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Effect of membrane fluidity upon binding of *Electrophorus* acetylcholinesterase to lipid vesicles

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A previous report (Watkins, M.S., Hitt, A.S. and Bulger, J.E. (1977) Biochem. Biophys. Res. Commun. 79, 640–647) has indicated that the asymmetric forms of *Electrophorus* acetylcholinesterase bind exclusively to sphingomyelin vesicles through interaction with the collagen-like 'tail' portion of the enzyme. We report here that acetylcholinesterase also binds to phosphatidylcholine vesicles containing saturated fatty acyl chains and to egg phosphatidylcholine vesicles containing cholesterol. This suggests preferential binding of acetylcholinesterase to membranes of lower fluidity. Surface charge of vesicles and density of zwitterionic lipid headgroups do not significantly affect binding of native acetylcholinesterase. The presence of chondroitin sulfate or hyaluronic acid slightly increases the binding of native acetylcholinesterase to sphingomyelin vesicles, while the presence of 1 M NaCl, bovine serum albumin, or tissue fractions enriched in basement membrane diminish binding. The dissociation constant for native acetylcholinesterase and sphingomyelin vesicles is $(1.0–1.5)\cdot 10^{-7}$ M, as measured by a flotation binding assay. The globular, 11S form of acetylcholinesterase also binds to lipid vesicles, although not to the same degree as native acetylcholinesterase. This suggests that the collagen tail of the enzyme enhances binding, but is not essential for binding to occur. These results are consistent with the location of acetylcholinesterase on the surface of the postsynaptic plasma membrane in vivo.

Introduction

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is an enzyme found in the nervous system and muscle of all vertebrates. It exists in a variety of molecular forms, the characteristics of which depend upon the species and tissue under investigation (for a review, see Ref. 1). These forms may be classified as asymmetric or globular, depending on the presence or absence of a collagen-like 'tail' [2–4]. The primary species of *Electrophorus* acetylcholinesterase are the 18, 14, and 9S asymmetric forms, and the 11S globular form which is a product of proteolysis.

Unlike the acetylcholine receptor, whose transmembrane topology has been definitively demonstrated (for a review, see Ref. 5), the precise intrasynaptic location of acetylcholinesterase has not been established. Perhaps this characteristic as well may depend on the particular species and tissue considered [1]. With respect to *Electrophorus* acetylcholinesterase, evidence exists for binding both to basal lamina components and to the postsynaptic plasma membrane. Early studies by Changeux suggested location of the enzyme on the surface of excitable microsacs prepared from the

electric organ [6]. Alterations in the properties of black lipid membranes and lipid monolayers upon addition of acetylcholinesterase were consistent with location on the postsynaptic plasma membrane [7–9]. Phospholipase C treatment has been shown to alter the sedimentation behavior of asymmetric forms of acetylcholinesterase [10]. Others have shown that asymmetric forms of acetylcholinesterase bind to sphingomyelin liposomes but not to phosphatidylcholine liposomes [11,12].

Evidence for binding of acetylcholinesterase to basal lamina has been reported in a variety of species, including frog [13], rat [14,15], quail [16], and *Torpedo* [17]. Binding of *Electrophorus* acetylcholinesterase to basal lamina is suggested by its aggregation by chondroitin sulfate [18], a glycosaminoglycan. More recent reports have shown both binding [19] and absence of binding [20] of *Electrophorus* acetylcholinesterase to basal lamina components.

In this report we examine the binding of *Electrophorus* acetylcholinesterase to purified lipid vesicles. The aspects of lipid specificity, surface charge, membrane fluidity, and stoichiometry are addressed. Additionally, effects of basal lamina components on lipid binding of acetylcholinesterase are explored.

Materials

The radionuclides [^{14}C]dipalmitoylphosphatidylcholine ([^{14}C]DPPC, 100 mCi/mmol) and ^{3}H-labeled diisopropylfluorophosphate ([^{3}H]DFP, 5.2 Ci/mmol) were purchased from New England Nuclear (Boston, MA). The di[^{3}H]isopropylfluorophosphate was found to be 96% pure by Sephadex G-10 chromatography [21].

The following lipids were purchased from Sigma Chemical Company (St. Louis, MO): bovine brain sphingomyelin, bovine brain ceramide, bovine heart cardiolipin, soybean phosphatidylinositol, L-α-dimyristoyl- and L-α-dipalmitoyl-phosphatidylcholine, L-α-dipalmitoyl-*N*,*N*-dimethylphosphatidylethanolamine, and cholesterol (99 + %). Egg sphingomyelin, L-α-dipalmitoylphosphatidic acid, and L-α-dimyristoylphosphatidylethanolamine were purchased from Calbiochem-Behring (San Diego, CA). Heptadecylamine was purchased from Aldrich Chemical Company (Milwaukee, WI). Chondroitin sulfate (from bovine nasal septum) was a kind gift from Dr. Art Mashburn. The human kidney glomeruli preparation was a kind gift of Dr. Saryu Dixit.

Methods

Acetylcholinesterase was assayed by the method of Ellman et al. [22]. Protein was assayed by the method of Lowry et al. [23] as described by Ji [24], using bovine serum albumin as a standard. Hydroxyproline was assayed by the method of Bergman and Loxley [25], after hydrolysis of the sample at 100°C for 18 h in 6 M HCl. Total amino acids were assayed using ninhydrin by the method of Rosen [26] after acid hydrolysis of the sample.

Acetylcholinesterase was purified by the method of Brooks et al. [27]. Egg phosphatidylcholine was purified from fresh eggs by the method of Singleton et al. [28]. It was found to be homogeneous by thin-layer chromatography on silica GF (Sigma Chemical Company) in the solvent systems chloroform/ acetone/ methanol/ acetic acid/ water (10 : 4 : 2 : 2 : 1, v/v, $R_F = 0.16$) and chloroform/ methanol/conc. ammonium hydroxide (80 : 30 : 6, v/v, $R_F = 0.38$). Its average molecular weight, based on percent phosphorus, was 766. Phosphorus was assayed by the method of Chen et al. [29].

Phospholipid vesicles were prepared by the method of Barenholz et al. [30], using a Branson W185D sonifier, followed by centrifugation at 39000 rpm for 1 h in a Beckman 50Ti rotor. Quantitation of the lipid yield after centrifugation was facilitated by incorporating a trace (less than 0.01 mole percent) of [^{14}C]DPPC into the sample prior to sonication.

Labeling of the active site of native acetylcholinesterase with di[^{3}H]isopropylfluorophosphate was accomplished by adding one volume of di[^{3}H]isopropylfluorophosphate (21.3 μM in anhydrous propylene glycol) to ten volumes of purified acetylcholinesterase (430 μg per ml) in 20 mM sodium phosphate/1 M NaCl (pH 7.0). The solution was kept at room temperature for 90 min, and then dialyzed exhaustively at 4°C against the same buffer to remove free label.

The 11S form of acetylcholinesterase was

formed by the action of trypsin against native, affinity-purified acetylcholinesterase [31] or crude *Electrophorus* electric organ homogenates [32], followed by affinity chromatography in 10 mM sodium phosphate/100 mM NaCl/0.01% NaN_3 (pH 7.0).

Binding of acetylcholinesterase to various phospholipids was assessed by flotation upon density gradient centrifugation. A 0.3 ml aliquot of phospholipid vesicles in 10 mM sodium phosphate buffer/100 mM NaCl (pH 7.0) was brought to 20% sucrose by the addition of solid sucrose. To this sample was added 30 μl of acetylcholinesterase stock solution in 20 mM sodium phosphate/1 M NaCl (pH 7.0). The weight ratio of lipid to protein was in excess of 100 : 1 in order to facilitate binding. The sample was layered over a 0.4 ml 40 percent CsCl cushion in a centrifuge tube, and a 0–15% (w/w) sucrose density gradient was constructed over the sample layer; sucrose solutions also contained 10 mM sodium phosphate buffer/100 mM NaCl (pH 7.0). The tube was centrifuged at 49000 rpm for 4 h in a SW 50.1 rotor at 23 ± 2°C. The gradient was then fractionated (fraction size, 0.3 ml) and the fractions analyzed by scintillation counting.

Results

Purification and labeling of acetylcholinesterase

Acetylcholinesterase was purified from *Electrophorus* electric organ to a specific activity of approximately 5000 units/mg protein [27]. Fig. 1 shows the results of analytical sucrose density gradient centrifugation of native acetylcholinesterase labeled with di[^{3}H]isopropylfluorophosphate. The distribution of molecular forms is identical to that seen for the unlabeled enzyme (data not shown), with a ratio of 9S/14S/18S forms of approximately 10 : 40 : 50, and no appreciable 11S (autolytic) form is seen.

Hydrodynamics of sphingomyelin vesicles

Lipid vesicles were prepared as described in Methods. When bovine brain sphingomyelin vesicles were subjected to sedimentation velocity ultracentrifugation, a sedimentation coefficient of 5.5S was obtained (raw data not shown). Utilizing previously reported values for average diameter of

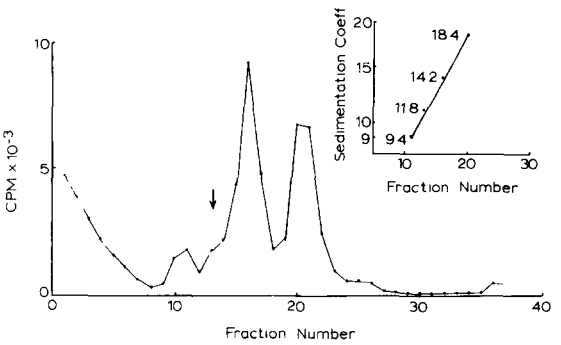

Fig. 1 Analytical sucrose density gradient centrifugation of affinity-purified native [^{3}H]DFP-acetylcholinesterase The top of the gradient is at the left Acetylcholinesterase was placed on top of a 5–20% sucrose density gradient in 20 mM sodium phosphate/1 M NaCl (pH 7 0). The tube was centrifuged in a Beckman SW 41 rotor at 39000 rpm at 4°C for 16 h Fraction size is 0.3 ml. The arrow indicates peak activity of 11S acetylcholinesterase run separately on an identical gradient. Counts above Fraction 10 are from free label due to incomplete dialysis Inset semi-log plot of sedimentation coefficient versus fraction number, including 11S acetylcholinesterase run separately on an identical gradient

similarly prepared vesicles [33], partial specific volume [30], and average molecular weight [34], we obtain a molecular weight of $3.04 \cdot 10^6$ for our vesicle preparation, with an approximate value of 4000 sphingomyelin molecules per vesicle. The corresponding values reported by Huang for egg phosphatidylcholine vesicles are: sedimentation coefficient, 1.76S; vesicular molecular weight $2.06 \cdot 10^6$; and number of molecules per vesicle, 2700 [35].

Flotation of bovine brain sphingomyelin vesicles by density gradient centrifugation with respect to time is shown in Fig. 2. Ultracentrifugation for four hours' duration was chosen as a convenient interval for assay of binding of acetylcholinesterase to lipid vesicles. Runs were made at ambient temperature (23 ± 2°C) since the samples are stable at this temperature and the results more relevant to the in vivo condition.

Binding of acetylcholinesterase to lipid vesicles of homogeneous composition

Fig. 3 illustrates the distribution of native acetylcholinesterase before and after density gradient centrifugation. This distribution should be compared with that of acetylcholinesterase centrifuged in the presence of various phospholipids.

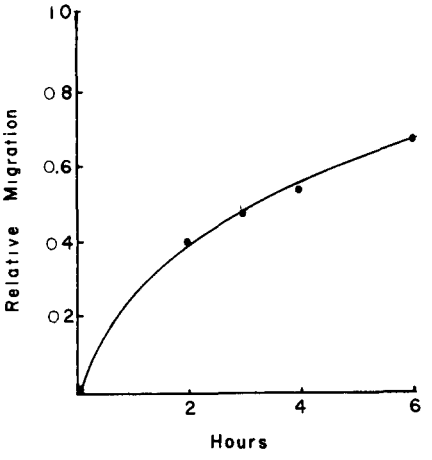

Fig 2. Relative migration of sphingomyelin vesicles upon density gradient centrifugation with respect to time A 0 3 ml sample of bovine brain sphingomyelin vesicles (7 9 mg/ml, doped with [^{14}C]DPPC) was brought to 20% (w/w) with solid sucrose, and layered upon a 0.4 ml 40% CsCl cushion A 0–15% sucrose density gradient was constructed above the sample, and the tube spun at 49000 rpm in an SW 50.1 rotor at 23°C. At the times indicated, a sample tube was fractionated and the fractions counted in a scintillation counter. Buffer· 10 mM sodium phosphate/100 mM NaCl (pH 7.0).

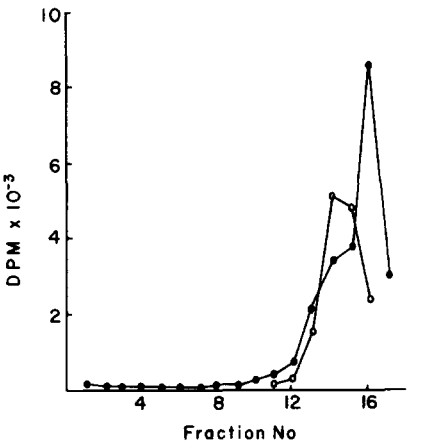

Fig 3. Distribution of native [^{3}H]DFP-acetylcholinesterase in density gradient before and after centrifugation The sample consisted of 0.3 ml of 10 mM sodium phosphate buffer/100 mM NaCl (pH 7.0), containing 20% (w/w) sucrose plus 30 μl of [^{3}H]DFP-acetylcholinesterase (403 μg/ml in 20 mM sodium phosphate buffer/1 M NaCl, pH 7.0) Amount of acetylcholinesterase in the sample was 12 μg. Open circles uncentrifuged; closed circles· centrifuged for 4 h at 49000 rpm in an SW 50.1 rotor at 23°C The top of the gradient is at the left

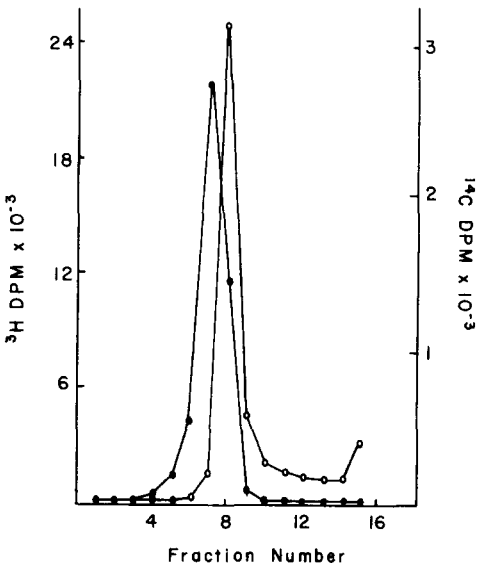

Fig 4. Density gradient centrifugation of native acetylcholinesterase in the presence of egg sphingomyelin vesicles. Sample contained 1 23 mg of [^{14}C]DPPC-doped egg sphingomyelin vesicles (closed circles) and 12 μg of [^{3}H]DFP-acetylcholinesterase (open circles) Distribution of vesicles alone was identical to that shown here

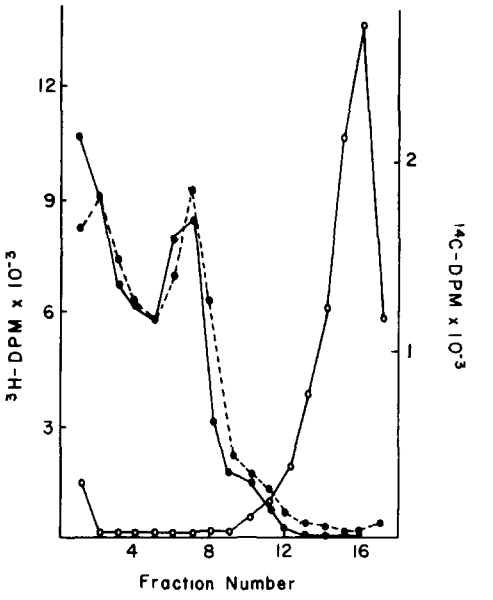

Fig. 5 Density gradient centrifugation of native acetylcholinesterase in the presence of egg phosphatidylcholine vesicles. Sample contained 2.07 mg of [^{14}C]DPPC-doped egg phosphatidylcholine vesicles (closed circles) and 12 μg of [^{3}H]DFP-acetylcholinesterase (open circles) Dashed line represents distribution of vesicles in the absence of acetylcholinesterase

TABLE I

SUMMARY OF DENSITY GRADIENT CENTRIFUGATION DATA FOR NATIVE ACETYLCHOLINESTERASE IN THE PRESENCE OF PHOSPHOLIPID VESICLES

Vesicle composition	Mol%	% Acetylcholinesterase above sample layer [a]
Bovine brain sphingomyelin	100	81
in 1 M NaCl	100	52
Egg sphingomyelin	100	89
Egg phosphatidylcholine	100	12
Dimyristoylphosphatidylcholine	100	75
in 1 M NaCl	100	42
Dipalmitoylphosphatidylcholine	100	91
in 1 M NaCl	100	75
N,*N*-Dimethyldipalmitoylphosphatidylethanolamine	100	82
Bovine heart cardiolipin	100	22
Bovine brain sphingomyelin	50	
Dipalmitoylphosphatidic acid	50	94
Bovine brain sphingomyelin	75 [b]	
Bovine heart cardiolipin	25 [b]	56
Bovine brain sphingomyelin	90	
Heptadecylamine	10	70
Bovine brain sphingomyelin	50	
Cholesterol	50	93
Bovine brain sphingomyelin	75	
Bovine brain ceramide	25	93
Bovine brain sphingomyelin	75	
Dimyristoylphosphatidylethanolamine	25	77
Bovine brain sphingomyelin	75	
N,*N*-Dimethyldipalmitoylphosphatidylethanolamine	25	90
Dimyristoylphosphatidylcholine	50	
Egg phosphatidylcholine	50	17
Dimyristoylphosphatidylcholine	85	
Egg phosphatidylcholine	15	59
Egg phosphatidylcholine	80	
Cholesterol	20	22
Egg phosphatidylcholine	50	
Cholesterol	50	50

[a] Standard deviation for these values is approximately 6.5.
[b] By weight.

The initial position of the sample layer corresponds roughly to fraction 15; a small amount of spreading does take place during the process of fractionating the gradient.

Representative density gradient centrifugations of native acetylcholinesterase in the presence of phospholipid vesicles of homogeneous composition are shown in Figs. 4 and 5. Note that native acetylcholinesterase does bind to and float with vesicles composed of egg sphingomyelin (Fig. 4), but not to vesicles composed of egg phosphatidylcholine (Fig. 5). The presence of more than one peak of phospholipid in the phosphatidylcholine-containing gradient indicates vesicle populations of differing size, density, or both differing size and density. Although sonication followed by preparative ultracentrifugation yielded homogeneous vesicles from most phospholipids, this was not true in all cases, especially with natural lipids.

The binding data for native acetylcholinesterase and various phospholipids is summarized in Table I. Note that of the vesicles of homogeneous composition, those containing egg phosphatidylcholine bound the least amount of acetylcholinesterase (12%). Binding to cardiolipin or phosphatidylinositol could not clearly be assessed using this method, since even in a denser gradient these two types of lipid vesicles did not float to any extent. This suggests that these two lipids form very small vesicles, since the small micellar structures formed by the detergent Triton X-100 or mixtures of Triton X-100 and sphingomyelin do not float under these conditions (unpublished observations). Addition of 1 M NaCl to both sample layers and gradients decreased the binding of acetylcholinesterase to bovine brain sphingomyelin and saturated phosphatidylcholines, but did not abolish it.

Binding of acetylcholinesterase to lipid vesicles of heterogeneous composition

Table I also includes the results of density gradient centrifugation of native acetylcholinesterase in the presence of phospholipid vesicles of heterogeneous composition. The mixed vesicles were used in order to examine: (1) lipids which do not form stable vesicle structures, such as phosphatidylethanolamine [36]; (2) the effect of surface charge on binding, by the inclusion of cardiolipin,

phosphatidic acid, or heptadecylamine; (3) the effect of membrane fluidity on binding, by the inclusion of cholesterol and alteration of fatty acid composition; and (4) the effect of density of zwitterionic lipid headgroups, by the inclusion of cholesterol or ceramide.

The inclusion of phosphatidylethanolamine in sphingomyelin vesicles only slightly decreased the binding of native acetylcholinesterase. The inclusion of heptadecylamine reduced binding by approximately ten percent. The inclusion of dipalmitoylphosphatidic acid enhanced binding, while cardiolipin decreased binding. These results suggest that while charge does not play a role in binding, increased fluidity of the vesicle membrane decreases binding. This is supported by the observation that cholesterol also enhanced binding. The presence of ceramide did not decrease binding, suggesting that a high concentration of zwitterionic lipid headgroups is not essential for binding to occur.

The concept of fluidity was further investigated by the addition of dimyristoylphosphatidylcholine or cholesterol to egg phosphatidylcholine vesicles The data in Table I shows that as increasing amounts of either components are included in egg phosphatidylcholine vesicles, more acetylcholinesterase is bound. A corresponding decrease in membrane fluidity was noted by measuring the motion of 7-doxylstearic acid in these vesicles (data not shown).

A comparison of the lipid binding of both native and 11S acetylcholinesterase as reported by several groups is presented in Table II. In contrast to the report of Watkins et al. [12], we observed that the 11S form does indeed bind to phospholi-

TABLE II

COMPARISON OF LIPID FLOTATION OF ACETYLCHOLINESTERASE BY SEVERAL AUTHORS

The data from other groups were originally published in graphic format, the percentages presented in this table are estimates from the authors' figures The data presented as + + or + were only mentioned in the text of the original article Some data from this report are also included in Table I. Buffers (1) 10 mM sodium phosphate/100 mM NaCl (pH 7 0), (2) 100 mM Tris-HCl (pH 8 0), (3) and 20 mM sodium phosphate (pH 7 0) Abbreviations Egg PC, egg phosphatidylcholine, DPPC, dipalmitoylphosphatidylcholine, DMPC, dimyristoylphosphatidylcholine, n.r , not reported.

	This report	Ref 48	Ref 33	Ref 12	Ref 45
Lipid preparation	Vesicles	Liposomes	Vesicles	Liposomes	Liposomes
Gradient type	Continuous	Continuous	Discont.	Discont	Discont.
Temperature (°C)	23	4	4	4	4
Buffer	1	2	3	1	1
Percent native acetylcholinesterase floated by					
(1) Sphingomyelin	81	65	42	75	+ +
(2) Sphingomyelin in 1 M NaCl	52	22	21	60	+ +
(3) Egg PC	12	21	16	0	n r
(4) Egg PC in 1 M NaCl	n r	15	6	n r	n r
(5) DPPC	91	n r	n r	n r	83
(6) DPPC in 1 M NaCl	75	n r	n r	n r	84
(7) DMPC	75	n r	16	n r	n r
(8) DMPC in 1 M NaCl	42	n r	7	n r	n r
Percent 11S acetylcholinesterase floated by					
(1) Sphingomyelin	72	n.r	n r	8 [a]	n r
(2) Sphingomyelin in 1 M NaCl	7	n.r	n r	n r	n r
(3) DPPC	36	n.r	n r	n r	+ +
(4) DPPC in 1 M NaCl	37	n.r	n r	n r	+
(5) DMPC	24	n.r	n r	n r	n r
(6) DMPC in 1 M NaCl	10	n.r.	n r	n r	n r

[a] 11S prepared by collagenase treatment of native acetylcholinesterase.

pid vesicles, although the effect of 1 M NaCl on binding is only evident with sphingomyelin vesicles.

Effect of other proteins and glycosaminoglycans on binding

The effect of the addition of various proteins and glycosaminoglycans on the flotation of native acetylcholinesterase by sphingomyelin vesicles was examined. The data in Table III indicate that both chondroitin sulfate and hyaluronic acid increased the degree of flotation by roughly the same extent (about 25%). Both bovine serum albumin and fatty acid-free albumin inhibited the binding of acetylcholinesterase. In a separate experiment, albumin itself bound directly to sphingomyelin vesicles, as evident by flotation.

The insoluble fractions of eel electric organ and human kidney glomeruli were utilized because of their relatively high collagen content (4.2 and 7.5 hydroxyproline residues per 100 amino acids, respectively). Neither of the tissue fractions bound to sphingomyelin, rather both pelleted to the bottom of the gradient. Both, however, were capable of binding a small amount of acetylcholinesterase (see Table III).

Stoichiometry of binding to sphingomyelin vesicles

In order to better define the stoichiometry of acetylcholinesterase binding to lipid vesicles, either native or 11S acetylcholinesterase was mixed in varying proportions with bovine brain sphingomyelin vesicles, and density gradient centrifugation was carried out as previously described. As

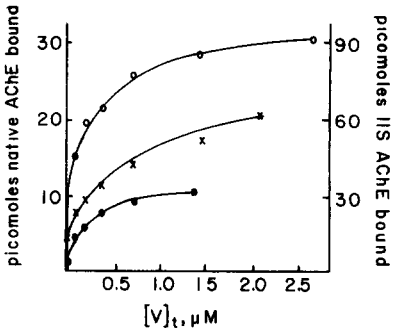

Fig 6 Dependence of flotation of native and 11S acetylcholinesterase (AChE) on amount of lipid. Either 12 μg (12.8 pmoles, closed circles) or 40 μg (43 pmoles, open circles) of native acetylcholinesterase, or 40 μg (120 pmoles, × ——— ×) of 11S acetylcholinesterase was mixed with an aliquot of bovine brain sphingomyelin vesicles, and density gradient centrifugation was carried out in 10 mM sodium phosphate buffer/100 mM NaCl (pH 7.0) as described in Methods. The amount of sphingomyelin in the sample layer is expressed as micromoles of lipid vesicles per liter, V_t. A double-reciprocal plot of the data for native acetylcholinesterase yielded an apparent dissociation constant, K_d, of $(1.0–1.2)\cdot10^{-7}$ M

TABLE III

EFFECT OF VARIOUS PROTEINS AND GLYCOSAMINOGLYCANS ON THE FLOTATION OF ACETYLCHOLINESTERASE BY SPHINGOMYELIN VESICLES

The sample layer contained 40 μg of native acetylcholinesterase, 640 μg of sphingomyelin, and 800 μg of the additional component

Additions	Percent acetylcholinesterase floated	Percent change
None	62	–
Chondroitin sulfate	78	+26
Hyaluronic acid	76	+23
Bovine serum albumin	53	−15
Bovine serum albumin, fatty acid-free	28	−55
Human kidney glomeruli, insoluble fraction [a]	45	−27
Eel electric organ, insoluble fraction [a]	54	−13

[a] Tissue was homogenized in 10 mM sodium phosphate buffer (pH 7.0) and centrifuged at 20000 rpm. The pellet was resuspended in buffer containing 1 M NaCl, and centrifuged. The final pellet was resuspended, dialyzed against distilled water, and lyophilized

shown in Fig. 6, as the concentration of lipid in the sample layer was increased, the result was an increase in the amount of enzyme floated. A double reciprocal plot yields an apparent dissociation constant, K_d, of $1.0 \cdot 10^{-7}$ M. A K_d for 11S acetylcholinesterase could not be obtained from these data. In a separate experiment, the amount of lipid was held constant while the amount of protein was varied. These data yielded a K_d of $1.5 \cdot 10^{-7}$ M, and Scatchard analysis was consistent with an estimate of one enzyme binding site per lipid vesicle.

Discussion

We have found that native acetylcholinesterase preferentially binds to lipid membranes of low fluidity. In his review, Shinitzky [37] considered the major determinants of membrane fluidity to be: cholesterol content, degree of unsaturation of acyl chains, phosphatidylcholine/sphingomyelin ratio, and the relative amount of integral membrane proteins. Cholesterol has the well-documented effect of decreasing membrane fluidity [38,39]. It should be noted, however, that the addition of cholesterol to a membrane does not alter the conformation of phospholipid headgroups [40].As the degree of unsaturation of acyl chains in phospholipids decreases, the fluidity of the membrane also decreases. The data in Table I show that as the mole percentage of dimyristoyl- (ditetradecanoyl)phosphatidylcholine in egg phosphatidylcholine vesicles increases from 0 to 50 to 85%, the flotation of native acetylcholinesterase by these vesicles increases from 12 to 17 to 59 %. The lack of linearity in this relationship is consistent with an asymmetric distribution of the saturated and unsaturated species [41].

The amide linkage in sphingomyelin and its hydroxyl group on C_3 permit the formation of both intra- and intermolecular hydrogen bonds, whereas phosphatidylcholine possesses only hydrogen bond donating capability [42]. This property in addition to the degree of fatty acyl unsaturation (only 5% unsaturation in egg yolk sphingomyelin, [34]) contributes to decreased fluidity in vesicles containing this phospholipid. Addition of ceramide to sphingomyelin vesicles increased the binding of native acetylcholinesterase. The hydroxyl groups on both C_1 and C_3 permit greater intermolecular hydrogen bonding than that present in a membrane of pure sphingomyelin and may further decrease the fluidity of the membrane in this region. Alternatively, the hydroxyl groups of both ceramide and cholesterol may provide additional binding sites for acetylcholinesterase directly.

The presence of integral membrane proteins, such as the acetylcholine receptor, decreases the fluidity of the membrane. Bourgeois et al. [43] utilizing autoradiography and electron microscopy, have determined that the density of receptors on the postsynaptic membrane in *Electrophorus* is very high. Therefore, the acetylcholine receptor may contribute to the localization of acetylcholinesterase on the surface of the postsynaptic membrane by decreasing the fluidity of the bilayer.

Discrepancies among the data in Table II for various investigators could be due to experimental technique. Most authors did not specify the protein-to-lipid ratio used in binding assays. Additionally, different methods were used to generate the lipid structures, which may have affected binding. Nonetheless, all investigators agree that acetylcholinesterase binds to sphingomyelin and binding to egg phosphatidylcholine is minimal. Watkins et al. [12] reported minimal binding of 11S acetylcholinesterase to sphingomyelin liposomes. They used collagenase treatment at 37°C to generate 11S from native acetylcholinesterase, while in this report and that of Kaufmann and Silman [44], trypsin was used. Although the hydrodynamic properties of 11S acetylcholinesterase produced with either protease are identical [45], trypsin might expose additional hydrophobic residues and thus enhance lipid binding. It would be useful to assay the binding of native acetylcholinesterase treated with collagenase at 20°C, since at this temperature only a portion of the collagen-like tail is digested [46]. Binding studies with purified 9S, 14S, and 18S forms of native acetylcholinesterase would further delineate the relative contributions of the globular and collagen-like portions of acetylcholinesterase to lipid binding.

The data in Table III indicate that the presence of either chondroitin sulfate or hyaluronic acid enhances the binding of native acetylcholinesterase

to sphingomyelin vesicles to the same extent. This observation may be due to one of two phenomena: either aggregation of acetylcholinesterase via its collagen tail enhances lipid binding, or binding of free water by the glycosaminoglycan effectively raises the concentration of protein and lipid, which facilitates their interaction. The latter possibility is more consistent with Massoulie's observation that chondroitin sulfate, but not hyaluronic acid, interacts with native acetylcholinesterase [18]. Massoulie et al. recently reported that native acetylcholinesterase does not bind to laminin, fibronectin, or collagen types IV or V [20]. Vigny et al., using acetylcholinesterase from Massoulie's laboratory, also reported no interaction with collagen type IV, but did report binding to collagen types I and V as well as laminin, fibronectin, and heparan sulfate [19]. The latter finding is not surprising, for "all glycosaminoglycans except hyaluronate and keratan sulfate bind collagen by electrostatic interaction at physiologic pH and ionic strength" [47]. If indeed acetylcholinesterase is anchored to the insoluble glycoprotein matrix within the synaptic cleft, soluble components could mediate its localization. The lack of binding constants in Vigny's report makes it impossible to discuss its significance with respect to our findings. Our data suggest that a significant portion of acetylcholinesterase in *Electrophorus* is located on the surface of the postsynaptic plasma membrane. This location would permit more efficient interaction of acetylcholine with its receptor.

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