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CHARACTERIZATION OF THE ASSOCIATION OF *ELECTROPHORUS ELECTRICUS* ACETYLCHOLINESTERASE WITH SPHINGOMYELIN LIPOSOMES

RELEVANCE TO COLLAGEN-SPHINGOMYELIN INTERACTIONS

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Electrophorus electricus **acetylcholinesterase is a large polymorphic enzyme. Its native forms 18 S, 14 S and 8.5 S posses a tail having a collagen-like structure. It was suggested that this tail is involved in the anchorage of the enzyme at the terminal of the synapse. Watkins et al. [1] showed that all forms of the enzyme having a collagen segment also bind to sphingomyelin liposomes with almost no binding to phosphatidylcholine (PC) liposomes. In agreement with the above results, the binding of acetylcholinesterase reported here was independent of the following liposomal parameters (a) curvature, (b) the physical state of the bilayer, (c) the gel to liquid crystalline phase transition of sphingomyelin, (d) stereospecificity of the sphingomyelin, (e) acyl chain of the sphingomyelin. The binding was reduced with increasing PC content in sphingomyelin vesicles. The binding has no effect on the bilayer integrity. The enzymatic activity can be released from the vesicles by incubation with collagenase. The association of the enzyme with the liposomes had minimal effect on its kinetic parameters (K_m, V_{max}). The only detectable effect was increasing enzyme stability at low enzyme concentration. This suggested that the binding of the enzyme to sphingomyelin liposomes reduced its surface denaturation. Such association was not unique to acetylcholinesterase since collagen showed similar behavior. Collagen binding to sphingomyelin liposomes was 5–10-times larger than to PC liposomes. The exact details of the interaction of collagen and collagen-like peptides with sphingomyelin bilayers are yet unknown although it differs from the well documented hydrophobic or electrostatic interactions [7]. This work proposes hydrogen bonding as a third mechanism which involves the interface region of sphingolipids molecules and the collagen or collagen-like tail of acetylcholinesterase. This binding is also of interest due to its correlation to the accumulation of sphingomyelin and collagen during aging and the development of atherosclerosis in blood vessels of mammals.**

Introduction

Membrane proteins are classified into two categories: peripheral and integral proteins (also described as extrinsic and intrinsic proteins) [2,3].

Abbreviations: DTNB, dithiobisnitrobenzoate; PC, phosphatidylcholine; sphingomyelin, sphingomyelin (D-erythro of bovine brain); C16 SPM, D,L-erythro-*N*-palmitoylsphingosylphosphorylcholine (fully synthetic).

This classification is based mainly on the procedures required to dissociate the protein from the membranous lipids [2,4]. Most of the studies on lipid-protein interactions in membranes or model systems deal with integral proteins [2–4]. Very little information is available on similar interactions of peripheral membrane proteins with lipid bilayers and membranes [3] and the biological significance of such interactions are still speculative.

One of the enzymes whose classification is not yet clear is acetylcholinesterase (EC 3.1.1.7). It is a polymorphic enzyme which exists in a number of molecular forms including collagen-tailed molecules as well as tailless or globular molecules. Homologous polymorphism exists in all vertebrate species (for review, see Refs. 5–7). This polymorphism is based mainly on hydrodynamic characterization. It may extend even further if fine details of the structure such as peptide primary sequence and carbohydrate analysis will be introduced [7,8]. The various forms of this enzyme differ in their intermolecular interaction and this may determine their localization either in plasma membranes or in basal laminae. The exact nature of these interactions and the relative contribution of electrostatic interactions, hydrogen bonding and so called hydrophobic interactions is not yet clear (for review, see Ref. 7). The acetylcholinesterase of erythrocytes membrane is classified as an integral membrane protein [9–11]. It interacts with membrane lipids by hydrophobic [10] and possibly also by electrostatic [11] interactions.

The classification of acetylcholinesterase involved in the synaptic transmission as a membrane protein is much less clear even for the acetylcholinesterase derived of the electric organ of *Electrophorus electricus* which seems to be the best characterized and the least complex (for review, see Refs. 5–7). The *Electrophorus electricus* acetylcholinesterase can exist in at least six distinct molecular forms of which three have the ~ 100 kDa triple helix collagen like tail which is approx. 50 nm long and three tailless forms (for more details on the exact structure of this enzyme see Refs. 5–7). For our purpose it is worth noting that the collagen like tail is responsible for the self aggregation of the native enzyme. This self aggregation is not fully understood though there are good indications for the involvement of a distinct aggregating agent possibly a non proteic polyanion of the glycosaminoglycans type such as chondriotin sulfate [7] or heparan sulfate [33]. It is also tempting to assume that such an aggregation factor is involved in the anchorage of the acetylcholinesterase to the synaptic cleft (basal laminae) [6,7]. However, hydrophobic interactions with the plasma membranes cannot be ruled out as suggested by the nonionic detergent (Triton X-100) enhanced enzyme solubilization and by the association of the collagen tailed forms with phospholipid liposomes in high salt concentration (for review, see Ref. 7). The latter suggest that possibility of stable hydrophobic interactions between the tail and the lipids in addition to the electrostatic interactions between the catalytic subunits of the enzyme and the charged phospholipids [12]. It seems that there is a major discrepancy between the findings of Watkins et al. [1] and the detailed studies of Ochoa [12]. Watkins et al. [1] demonstrate a tail-dependent specific interaction of *Electrophorus* acetylcholinesterase with sphingomyelin liposomes but not with liposomes made of phosphatidylcholine (PC) the other major choline phospholipid (for more details on the properties of these two lipids, see Refs. 13 and 14). Ochoa [12] clearly demonstrates interaction of tailed acetylcholinesterase with PC liposomes. Our paper will deal with this discrepancy being directed to get a better insight into the interaction of acetylcholinesterase with lipid bilayer in terms of lipid specificity and other membrane basic properties which may affect this interaction.

Materials and Methods

Materials

Enzymes. Native *Electrophorus electricus* acetylcholinesterase (EC 3.1.1.7) type III, above 1000 units/mg in ammonium sulfate solution and collagenase (EC 3.4.24.3) type II, 2000 units/mg were purchased from Sigma Chem. Co., St. Louis, MO).

Chemicals. Dithiobis(2-nitrobenzoate) (DTNB) and acetylthiocholine were obtained from Sigma (St. Louis, MO). D-Erythro bovine brain sphingomyelin was prepared and analyzed as described by Barenholz et al. [15]. Its sphingose

base composition and acyl chain composition very similar to that described [15]. The fully synthetic D,L-erythro-*N*-palmitoylsphingosylphosphorylcholine (C16 SPM) was a generous gift of Professor D. Shapiro of the Weizmann Institute of Sciences. It was further purified by silicic acid chromatography. *N*-Acylsphingosinephosphoryl[^{3}H]choline ([^{3}H]SPM) 2000 dpm/nmol (of bovine brain) was a generous gift of Professor S. Gatt, the Hebrew University Hadasah Medical School. Egg phosphatidylcholine (PC) was either purchased from Makor Chemicals (Jerusalem, Israel) or prepared as described elsewhere [16]. Its acyl chain composition was almost identical to that described elsewhere [16]. All lipids had greater than 99.0% purity based on thin-layer chromatography using 1 mg load for 2 cm strip of 20 cm long, 0.25 mm thick silica gel G plates [17]. All sphingomyelin or PC preparations were ninhydrin negative. All other reagents or solvents used were of analytical grade or better.

Methods

Preparation of liposomes. Multilamellar large vesicles (MLV) of: sphingomyelin, Cl6 SPM, egg PC or mixtures of sphingomyelin with egg PC were prepared in the desired mole ratio in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and 1.2 M sucrose as described elsewhere [18]. The complete removal of traces of the chloroform-methanol solution was accomplished using high vacuum pump (0.1 mmHg) for 2 h. Small unilamellar vesicles (SUV) were prepared from the multilamellar large vesicles as described by Barenholz et al. [19].

Association experiments. The association of acetylcholinesterase to liposomes was determined as described by Watkins et al. [1]. Either multilamellar large or small unilamallar vesicles of the desired lipid composition in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and 1.2 M sucrose (see preparation of liposomes) were mixed with acetylcholinesterase. The mixture was incubated at the desired temperature (usually 4°C) for the desired time (usually 30 min). Flotation type binding assay was used to determine the extent of acetylcholinesterase association with the various types of liposomes [1]. This method is based on the large differences in density of free acetylcholinesterase (1.183–1.132 g/ml having most of the free enzyme at density 1.1602 g/ml) and of acetylcholinesterase associated with bovine brain sphingomyelin liposomes (1.0886–1.0061 g/ml with most of the associated enzyme at a density 1.0296 g/ml). The mixtures were applied to discontinuous sucrose gradients consisted of the following four layers: 0.5 ml of 2 M sucrose in the bottom of the tube; 0.7 ml of acetylcholinesterase: liposomes incubation mixture in 1.2 M sucrose; 3.1 ml of 1.0 M sucrose; and on the top 0.5 ml sucrose free buffer. All sucrose solutions were prepared in 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl. The NaCl was added to reduce any nonspecific binding due to electrostatic interactions. Centrigufation was carried out at 50 000 rpm ($204\,000 \times g_{av}$) using a Beckman 50.1 rotor for 3 h at 4°C. Fractions of 0.4 ml were collected using a Harvard Apparatus variable speed preistaltic pump model 1210. Routinely each fraction was assayed immediately after collection for its liposomes content (a), acetylcholinesterase activity (b) and density (c).

(a) The liposome content was determined routinely from turbidity profile of the gradient fractions at 400 nm for multilamellar large vesicles [16] and at 300 nm for small unilamellar vesicles [19]. In some experiments [^{3}H]SPM (see Materials) was used for liposome preparation and its distribution on the gradient was used to assess sphingomyelin distribution profile. The latter method is related to [^{3}H]SPM content and is not affected by factors such as liposome size which affect the determination by turbidity.

(b) The acetylcholinesterase content in each fraction was determined from its enzymatic activity at 23°C. Incubation mixtures in a final volume of 3 ml 0.01 M sodium phosphate buffer (pH 8.0) contained the desired amount of tested enzyme solution, 0.1 μmol DTNB and 1.5 μmol substrate (acetylthiocholine chloride). At this concentration (0.5 mM) the substrate was at saturation so that the rate of enzymatic activity was independent of substrate concentration and described V_{max} values which were proportional to the concentration of the active enzyme. The rate of the enzymatic activity was determined from the initial velocity of the time-dependent release of thiocholine. K_m and V_{max} for the enzymatic reaction were determined under

the same conditions except that the substrate concentration was varied. Recovery of enzymatic activity throughout the gradient when measured immediately after collecting the fractions was always better than 80% of the total acetylcholinesterase activity applied to the gradient. After incubation with sphingomyelin liposomes the recovery of enzymatic activity was better than 90%. The consistent difference in activity recovery was due to the stabilization of the enzyme by its association with sphingomyelin liposomes (see the results in Table IV).

(c) The density of each fraction was measured using a Bausch and Lomb refractometer, and calculated from critical tables.

Collagenase treatment. The effect of collagenase treatment on acetylcholinesterase (AcCE) association with liposomes was tested on free enzyme and on enzyme associated with sphingomyelin liposomes (the so-called 'SPM-AcCE complex'), conditions similar to those previously described [1] were used.

For treatment of the free acetylcholinesterase, incubation mixtures in final volume of 0.1 ml of 0.01 M sodium phosphate buffer (pH 7.0) containing 1 M NaCl, 0.5 mM $CaCl_2$ and 0.3 mM $MgCl_2$, 4 μg collagenase and 2 μg acetylcholinesterase were incubated for 1 h at 37°C. The reaction was retarded by cooling the incubation mixture to 4°C. Aliquots were removed immediately after cooling and assayed for binding of the acetylcholinesterase to various liposomes by the flotation assay described in Association experiments.

Treatment of acetylcholinesterase associated with sphingomyelin liposomes was performed under similar conditions except that 'SPM-AcCE complex' having an acetylcholinesterase activity equivalent to 2 μg of free acetylcholinesterase was incubated with the collagenase. Aliquots of this incubation mixture were applied to the same flotation assay used for the association experiments to test the effect of the collagenase treatment on the association of acetylcholinesterase to the sphingomyelin liposomes. The control experiment was performed under identical conditions except that collagenase was omitted from the incubation mixture.

Association of collagen with liposomes. Lyophilized powder of biologically ^{14}C-labelled collagen 20000 dpm/mg was a generous gift of Professor Ben Shushan of the Hebrew University-Hadassah Dentistry School. 5 mg of this powder were dissolved in 0.6 ml 0.001 M acetic acid, then 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl was added. The final pH of the collagen solution was 7.0. To study the association of collagen with either egg PC multilamellar large vesicles or with sphingomyelin multilamellar large vesicles, ^{14}C-labelled collagen in final concentration of 0.25 mg/ml was incubated with multilamellar large vesicles (10 μmol/ml lipid). After 30 min incubation at 4°C aliquots of the incubation mixture were processed by the same procedure used for the binding experiments of acetylcholinesterase. The distribution of collagen on the gradient was determined from its radioactivity and of the liposomes by turbidity at 400 nm.

Results and Discussion

I. Preferential association of acetylcholinesterase with sphingomyelin liposomes: effect of PC content

Fig. 1 demonstrates the distribution of acetylcholinesterase activity along the sucrose gradient (curve ●——●). It is clear that all the recovered acetylcholinesterase activity (> 83% of total activity applied) was present at the bottom of

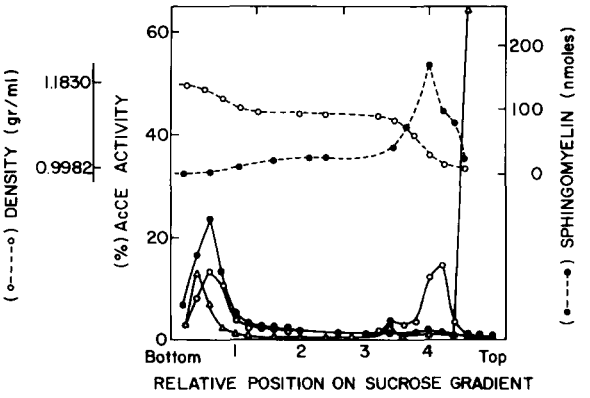

Fig. 1. The association of acetylcholinesterase with sphingomyelin liposomes as determined by the flotation association assay (see Methods). ●——●, no liposomes present (see legend to Table I and text); ○——○, in the presence of bovine brain sphingomyelin liposomes (see legend to Table I and text); △——△, in the presence of D,L-erythro C16 SPM liposomes (see legend to Table IV and text).

the gradient (density range 1.183–1.132 g/ml). Incubation with egg PC multilamellar large vesicles had little effect on this distribution (Table I) since only approx. 3.5% of the recovered activity (82% of the applied) was associated with the egg PC liposomes. These results are in good agreement with those of Ochoa [12] regarding the minimal association of the enzyme with the PC preformed liposomes. Ochoa [12] also demonstrates incorporation of the enzyme into the bilayer of PC liposomes. However, this reconstitution procedure requires solubilization followed by removal of a detergent (Triton X-100) and can be accomplished using tailless enzyme, therefore it is very different from the experimental design described here. Incubation of the enzyme with sphingomyelin multilamellar large vesicles (curve ○——○) cause large changes in the distribution pattern of the enzyme on the gradient. More than 50% of the recovered activity (> 90% of the applied) floated at the same density as the liposomes, which was density range 0.9982–1.0886 g/ml with most of the acetylcholinesterase at density of 1.0296 g/ml for bovine brain sphingomyelin liposomes (curve ○——○) and at 1.0061 g/ml for C16 SPM liposomes (curve △——△). These results are described quantitatively in Table I. It confirms the results previously described by Watkins et al. [1]. Table I also showed that increasing mole % PC in multilamellar large vesicles made of mixtures of bovine brain sphingomyelin and egg PC reduced the acetylcholinesterase association to the multilamellar large vesicles. At 33 mole % egg PC the acetylcholinesterase activity associated with the liposomes was reduced by more than 90% relatively to that obtained for liposomes made of pure sphingomyelin. The acetylcholinesterase activity remaining in the bottom fractions after incubation with sphingomyelin liposomes was not associated with sphingomyelin liposomes even when the incubation with sphingomyelin liposomes was repeated. Most of the associated enzyme ('SPM-AcCE complex', always more than 65%) remained associated with the sphingomyelin liposomes after repeating the floatation assay on 'SPM-AcCE complex'.

TABLE I

EFFECT OF LIPOSOME COMPOSITION ON LIPOSOME-AcCE ASSOCIATION

All incubation mixtures contain multilamellar large vesicles (3.33 μmol bovine brain sphingomyelin) the specified mol% egg PC and 0.14 μg acetylcholinesterase. Incubation was performed for 30 min at 4°C. For more details see Methods.

Small unilamellar vesicles composition		Liposome-associated acetylcholinesterase (%)
Bovine brain sphingomyelin (mol%)	Egg PC (mol%)	
100	0	57.5 ± 4.0
83.4	16.6	39.3 ± 4.0
66.7	33.3	12.2 ± 4.0
0	100	8.6 ± 4.0
No lipid		4.9 ± 4.0

All other experiments were designed to get a better understanding of the various factors involved in this preferential association of acetylcholinesterase with sphingomyelin liposomes.

II. Factors involved in the association of acetylcholinesterase with sphingomyelin liposomes

Parameters which may affect the association of acetylcholinesterase with sphingomyelin liposomes are classified into two categories: one related to liposomes in general and another which involves properties of sphingomyelins in particular. In the first class we include liposome curvature and ratio of lipid to protein. Liposome curvature may affect many of the physical properties of liposomes (Refs. 21,22 and references listed therein) primarily through its effect on the packing of lipid molecules in the bilayer. This may have major impact on the thermotropic behaviour, fusion capability, stability, intactness and also protein insertion into the lipid bilayer. Table II demonstrates only minimal effect of liposomes size on their association with acetylcholinesterase. The highly curves sphingomyelin small unilamellar vesicles are vesicles of minimal size and maximal curvature (approx. 120 Å radius, see Ref. 19) resemble in their behavior the multilamellar large vesicles, which are very large vesicles with almost no curvature. Table II also shows that there was almost no effect of lipid concentration on this association in the concentration range of 1.3–13.3 μmol/ml sphingomyelin. Increasing the acetylcholinesterase concentration

TABLE II

EFFECT OF LIPOSOME CURVATURE AND SPHINGOMYELIN CONCENTRATION ON THE SPHINGOMYELIN LIPOSOMES-acetylcholinesterase ASSOCIATION

Multilamellar large vesicles and small unilamellar vesicles were prepared from bovine brain sphingomyelin as described in Methods. Each incubation mixture contains the specified bovine brain sphingomyelin content as either multilamellar large vesicles or as small unilamellar vesicles and 0.14 μg of acetylcholinesterase in 1 ml. For more details see Table I and Methods.

Sphingomyelin small unilamellar vesicles (μmol/ml)	Liposome-associated acetylcholinesterase (%)	Sphingomyelin multilamellar large vesicles (μmol/ml)	Liposome-associated acetylcholinesterase (%)
1.33	50.0 ± 4.0	–	–
3.33	57.5 ± 4.0	3.33	35.8 ± 4.0
6.66	58.1 ± 4.0	6.66	56.3 ± 4.0
–	–	13.32	44.5 ± 4.0

100-fold (from 0.14 μg to 14 μg) had only minimal effect either for D-erythro bovine brain sphingomyelin small unilamellar vesicles (compare association of 57.5% ± 4.0% to 39.1% ± 4.0%) or for D,L-erythro C16 SPM small unilamellar vesicles (compare association of 45.8% ± 4.0% to 64.8 ± 6.0%). This can be explained by the following calculation. From the molecular weight of the intact enzyme (1.150 kDa for the A_{12} form, see Ref. 7), and an average of about 4000 sphingomyelin molecules per single small unilamellar vesicle, the percentage acetylcholinesterase associated with small unilamellar vesicles can be calculated. For 14 μg of enzyme and 3.3 μmol of sphingomyelin there are $7.3 \cdot 10^{12}$ molecules of acetylcholinesterase per $4.95 \cdot 10^{14}$ small unilamellar vesicles yielding a maximum 1.5% of the small unilamellar vesicles associated with an acetylcholinesterase. For multilamellar large vesicles, assuming an average 100-fold greater molecular weight than for small unilamellar vesicles, a maximum of 1.5 molecules of acetylcholinesterase can be associated on the average with a single vesicle (MLV). In conclusion, in the case of the small unilamellar vesicles due to their large excess and in the case of the multilamellar large vesicles due to their large surface area available for the association, the ratio of acetylcholinesterase to lipid used is much below saturation and its effect on the 'binding' of acetylcholinesterase to the liposomes is minimal.

The fact that only approx. 60% of the acetylcholinesterase is associated with the liposomes may be explained by the polymorphison of this enzyme [5–7]. Possibly the tailless forms of the enzyme such as the G_4 acetylcholinesterase [7] do not associate with sphingomyelin liposomes. This assumption is supported by the finding that the excess of enzyme remaining after interaction with sphingomyelin liposomes in the bottom fraction of the gradient have no tendency to become associated with sphingomyelin in a repeated incubation and also by the effect of collagenase treatment on either preventing the association or releasing the liposome-associated acetylcholinesterase (see Table III).

The second class of factors involved in this association are more specific and related to the sphingomyelins themselves. These parameters can be divided into chemical factors such a stereospecificity of the sphingomyelins or its acyl chain composition and to physical properties such as their physical state. Both types of sphingomyelin properties were studied. (For detailed reviews on sphingomyelin properties and its comparison with PC see Refs. 13 and 14). We studied the relation of sphingomyelin stereo configuration and SPM-AcCE association. Somparison of small unilamellar vesicles composed of bovine brain sphingomyelin, which like all naturally occurring sphingolipids is of the D-erythro configuration [23], to the small unilamellar vesicles composed of fully synthetic racemate D,L-erythro-*N*-palmitoylsphingosylphosphorylcholine show that the stereo configuration had almost no effect on the association. (Compare association of 57.5% ± 4.0% and 45.8% ± 4.0%, respectively.) The same data also showed that there was no effect of acyl chain composition

TABLE III

EFFECT OF acetylcholinesterase TREATMENT WITH COLLAGENASE ON ITS ASSOCIATION WITH SPHINGOMYELIN LIPOSOMES

All experiments were performed using bovine brain sphingomyelin (3.3 μmol/ml) multilamellar large vesicles.

Treatment	Liposome-associated acetylcholinesterase (%)
Repeated fun for AcCE-SPM complex	62.7 ± 4.0 [a]
Collagenase treated AcCE-SPM complex	9.0 ± 4.0 [a]
Acetylcholinesterase-sphingomyelin	57.0 ± 4.0 [b]
Sphingomyelin liposomes + collagenase treated acetylcholinesterase	7.4 ± 4.0 [b]
Sphingomyelin liposomes + acetylcholinesterase nonassociated with sphingomyelin liposomes	7.0 ± 4.0 [b]
No lipid	4.5 ± 4.0 [b]

[a] The 100% is the total activity associated with sphingomyelin after the first gradient. Therefore the 62.7% is a minimal value only.

[b] The distribution of acetylcholinesterase on the gradient is expressed in % of total enzymatic activity applied to the gradient.

since the liposomes prepared from the synthetic *N*-palmitoylsphingomyelin behaved almost identically to the liposomes prepared from the bovine brain sphingomyelin which is a mixture of sphingomyelins of different acyl chains of which the *N*-palmitoylsphingomyelin is only a minor component [14,15].

We selected the structural states of gel or liquid crystalline for sphingomyelin bilayers and the transition between these two states to study the impact of sphingomyelin organization on the association with acetylcholinesterase. This phase transition is a major physicochemical event of a lipid bilayer in general. Many of the other physical properties are secondary and derived from the structural state (for review see Refs. 13, 14, 24 and 25) including lipid-protein interaction and association (Refs. 26, 27, 28 and references listed there). For example, protein insertion into a lipid bilayer in many cases is maximal in the range of coexistence of gel and liquid crystalline domains in the same bilayer (phase transition or phase separation) (Refs. 27 and references listed there). This property may be of some relevance in the case of sphingomyelins which have their gel to liquid crystalline phase transition at the physiological temperature range [13,14]. It is clear that the association of acetylcholinesterase with sphingomyelin bilayer was unaffected by the physical state of the sphingomyelin bilayer. The association at the phase transition temperature for both bovine brain sphingomyelin and C16 SPM was almost identical to that observed at the gel state. (Compare 47.8% ± 4.0% to 48.8% ± 4.0% for D-erythro bovine brain sphingomyelin small unilamellar vesicles and 82.4% ± 6% to 64.8 ± 4.0% for D,L-erythro C16 SPM small unilamellar vesicles at 37°C-phase transition range and 4°C-gel state, respectively.) This may suggest that insertion of the acetylcholinesterase deep into the sphingomyelin bilayer did not occur. This possibility was further supported by the observation that the leakage of 6-carboxyfluorescein (measured as described elsewhere [29]) was not affected by the liposome-acetycholinesterase association even at the phase transition range in which many proteins will drastically increase the rate of 6-carboxyfluorescein leakage [29]. It is worth noting that measurement of acetylcholinesterase binding at temperature in which all sphingomyelin molecules are in the liquid crystalline phase are problematic due to the thermal inactivation of both free enzyme and of acetylcholinesterase-associated with sphingomyelin liposomes (see Fig. 2).

III. The role of the collagen-like tail of acetylcholinesterase in its association with sphingomyelin liposomes

Watkins et al. [1] suggest that the sphingomyelin binding site (or sites) of 18 S acetylcholinesterase reside on its collagen like tail. This assumption is based on the fact that the tailless 21.4 S acetylcholinesterase and the 18 S acetylcholinesterase after treatment with collagenase failed to bind to sphingomyelin liposomes [1]. Our results (Table III) support the above conclusion. Not only that collagenase-treated acetylcholinesterase did not associate with sphingomyelin liposomes but also the isolated 'AcCE-SPM complex' released most of the enzymatic activity when treated with collagenase (Table III). Another indication to the

involvement of the collagen like tail in this association is the preferential association of collagen with sphingomyelin liposomes. While there is almost no association of collagen to egg PC liposomes, 32.2% ± 5% of the collagen become associated with the sphingomyelin liposomes. This not only supports the direct involvement of the collagen like tail in the sphingomyelin-acetylcholinesterase association but also indicate that such associations are of a broader specificity and may involve other collagens or collagen-like peptides.

One of the main possible biomedical implications of such interactions is the parallel accumulation of collagen and sphingomyelin in the aortic intima during aging and the development of atherosclerosis [14,30].

IV. The effect of acetylcholinesterase-sphingomyelin association on the enzymatic properties of acetylcholinesterase

The enzymatic properties of acetylcholinesterase (EC 3.1.1.7) associated with sphingomyelin liposomes were compared with those of the free enzyme. The acetylcholinesterase of *Electrophorus* electricus catalyzes a reaction involving one substrate – the hydrolysis of acetylcholine or its analogs such as the acetylthiocholine. The products of this reaction are acetate and choline or thiocholine, respectively. This monosubstrate reaction can be characterized by its K_m and V_{max} values which are used routinely to examine changes in enzymatic activity. We found that the acetylcholinesterase associated with sphingomyelin liposomes had almost identical K_m values for acetylthiocholine as the free enzyme. The K_m value for the enzyme associated with C16 SPM small unilamellar vesicles was 0.037 ± 0.005 mM compared with 0.038 ± 0.004 mM for the free enzyme. The identity in the K_m values suggest that the association of the enzyme with sphingomyelin liposomes does not affect its interaction with the substrate. Removal of either part or most of the acetylcholinesterase collagen like tail was obtained by treatment with collagenase at 20–25°C or 37°C, respectively [5–7]. Such treatment also had minimal effect on the K_m of the enzyme (Refs. 1, 5, and this work). The above observations support the hypothesis that the collagen-like tail which is responsible for the association with sphingomyelin liposomes is not involved directly in the enzymatic reaction.

Comparison of V_{max} values for this reaction is much more complicated due to the loss of enzyme activity with time which occurs when acetylcholinesterase (free) is present at low concentration even a 4°C as was previously reported [1,31]. Table IV demonstrates that the acetylcholinesterase associated with sphingomyelin liposomes is much more stable than the free enzyme. 24 h at 4°C were sufficient to inactivate 70% of the free enzyme in contrast to no loss of activity of the enzyme associated with C16 SPM small unilamellar vesicles. Even after 26 days at 4°C the acetylcholinesterase associated with C16 SPM small unilamellar vesicles retained 37% of its original activity while free enzyme lost all its activity (Table IV). Similar results were obtained for bovine brain sphingomyelin liposomes (data not shown). The full explanation for this stabilization is not yet available. Watkins et al. [1] report on stabilization of the enzymatic activity by 1% (w/v) sodium cholate. Based on these two different stabilization phenomena phenomena, one can assume that the inactivation with time is due to protein surface denaturation. 1% sodium cholate which is above the detergent critical micellar concentration caused large increases in surface pressure (above −40 dyn/cm). At this high pressure very small amounts of enzyme can become associated with the air/water interface [32] and therefore surface denaturation of the enzyme is dramatically reduced. The association of the enzyme with the

TABLE IV

STABILIZATION OF acetylcholinesterase BY ITS ASSOCIATION WITH SPHINGOMYELIN LIPOSOMES

The effect of storage at 4°C on the enzymatic activity (V_{max}) of acetylcholinesterase associated with C16 SPM small unilamellar vesicles and of free enzyme was compared as described in the text.

Time at 4°C (days)	Enzyme stability	
	Associated acetylcholinesterase (%)	Free acetylcholinesterase (%)
0	100 ± 4.0	100 ± 4.0
1	100 ± 4.0	31 ± 4.0
4	85 ± 4.0	15 ± 4.0
26	37 ± 4.0	1 ± 4.0

sphingomyelin liposomes may also prevent surface denaturation possibly because much less enzyme can become associated with the surface due to its association with the liposomes. The tendency of the enzyme to interact with the interface resides in the catalytic part of the enzyme and not in the collagen-like tail [32].

The temperature dependence of acetylcholinesterase activity can serve as a delicate tool to compare various enzyme preparations. This is best described by Arrhenius plots in which the natural log of the activity is plotted versus the reciprocal of the absolute temperature ($1/T$). For human erythrocyte membrane, acetylcholinesterase reconstituted into lipid vesicles [10] such Arrhenius plots show breaks which are dependent of its lipid environments. Fig. 2 shows clearly that this is not the case for our *Electrophorus electricus* acetylcholinesterase-sphingomyelin system. The Arrhenius plots of the activity of acetylcholinesterase associated with C16 SPM liposomes (upper curve) and the free enzyme (lower curve) had almost identical profiles. Both curves have a 'break' at 44–45°C with a negative slope (activation) at the low temperature side of −1.10 and −1.02 (arbitrary units) and positive slopes (inactivation) at the high temperature side of 10.20 and 10.01 (arbitrary units) for the C16 SPM-associated enzyme and the free enzyme, respectively. This profound similarity suggests that the discontinuity in the Arrhenius plots is due to intrinsic properties of the enzyme catalytic activity. This again supports the assumption that the binding of the enzyme to sphingomyelin liposomes does not involve and does affect the catalytic portion of the acetylcholinesterase molecule. The effect of the lipid environment on the Arrhenius plots of red blood cell membrane acetylcholinesterase activity is a reflection of the hydrophobic association between this enzyme and the lipid bilayer [10]. The intact *Electrophorus electricus* acetylcholinesterase interact with sphingomyelin bilayers through its collagen like tail in a different way and therefore its catalytic activity is almost unaffected by the lipid properties.

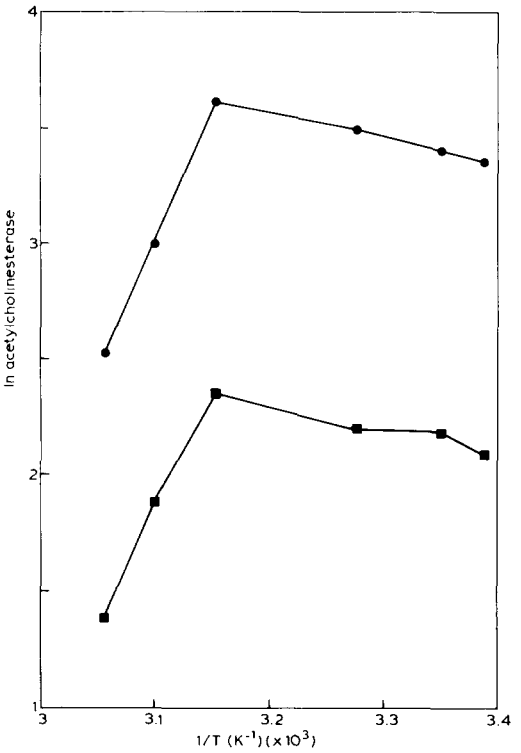

Fig. 2. Arrhenius plots describing the natural log of acetylcholinesterase activity (arbitrary units) versus the reciprocal of absolute temperature (T^{-1}). Incubation mixtures without the substrate (see Methods) containing either free acetylcholinesterase (lower curve ■———■) or acetylcholinesterase associated with D,L-erythro C16 SPM small unilamellar vesicles (upper curve ●———●) were preincubated for 2 min at the desired temperature to ensure thermal equilibratoin. Then substrate (1.5 μmol of acetylcholinesterase) was added and the initial velocity of the enzymatic hydrolysis was determined as described in Methods.

Conclusions

The association of tailed acetylcholinesterase and collagen with sphingomyelin. Possible explanation and implications.

It is accepted that the acetylcholinesterase involved in the synaptic transmission can interact with plasma membrane by hydrophobic interactions and with the basal laminae through ionic interaction [5–7]. It seems that these two types of interactions involved two different parts of the enzyme. The hydrophobic interactions involved mainly the globular portion of this enzyme in which its catalytic activity resides. While the ionic interactions required the collagen like tail of the enzyme [5–7]. This is supported by the following observations:

(1) Similarity in the amino acid composition of the globular portion of the synaptic acetylcholinesterase to that of the highly hydrophobic erythrocyte enzyme which is an integral membrane protein [7,10,11].

(2) The adsorption of the synaptic acetylcholinesterase to the air/water or lipid/water interface which are hydrophobic in nature are not required the collagen like tail [32], so is its reconstitution into the bilayer of liposomes [12].

(3) The collagen like tail is required for the interaction of this enzyme with the basement membrane components [7]. Recent results indicate that the best association is obtained with heparan sulfate proteoglycan [33]. This efficient binding may explain the high concentration of the enzyme at the neuromuscular junction region.

Based on our results we would like to propose a third type of association between acetylcholinesterase and membranes. This proposed interaction requires a collagen-like tail for the acetylcholinesterase and lipid domains or micro domains enriched in sphingolipids in the membrane. It involves hydrogen bonding between the hydroxyproline and/or hydroxylysine residues (which characterize the collagen like tail [5–7]) and the interface region of the sphingolipid molecule. This region has two good candidates for hydrogen bonding. The free hydroxyl group at carbon No. 3 and the amide bond at carbon No. 2 of the sphingosine residue, respectively [13,14,34]. It is worth noting that such hydrogen bonding can be partially stabilized by the intermediate dielectric constant of the sphingolipid interface region [13,14,34]. Another factor in controlling the strength of this association is the possibility of varying the number of hydrogen bonds by which a collagen-like peptide is attached to the membrane. Such an interaction does not require the insertion of enzyme into the lipid bilayer. The main advantages of this association is its dynamic nature; it can easily be manipulated by the exact chemical composition of the membrane as well as by its physical properties. Fast processes such as changes in number and size of lipid domain may change the number of enzyme molecules associated with the membrane, their distribution pattern on the surface, as well as the strength of the association. Such association will have no effect on the enzyme activity though it will affect the location of the activity and also the enzyme local concentration, both of which are very important for the synaptic transmission.

Another important implication is the involvement of hydrogen bonding in the process of the parallel accumulation of sphingomyelin and collagen in the arota during aging and development of atherosclerosis [13,14,30]. In this case the whole molecule of the collagen may be involved in the association with the large domains of sphingomyelin in the plasma membrane of intima cells as well as with sphingomyelin present extracellularly (for review, see Refs. 13 and 14). More studies are required to characterize the effect of the type of the collagen and its exact secondary and tertiary structure on collagen-sphingomyelin association.

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