificity of the catalytic and peripheral sites for these ligands, the chemical organization and homogeneity of these sites remain unclear at present. The ligands have been placed into four groups (Table 2). Those in groups 1 and 2 have higher affinity for the peripheral anionic site than for the catalytic anionic site. Ligands in group 1 accelerate acetylcholine hydrolysis when bound to the peripheral site, whereas ligands in group 2 have little effect when bound. Ligands in group 3 have greater affinity for the catalytic anionic site, being substrates or substrate analogues, and inhibit catalytic activity when bound to the peripheral anionic site. This inhibition accounts for part of the substrate inhibition observed with acetylcholine. Ligands in group 4 have little or no affinity for the peripheral anionic site.

No simple correlation between affinity for the peripheral anionic site and pharmacological properties of the ligands was found among this group. The ligands with affinity for the peripheral site include nicotinic agonists (nicotine, TMA) and antagonists (hexamethonium, pentamethonium, TEA), muscarinic antagonists (atropine),

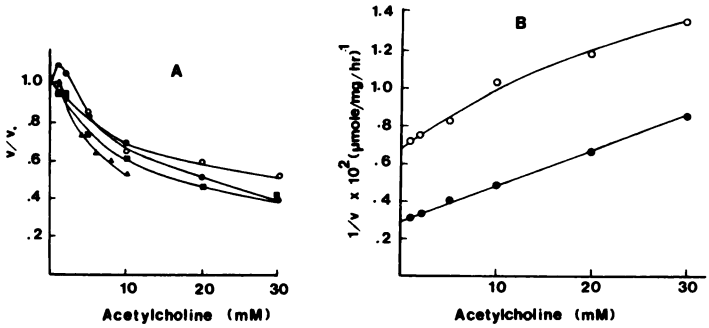

FIG. 8. *Effect of $CaCl_2$ on substrate inhibition of acetylcholinesterase by acetylcholine*

The velocities are corrected for nonenzymatic hydrolysis at high substrate concentrations. A. v/v_0 is defined in Fig. 2. O——O, in the absence of $CaCl_2$; Δ——Δ, 0.2 mM $CaCl_2$; ■——■, 0.1 mM TEA. B. O——O, in the absence of $CaCl_2$; ●——●, 5 mM $CaCl_2$.

and neuromuscular blocking agents (gallamine), as well as inorganic salts ($CaCl_2$, $MgCl_2$, NaCl) and compounds such as the water-soluble carbodiimides (EDAC, CMC). However, depolarizing agents (acetylcholine, TMA, nicotine, and decamethonium) do not stimulate acetylcholine hydrolysis when bound to the peripheral site, whereas receptor antagonists, with the exception of *d*-tubocurarine,[4] increase the maximum velocity of acetylcholine hydrolysis when bound. Thus the dynamic changes in catalytic activity of acetylcholinesterase resulting from the interaction of ligands with the peripheral site appear to correlate with the pharmacodynamic *efficacies* of the ligands at the receptor level.

It appears that the peripheral regulatory site consists of one or more carboxyl groups (13) in a more polar environment than that of the catalytic anionic site(s). Thus the ligands that bind to the peripheral site are relatively polar cationic salts ($CaCl_2$, TEA, hexamethonium), some of which are attached to an aromatic ring (atropine, gallamine) which may bind to an accessory site (10, 20). Compounds with low affinity for the peripheral site are generally highly hydrophobic (chlorpromazine, tetracaine) or have groups which specifically direct the molecule to the catalytic anionic site, as in the case of the *m*-hydroxy group in edrophonium (21) or the ester group in butyrylcholine (12). These data suggest that the peripheral site is readily accessible to polar ligands and is localized near the protein surface or in a highly solvated cavity. Conversely, the catalytic site(s) must be either situated in a nonpolar environment near the surface (22) or buried in a nonpolar region. It is pertinent that the binding of the quaternary ammonium head of acetylcholine to the catalytic anionic site involves hydrophobic as well as ionic interactions (23). Further supporting a difference in polarity, water-soluble carbodiimides selectively inhibit the peripheral anionic site without blocking substrate binding to the catalytic anionic site, whereas hydrophobic carboxyl group reagents such as dicyclohexylcarbodiimide and *N*-ethoxycarbonyl-2-2-ethoxy-1,2-dihydroquinoline are potent inhibitors of the catalytic anionic site (13).

Biphasic nonlinear double-reciprocal plots similar to those observed for substrate hydrolysis by the EDAC-modified enzyme in Fig. 1 were recently observed by Désiré *et al.* (16) with the free enzyme studied at very low substrate concentrations at low ionic strength. This biphasic dependence was lost in the presence of inorganic salts. Those authors suggested that the results could be explained by substrate activation at a peripheral site. Since we observed a similar nonlinear reciprocal plot for substrate hydrolysis after modification of the peripheral site by EDAC (Fig. 1), substrate activation, if it occurs, cannot take place by binding to the peripheral site sensitive to $CaCl_2$ and other salts under investigation here. Acetylcholine inhibits hydrolysis when bound to the carbodiimide-sensitive peripheral site, since substrate inhibition is much delayed when the peripheral site is modified and butyrylcholine inhibition of the free enzyme has been shown to occur at the peripheral site as well as the catalytic site. Evidence from our laboratory suggests rather that the biphasic response in the absence of salts is due to the ligand-dependent interconversion of two previously identified conformational states of the enzyme (24), while in the presence of salts one form of the enzyme predominates (24). Recent kinetic studies on the EDAC-modified enzyme support this suggestion.[5]

Finally, it is tempting to speculate on the possible mechanisms by which ligands modulate the altered reactivity of the esteratic site when bound at the peripheral site and the possible relationship of this dynamic property to the excitation of cholinoreceptive proteins regulating ion flow (25). The peripheral anionic site exhibits structural specificity, as discussed above. Activation of the esteratic site requires a

[4] In the enzyme from *Electrophorus electricus* both *d*-tubocurarine and gallamine accelerate deacylation (9), whereas in the bovine erythrocyte enzyme the former agent does not accelerate. It is possible that differences in specificity of the peripheral sites of enzymes from different tissues may be related to differences in pharmacological specialization of those tissues, but evidence for this is not available.

[5] B. D. Roufogalis and V. M. Wickson, manuscript in preparation.

quaternary ammonium group of certain geometry. A striking selectivity for activation of esteratic site activity has been demonstrated with the relatively rigid spiran quaternary ammonium compounds, where activation of esteratic site activity is highly dependent on the position of methyl groups on the ring (26, 27). Belleau and DiTullio (28) suggested that compensation between enthalpy and entropy of interaction of a variety of compounds with the erythrocyte enzyme may be related to differences in molar volumes and flexibilities of the TMA substituents, which effect changes in the structure of enzyme-bound water. The possibility that changes in the structure of water may be the determinant in the regulation of the protein structure responsible for increased esteratic site reactivity is attractive. It is of particular interest that the peripheral anionic site of acetylcholinesterase selectively recognizes the same cholinergic ligands recognized by cholinergic receptors, and undergoes changes in response parallel to those found in those receptors. Thus similar principles may regulate the conformational response of both proteins. However, the possible physiological role of the peripheral site of acetylcholinesterase resulting from its unique structure and topography remains to be determined.

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