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Membrane-Bound Acetylcholinesterase: Comparison of Enzymes in Sarcoplasmic Reticulum and Sarcolemma from Striated Muscle

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SUMMARY

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Membrane fractions of sarcolemma and sarcoplasmic reticulum prepared from rat diaphragm contain membrane-bound acetylcholinesterase and butyrylcholinesterase. The specific activity of sarcoplasmic reticulum acetylcholinesterase is 4-fold greater than that of sarcolemma, while their butyrylcholinesterase activities are comparable. The innervated portion of the sarcolemma shows enrichment of acetylcholinesterase, while the noninnervated portion shows enriched butyrylcholinesterase activity. Treatment of membranes with solubilizing agents gave selective solubilization of sarcolemma acetylcholinesterase by collagenase or by 0.5% Triton X-100 in 1 M NaCl, and of sarcoplasmic reticulum acetylcholinesterase by EDTA. Solubilization extracted 70% of the total membrane acetylcholinesterase, with a 2-6-fold increase in specific activity. Membrane-bound acetylcholinesterases were inhibited by Ca^{2+}, Mg^{2+}, and NaCl. Inhibition by Mg^{2+} was dependent on the substrate concentration and occurred in both sarcolemma and sarcoplasmic reticulum at substrate concentrations below 0.5 mM. Membrane acetylcholinesterase was activated by Mg^{2+} only in sarcolemma membranes when the substrate was above 0.7 mM. The properties of membrane-bound acetylcholinesterase are different from those of the soluble enzyme and also vary with respect to membrane type.

INTRODUCTION

Striated muscle contains considerable cholinesterase activity, exclusive of the very high activity associated with the neuromuscular junction. Acetylcholinesterases have been demonstrated by histochemical methods in the sarcolemma beyond the end plate (1) and in the sarcoplasmic reticulum (2). The occurrence of acetylcholinesterase in both types of muscle membrane has been confirmed by biochemical assays on isolated, purified membrane fractions (3, 4). In both instances the enzyme appears to be firmly attached and may constitute an integral part of the membrane structure. In this respect it is quite different from soluble isoenzymes present in muscle homogenates (5), as well as the acetylcholinesterase associated with myosin (6). The membrane-associated acetylcholinesterases may therefore represent specific isoenzymes which have unique distributions, catalytic properties, and biological half-lives. The significance of specific isoenzymes

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is indicated by changes in muscle cholinesterases associated with denervation and dystrophic muscle atrophy (7–9).

The allosteric nature of acetylcholinesterase has been investigated in a number of recent studies (10–14), which demonstrated that partially purified enzymes from erythrocytes and *Electrophorus* contain a cholinergic drug-binding site which modulates the activity of the catalytic center. In view of the often vigorous treatment used to solubilize acetylcholinesterase, it is quite likely that important changes, particularly with regard to allosteric relationships, could be induced during purification. The object of this study is a comparison of membrane-bound acetylcholinesterases with regard to catalytic and also allosteric properties. With this in mind, mild methods of membrane preparation have been utilized to keep the enzyme in its native state as closely as possible.

EXPERIMENTAL PROCEDURE

Membrane preparation. Diaphragm muscles were excised from recently killed male Sprague-Dawley rats (250–350 g) and rinsed in 0.9% NaCl. When required, the end plate region (inner portion) was separated from the noninnervated region by cutting 25% of the muscle away from each edge at right angles to the muscle fibers. All operations were carried out in polypropylene vessels at 4°, and centrifugations were performed at 3000 × *g* for 20 min unless otherwise stated. Diaphragms from three or four rats were first minced and then homogenized in 0.6 M KCl (20 ml) in a Polytron PT-10 homogenizer (Brinkmann Instruments) at setting 2 for 2 min. After centrifugation, the pellet or supernatant fraction was used for preparing sarcolemma or sarcoplasmic reticulum, respectively.

Sarcolemma membrane. Sarcolemma was prepared by a simplification of the method described by Namba and Grob (4), modified to obtain the sarcoplasmic reticulum from the same muscle. The pellet obtained after homogenization as described above was first washed in 20 ml of 0.6 M KCl and centrifuged (wash repeated five times) and then incubated in 20 ml of 0.6 M KCl at 37° for 30 min. The pellet obtained by centrifuging the incubation mixture was washed with six 20-ml aliquots of distilled water and finally stored as a suspension in 2–3 ml of 0.32 M sucrose–5 mM histidine buffer, pH 7.6. The protein concentration was approximately 2 mg/ml, with a yield of 0.7 mg of protein per hemidiaphragm as determined by the method of Lowry *et al.* (15).

Sarcoplasmic reticulum membrane. The supernatant fraction from the homogenization step above was diluted 10-fold with water and stored at 4° for 18 hr. It was then centrifuged at 15,000 × *g*, and the pellet was discarded. The final supernatant fraction was centrifuged at 45,000 × *g*, and the resulting pellet was stored as a suspension in 4–5 ml of 0.32 M sucrose–5 mM histidine buffer, pH 7.6. The yield was 1.1 mg of protein per hemidiaphragm.

Erythrocyte ghosts. These membranes were prepared from human blood by the procedure of Steck *et al.* (16).

Cholinesterase assays. Suspensions of the membrane preparations in 10–20 ml of buffer (approximately 0.2 mg of protein per milliliter) were assayed by the radiometric procedure described previously (17, 18). The final composition of the assay buffer was 0.1 mM Tris-HCl–1 mM histidine HCl–64 mM sucrose, pH 7.6, containing $CaCl_2$, $MgCl_2$, or NaCl at appropriate concentrations when required. The specific substrates DL-[1-^{14}C]-acetyl-β-methylcholine iodide for acetylcholinesterase and [1-^{14}C]butyrylcholine iodide for butyrylcholinesterase were purchased from New England Nuclear Corporation and were used at a final concentration of 5 μM. The use of low substrate concentrations avoids anomalous effects on substrate hydrolysis caused by local pH changes in the vicinity of the membrane-bound enzyme (19). Aliquots of the assay suspension were analyzed by trapping unhydrolyzed choline substrate on a short column of cation exchange resin. Each assay comprised five points and was continued for an additional five points when additions were made to the incubation medium (e.g., 2 mM $CaCl_2$ or 3 μM *d*-tubocurare).

The kinetics of substrate hydrolysis followed a pseudo-first-order rate equation at substrate concentrations well below the K_m.

This assay procedure was convenient and consistent, resulting in a rate constant with units of liters per gram of protein per minute, independent of variations in the substrate concentration below 0.1 mM (20). Specific activities were calculated from the assay rate constant and are expressed as micromoles per minute per gram of protein for a substrate concentration of 0.1 mM.

The soluble acetylcholinesterase used for comparative purposes was a partially purified preparation from *Electrophorus electricus* obtained from Sigma Chemical Company, with an activity of 1000 μM units/mg of protein.

Cholinesterase staining. A portion of the sarcolemma membrane suspension was recovered by suction on a 13-mm cellulose acetate filter (Millipore, 5.0-μ pore size) and fixed for 20 sec in formalin vapor. Cholinesterase staining was done at room temperature by the method of Karnovsky and Roots (21) in a medium containing 2 mM acetylthiocholine iodide, 65 mM sodium phosphate buffer (pH 6.0), 5 mM sodium citrate, 3 mM cupric sulfate, and 0.5 mM potassium ferricyanide. The filter disc was dehydrated in graded concentrations of ethanol, cleared in xylene, and mounted in Canada balsam.

ATPase assay of sarcoplasmic reticulum membrane. Mg^{2+}-activated ATPase was assayed by the procedure of Martonosi and Feretos (22) in a medium containing 100 mM KCl–5 mM histidine buffer (pH 7.4), 4 mM $MgCl_2$, 4 mM ATP, and 0.05 mM $CaCl_2$. After 30 min at 37°, the reaction was stopped with 10% trichloracetic acid, and 0.4 mM ammonium molybdate was added. The phosphomolybdate complex was extracted into butyl acetate (4 ml) and read in a spectrophotometer at 340 nm. ATPase activity was determined from a standard curve, using KH_2PO_4 as standard.

Calcium uptake of sarcoplasmic reticulum membrane. Ca^{2+} uptake was estimated according to the procedure of Martonosi and Feretos (23) on sarcoplasmic reticulum suspensions in the same buffer used for ATPase determination, which also contained 0.1 mM $^{45}CaCl_2$. Aliquots of the incubation medium were filtered by suction on 13-mm cellulose acetate filters (Millipore, 0.45-μ pore size). The filters were dissolved in 2-ethoxyethanol (2 ml), and ^{45}Ca was counted in a liquid scintillation counter. Bound Ca^{2+} was calculated by correcting for radioactivity of the filtrate adhering to the filter disc.

Solubilization of acetylcholinesterase from sarcoplasmic reticulum and sarcolemma membranes. The sarcolemma membrane preparation from two diaphragms (3–4 mg of protein) was treated with 2 ml of the extraction medium (see Table 4) in a shaking incubator for 30 min at 37°. The mixture was centrifuged at 5000 × *g*, and the acetylcholinesterase activity and protein content of the supernatant and pellet were assayed to determine the degree of solubilization and specific activity. Collagenase used on sarcolemma membranes was a preparation obtained from *Clostridium histolyticum* (Sigma type III, fraction A, chromatographically purified) with an activity of 500 units/mg.

Solubilization of sarcoplasmic reticulum membrane-bound acetylcholinesterase was determined similarly, except that the incubation mixture was centrifuged at 100,000 × *g* for 20 min.

RESULTS

The sarcolemma fraction obtained by the method described is a fluffy white suspension which aggregates on standing. The material can be trapped on a cellulose acetate filter and stained for cholinesterase by the method of Karnovsky and Roots (21). Many unbroken end plates are readily identified, together with end plate fragments (Fig. 1).

The preparation of sarcoplasmic reticulum from the same muscle used for the sarcolemma fraction requires the use of 0.6 M KCl. Some properties of sarcoplasmic reticulum obtained by this method have been compared with those of a more conventional preparation (22, 23). There is no significant difference between the two preparations with respect to Ca^{2+} uptake, Mg^{2+}-activated ATPase, or acetylcholinesterase activity (Table 1).

Specific activities of acetyl- and butyrylcholinesterase for the three types of membrane preparation are given in Table 2. The acetylcholinesterase activity of the sarcoplasmic reticulum is about 4 times that of the

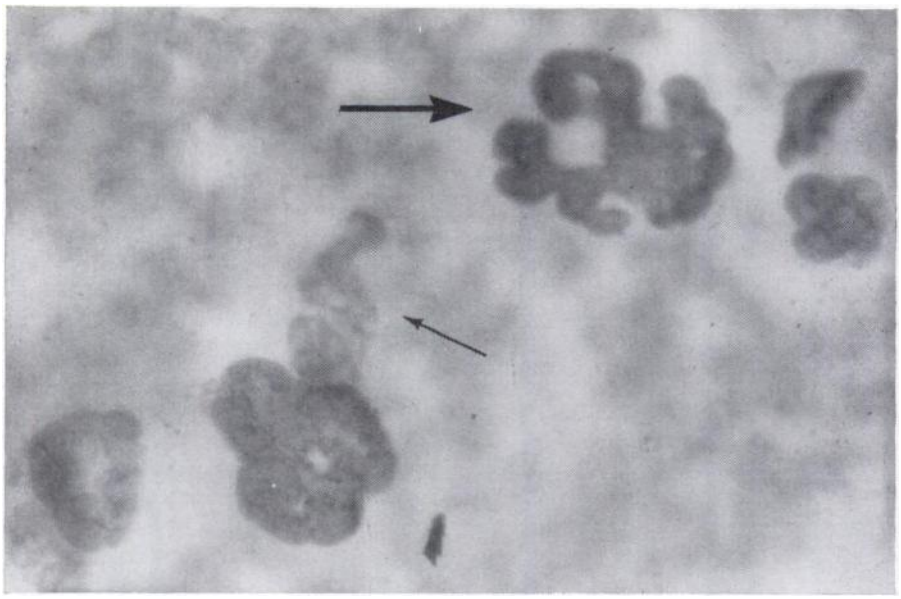

FIG. 1. *Motor end plates in membrane preparation of sarcolemma mounted on a cellulose acetate filter and stained for cholinesterase by the method of Karnovsky and Roots (21)*

Whole end plates (large arrow) and fragments (small arrow) are present. × 1200.

TABLE 1

Mean activities for ATPase, Ca^{2+} uptake, acetylcholinesterase, and butyrylcholinesterase at 30° in sarcoplasmic reticulum

Preparation A was obtained as described under EXPERIMENTAL PROCEDURE; preparation B was that of Martonosi and Feretos (22). Values are are averages of two determinations.

Activity	Preparation A	Preparation B
ATPase[a]	11.5	16.5
Ca^{2+} uptake[b]	6.25	11.7
Acetylcholinesterase	2.4	1.9
Butyrylcholinesterase	3.9	3.0

[a] Units of activity are micromoles of P_i per milligram of protein in 30 min.

[b] Units are micromoles per gram of protein in 30 min.

TABLE 2

Specific activities of acetylcholinesterase and butyrylcholinesterase in sarcolemma, sarcoplasmic reticulum, and human erythrocyte membrane preparations

Results are the means ± standard errors of the number of determinations shown in parentheses, and refer to hydrolysis of acetyl-β-methylcholine (0.1 mM) at 37°.

Membrane	Acetylcholinesterase	Butyrylcholinesterase
	μmoles/min/g protein	
Sarcolemma	1.19 ± 0.3 (5)	3.5 ± 1.85 (3)
Sarcoplasmic reticulum	4.6 ± 0.2 (5)	5.8 ± 0.52 (4)
Erythrocyte	180.0 ± 11.5 (11)	

entire muscle sarcolemma membrane, whereas the butyrylcholinesterase activities are comparable. However, both types of muscle membrane have much lower acetylcholinesterase specific activities than red cell ghosts.

Table 3 compares specific activities of the sarcolemma membrane after separation of the diaphragms into an inner portion, containing most of the end plates, and an outer portion, consisting of noninnervated sarcolemma (24). The ratio of specific activities for the two regions shows the preferential location of acetylcholinesterase in the inner region, presumably associated with end plates. The entire muscle membrane contains a general background of acetylcholinesterase ac-

TABLE 3

Specific activities at 30° of acetylcholinesterase and butyrylcholinesterase in sarcolemma from inner (end plate) and outer (noninnervated) regions of rat hemidiaphragm

Values are the means ± standard errors of five determinations.

Region	Protein yield	Acetylcholinesterase (A)	Butyrylcholinesterase (B)	A:B
	mg/hemidiaphragm	*μmoles/min/g protein*		
Inner	0.31 ± 0.03	1.028 ± 0.06	0.80 ± 0.10	1.44
Outer	0.44 ± 0.05	0.58 ± 0.04	1.37 ± 0.13	0.42
Ratio of inner to outer	0.71	1.84	0.61	

TABLE 4

Degree of solubilization[a] of sarcolemma membrane-bound acetylcholinesterase after various treatments for 30 min at 37°

Values are the averages of two determinations.

Treatment	Specific activity	Distribution of activity
	μmoles/min/g protein	%
Control		
Soluble	0	0
Membrane-bound	1.25	100
Collagenase, 30 units/ml		
Soluble	3.33	70
Membrane-bound	0.56	30
EDTA, 2.5 mM		
Soluble	0	0
Membrane-bound	1.40	100
Triton X-100, 0.5%		
Soluble	0	0
Membrane-bound	1.1	100
Triton X-100, 0.5%, in 1 M NaCl		
Soluble	2.06	67
Membrane-bound	0.28	32

[a] Not sedimented at 5000 × *g*.

TABLE 5

Degree of solubilization[a] of sarcoplasmic reticulum membrane-bound acetylcholinesterase after various treatments for 30 min at 37°

Values are the means of five determinations.

Treatment	Specific activity	Distribution of activity
	μmoles/min/g protein	%
Control		
Soluble	7.88	52
Membrane-bound	3.02	48
Collagenase, 30 units/ml		
Soluble	3.50	68
Membrane-bound	1.89	32
EDTA, 2.5 mM		
Soluble	18.0	64
Membrane-bound	2.48	36
Triton X-100, 0.5%		
Soluble	0.85	18
Membrane-bound	2.70	82

[a] Not sedimented at 100,000 × *g*.

tivity as well as butyrylcholinesterase. The data indicate that the distribution of membrane-bound butyrylcholinesterase is opposite to that of acetylcholinesterase; i.e., the former is preferentially located outside the end plate-containing region.

The stability of the membrane preparation was investigated by storage of the suspensions at 4° for 18 hr, followed by incubation at 37° for 30 min. Enzyme activity is gradually released into the medium from the sarcoplasmic reticulum, but none is released from the sarcolemma (Tables 4 and 5, controls). These results suggest that the modes of attachment of acetylcholinesterase to the various membrane types might be different. Collagenase, EDTA, and 0.5% Triton X-100 (alone and in 1 M NaCl) are all known solubilizing agents for acetylcholinesterase and were investigated on sarcoplasmic reticulum and sarcolemma fractions. Collagenase was most effective on the sarcolemma membrane, releasing 70% of the enzyme activity with a 3-fold increase in specific activity (Table 4). Triton X-100 (0.5%) alone was ineffective,

but Triton X-100 in 1 M NaCl was almost as effective as collagenase. In sarcoplasmic reticulum, collagenase and EDTA increased the release of enzyme somewhat above controls. However, only EDTA brought about a more selective solubilization of acetylcholinesterase, raising the specific activity 6-fold (Table 5). Successive EDTA treatments extracted progressively less enzyme, with decreasing specific activity. About one-third of the sarcoplasmic reticulum-bound acetylcholinesterase could not be extracted by EDTA. In most cases the various treatments of sarcolemma and sarcoplasmic reticulum did not release or destroy occluded enzyme, since recoveries averaged 103 ± 4.5%. The exception to this was Triton X-100 treatment of sarcoplasmic reticulum, where only 60% of the total acetylcholinesterase activity was recovered.

The effects of Ca^{2+}, Mg^{2+}, and NaCl on the membrane-bound acetylcholinesterases was compared with their effects on a partially purified enzyme preparation from *Electrophorus*. All three cations decreased acetylcholinesterase activity of the membrane preparations in a concentration-dependent manner (Table 6). By contrast, the soluble enzyme from *Electrophorus* was activated by all three ions, in agreement with numerous previous observations. Since divalent cations are more effective in altering enzyme activity, the effect of Mg^{2+} was studied as a function of substrate concentration.

Typical double-reciprocal plots for the sarcolemma membrane-bound acetylcholinesterase are shown in Fig. 2 for substrate concentrations ranging from 0.5 to 2 mM. The plot is a straight line in the absence of added $MgCl_2$, which extrapolates to K_m = 2.6 ± 0.38 mM. In the presence of 10 mM $MgCl_2$ the plot is a curve crossing the control line at 690 ± 27 μM. Therefore at substrate concentrations below this value Mg^{2+} inhibits acetylcholinesterase activity, whereas above this level activation is observed.

Results of similar experiments on sarcoplasmic reticulum membranes are shown in Fig. 3. The apparent K_m is 3.9 ± 0.81 mM, and the plot in the presence of 10 mM Mg^{2+} is also a curve, which diverges from the control line at substrate concentrations below 560 ± 220 μM. In this case Mg^{2+} does not affect acetylcholinesterase activity at substrate concentrations above 0.6 mM, whereas inhibition is observed when the substrate is below this value.

TABLE 6

Effects of $CaCl_2$, $MgCl_2$, and NaCl on acetylcholinesterase bound to sarcoplasmic reticulum and sarcolemma membranes and on soluble, partially purified acetylcholinesterase from Electrophorus

Enzyme activities are relative to controls and were assayed with 5 μM acetyl-β-methylcholine.

Added ion	Concentration	Sarcoplasmic reticulum[a]	Sarcolemma[a]	Purified eel enzyme
		%	%	%
Ca^{2+}	0	100.0	100.0	100
	0.2	84.2 ± 6.2	72.1 ± 8.5	150
	0.8	71.6 ± 8.6	52.7 ± 10.0	158
	2.0	59.5 ± 8.3	47.9 ± 6.4	160
Mg^{2+}	0	100.0	100.0	100
	0.2	82.5 ± 2.0	79.2 ± 13.5	195
	0.8	74.5 ± 5.8	65.8 ± 4.1	262
	2.0	62.6 ± 10.5	56.2 ± 7.1	283
Na^{+}	0	100.0	100.0	100
	0.6	93 ± 4.3	89.5 ± 4.5	81
	6.0	74 ± 4.9	95.5 ± 5.5	83
	300.0	20 ± 1.0	45.5 ± 2.5	329

[a] Values are the means ± standard errors of four determinations.

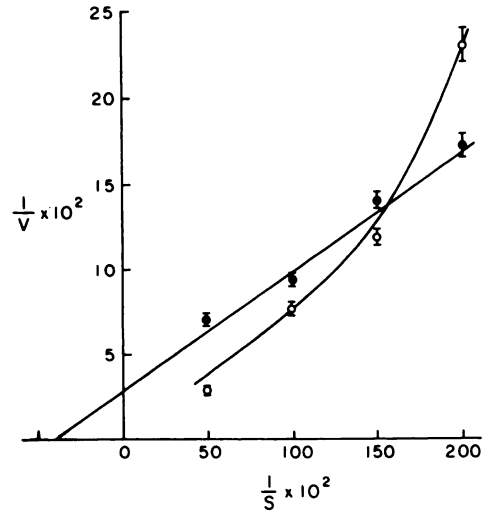

FIG. 2. *Plot of reciprocals of hydrolysis rate (V, micromoles per minute per gram of protein) against acetyl-β-methylcholine concentration (S, millimolar) for acetylcholinesterase bound to sarcolemma with* (○) *and without* (●) *10 mM $MgCl_2$ added to buffer*

Points are the means ± standard errors (vertical bars) of three determinations.

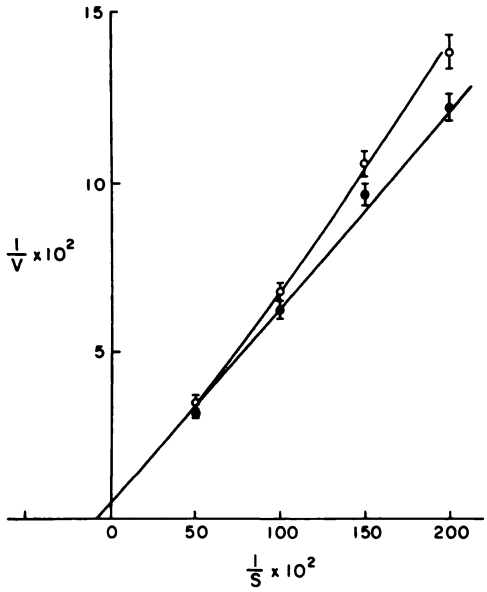

FIG. 3. *Plot of reciprocals of hydrolysis rate (V, micromoles per minute per gram of protein) against acetyl-β-methylcholine concentration (S, millimolar) for acetylcholinesterase bound to sarcoplasmic reticulum with* (○) *and without* (●) *10 mM $MgCl_2$ added to buffer*

Points are the means ± standard errors (vertical bars) of three determinations.

DISCUSSION

Most studies of acetylcholinesterase have been conducted on soluble, partially purified enzymes prepared from the electric organ of *Electrophorus* or bovine erythrocytes. However, in a number of studies, differences have been observed between acetylcholinesterase *in situ* and in solution, not only in terms of affinities of drugs (4, 25, 26) but also with regard to the mechanism of interaction (27–29). In the present study we have attempted to compare the acetylcholinesterases attached to two different membrane types prepared from striated muscle with a minimal degree of membrane destruction. The presence of unbroken end plates in sarcolemma and the Ca^{2+} uptake and ATPase activities of sarcoplasmic reticulum indicate that membrane integrity was largely retained.

The various types of membrane preparation show wide differences in acetylcholinesterase specific activities and also differences in distribution of enzyme types. A considerable butyrylcholinesterase activity associated with the sarcolemma membranes supports similar findings reported by Barnard and Rogers (30). Membrane-bound butyrylcholinesterase in the sarcolemma is distinguished from acetylcholinesterase by the specific substrate butyrylcholine, and can be demonstrated in whole diaphragm muscles *in vitro* (18). Barnard and Rogers (30), using different methodology (radioautography of labeled cholinesterase inhibitors), concluded that approximately one-third of the cholinesterase substrate sites in rat diaphragm end plates were butyrylcholinesterase. Recently Hall and Kelly (31, 32) reported that over 80% of the cholinesterases extractable from whole rat diaphragm muscle is acetylcholinesterase. This estimate was based on the use of specific enzyme inhibitors with 1 mM acetylcholine as the substrate, rather than specific substrates. Thus their results are not directly comparable to the present study of butyrylcholinesterase associated with muscle membranes. This enzyme appears to be preferentially located in the noninnervated part of the sarcolemma, while the distribution of acetylcholinesterase shows the expected association with the innervated part of the muscle. Based on Table 3, approximately one-third of the total sarcolemma

acetylcholinesterase is associated with end plates, comprising 0.3% of the membrane surface (33). The density of this enzyme in the end plate is therefore estimated to be approximately 200-fold higher than in the rest of the muscle membrane. Total sarcolemmal acetylcholinesterase activity is 575 ± 40 pmoles/min/hemidiaphragm, which compares with 460 ± 155 for the surface enzyme (18) of whole hemidiaphragms (acetyl-β-methylcholine substrate at 0.1 mM, 30°). The similarity between the activity of the isolated sarcolemma membrane and that of whole muscle suggests that most of the acetylcholinesterase is located on the external surface of the sarcolemma membrane.

The stability and mode of binding of acetylcholinesterase to sarcolemma and sarcoplasmic reticulum membranes appear to be different. The enzyme is firmly attached to sarcolemma membranes and is not detached by successive extractions at high ionic strength (0.6 M KCl). Collagenase, as previously reported by Hall and Kelly (31), selectively releases the enzyme from sarcolemma membranes. Part of the sarcoplasmic reticulum acetylcholinesterase is solubilized at low ionic strength, particularly in the presence of EDTA, suggesting the involvement of Ca^{2+} or Mg^{2+} in binding, as also observed for bovine erythrocyte acetylcholinesterase (29). About 30% of the acetylcholinesterase in this membrane is not extracted by EDTA, indicating at least two types of enzyme or different modes of attachment. Whole brain and erythrocytes show similar behavior (29, 34); about 50% of the enzyme can be solubilized by EDTA. Triton X-100 alone is relatively ineffective in solubilizing acetylcholinesterase from both muscle membrane fractions, whereas the enzyme from brain tissue and also from erythrocytes is completely solubilized by this detergent (35, 36). However, Triton X-100 combined with high salt (1 M NaCl) is as effective as collagenase in detaching acetylcholinesterase from sarcolemma membranes (32). These results suggest that different membranes containing acetylcholinesterase may each have a specific mode of binding the enzyme.

Interpretation of the effects of divalent cations on membrane-bound acetylcholinesterases is complicated by a number of factors. The anomalous behavior shown in Figs. 2 and 3 could result from a mixture of two or more membrane-bound isozymes with different K_m values and binding affinities for cations. A second complication may be cation binding by other structures in the membrane, particularly of divalent ions, which could influence the enzyme indirectly. However, results on the membrane-bound enzymes can be interpreted on the basis of cation binding sites for divalent ions and bisquaternary drugs known to be present in purified acetylcholinesterase preparations.

Part of the cation activation of soluble acetylcholinesterase is due to an increase in ionic strength, which disaggregates large molecular weight complexes (37, 38). Activation also depends on the nature of the cation, divalent metal ions and small quaternary nitrogen cations being more effective than NaCl (12, 39). Furthermore, activation is maximal under conditions when the deacetylation step is made rate-limiting by the use of a specific substrate (phenyl acetate) or when optimal acetylcholine concentrations are employed (40). The activation mechanism is thus acceleration of the rate of deacetylation by binding of cations to an anionic site of the acetylated enzyme intermediate.

A study by Wins *et al.* (28) of membrane-bound acetylcholinesterase prepared from *Electrophorus* also showed activation by Mg^{2+} and Ca^{2+} in the presence of optimal substrate concentrations. The Mg^{2+} activation phenomenon was found in the present study for the sarcolemma enzyme, but only at substrate concentrations exceeding 0.7 mM. Activation was not observed for the sarcoplasmic reticulum membrane, suggesting weak or absent binding of Mg^{2+} to this enzyme species.

At substrate concentrations below 0.5 mM, Mg^{2+} inhibits acetylcholinesterase in both muscle membrane preparations. This phenomenon also occurs with soluble acetylcholinesterase from bovine erythrocytes but is not observed in the soluble enzyme from *Electrophorus*. The eel enzyme shows only activation by Ca^{2+} and Mg^{2+} over the range of substrate concentration from 1 μM to 1 mM (Table 6).

Inhibition of erythrocyte acetycholinesterase by cations is observed under conditions in which the rate-limiting step is acetylation of the enzyme, e.g., by the use of a specific substrate (dipropylacetylcholine) or at low concentrations of acetylcholine (39). The effects of quaternary nitrogen cations on reactions analogous to acetylation of the enzyme, namely, carbamylation and sulfonylation, have been studied extensively (11–13, 41–44). The rates of these reaction steps are markedly altered, especially by large bulky bisquaternary drugs. These studies have led to the concept that acetylcholinesterase is an allosteric enzyme which, in addition to the catalytic center, possesses anionic sites having a high binding affinity for bisquaternary drugs such as *d*-tubocurare (11, 45, 46) and gallamine (13). It is significant that the regulatory effects of curare and gallamine on soluble as well as membrane-bound acetylcholinesterases are antagonized by Mg^{2+} and Ca^{2+} (10, 13, 28), suggesting that the inorganic cations bind at the allosteric site of the enzyme.

A similar antagonism between curare and Ca^{2+} was found for membrane-bound acetylcholinesterase of sarcoplasmic reticulum and sarcolemma. The addition of 2 mM Ca^{2+} to membrane suspensions in the presence of 3 μM *d*-tubocurare caused no enzyme inhibition, in contrast to the approximately 50% inhibition observed without previous curare treatment for both enzyme preparations (Table 6). We thus attribute the inhibition of membrane-bound acetylcholinestrases by divalent cations to binding at an allosteric binding site.

The present study demonstrates that different membrane-bound acetylcholinesterases, even from the same tissue, possess properties that are distinct from one another and from those of soluble acetylcholinesterases. Some of these differences appear to be enzyme-specific, such as the presence of different cation binding sites, possibly due to different isozymes. Other differences, such as the mode of attachment, might be specific to the structure and function of the membrane. Taken together, the results support the possibility that acetylcholinesterase may have physiological functions in membranes other than a role in synaptic transmission.

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