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INTERACTION OF HUMAN RED CELL MEMBRANE ACETYLCHOLINESTERASE WITH PHOSPHOLIPIDS

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Summary

Liposomes of phospholipids fully sustain the enzyme activity of the amphiphile-dependent dimers of human erythrocyte membrane acetylcholinesterase; no head group specificity exists. Diacylglycerides, glycerophosphorylcholine, or free fatty acids do not sustain the catalytic activity. It could be shown that the dimeric acetylcholinesterase with an exposed hydrophobic region can penetrate the lipid bilayer of liposomes and thus becomes stabilized by the surrounding phospholipid molecules.

The lipid dependency of a number of membrane enzymes is a well known fact [1]; however, little is known so far about specific interactions of pure human erythrocyte membrane acetylcholinesterase with lipids. This purified enzyme [2,3] exists as a 6.5 S dimer in the presence of detergents at their micellar concentrations. It could be shown that the catalytic activity of the dimeric form not only depends on the presence of amphiphilic molecules [3] but is also strongly modulated by the hydrophobic environment [4]. In this study we investigated the interaction of phospholipids and related substances with the enzyme as well as their effect on catalytic activity. The dimeric form of acetylcholinesterase was obtained as described previously [3] except that 1.5% β-D-octylglucoside (Calbiochem, San Diego) was used instead of Triton X-100. Acetylcholinesterase activity was determined according to the method of Ellman et al. [5]. All enzyme assays were carried out in 100 mM sodium phosphate buffer, pH 7.4. Membrane lipids were extracted from human eryth-

rocyte ghosts [6] following the procedure of Foch et al. [7]. Individual phospholipids were separated according to the method of Comfurius and Zwaal [8]. Purity was checked by two-dimensional thin-layer chromatography according to the method of Broekhuyse [9]. Cholesterol was determined as described by Watson [10]. Liposomes were prepared by sonication (1 h, 25°C) [3] or by gel filtration according to the method of Brunner et al. [11]. Acetylcholinesterase was co-reconstituted with phospholipids either by gel filtration [11] or by removal of Triton X-100 with the hydrophobic resin, Amberlit XAD-2 [12].

The effect of phospholipids on the activity of acetylcholinesterase was investigated by adding dimeric enzyme to preformed liposomes. Fig. 1 shows the enzyme activity as a function of the concentration of different lipids. With total erythrocyte membrane lipids, full activity was preserved at 3.9 mM lipid phosphorus and 3.0 mM cholesterol. Cholesterol-depleted erythrocyte membrane lipids sustained activity to the same degree. Similar results were obtained with phosphatidylcholine (Koch-Light Ltd., London). On the other hand, phosphatidylserine from bovine spinal cord (Lipid Products, Nutfield) alone or its mixture with cholesterol (1 : 1, mol/mol) was essentially unable to sustain the catalytic activity. The effect of phosphatidylethanolamine from erythrocyte membranes, which does not form liposomes [13], was investigated in mixtures with phosphatidylcholine or phosphatidylserine at molar ratios of 1 : 1. The results are summarized in Table I. To check the effect of diacylglycerides on the catalytic activity of acetylcholinesterase, liposomes prepared

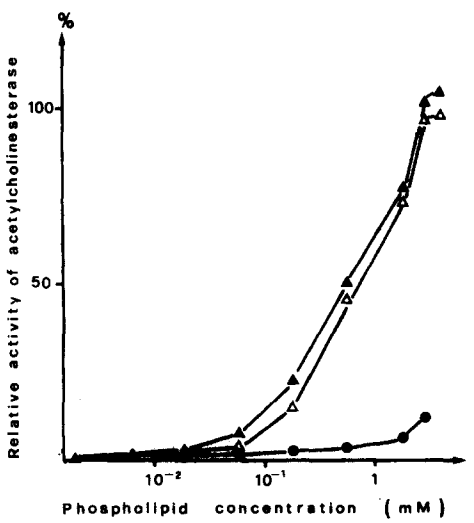

Fig. 1. Effect of phospholipids on the relative activity of acetylcholinesterase. The dimeric form of acetylcholinesterase (0.02 I.U. in 100 mM phosphate buffer, pH 7.4, and 1.5% β-D-octylglucoside) was diluted 1000-fold in 1 ml of liposomal suspensions of various lipid concentrations. The mixtures were incubated for 30 min at 25°C and activity was then measured by adding 2 μl of a solution of 0.52 M acetylthiocholine iodide in water and 30 μl of 5.05 mM 3,3'-dithiobis(6-nitrobenzoic acid) in buffer. Phospholipid concentrations are expressed in terms of lipid phosphorus which was assayed by using the method of Rouser et al. [14] after lipid extraction according to the method of Renkonen et al. [15]. The activity was related to the value obtained in 0.05% Triton X-100 (see Fig. 3, Ref. 3). Egg lecithin (▲———▲), erythrocyte phospholipids (△———△), phosphatidylserine (●———●).

TABLE I

EFFECT OF VARIOUS PHOSPHOLIPIDS ON THE CATALYTIC ACTIVITY OF ACETYLCHOLINESTERASE DIMERS

The liposomes were prepared by sonication (A) or by gel filtration (B). Samples contained 3.9 mM phospholipids. The phosphatidylcholine derivatives contained 3.9 mM phosphorus and were water soluble. The fatty acids (7.8 mM) were sonicated for 30 min at 35°C. The dimeric form of acetylcholinesterase (0.02 I.U. in 100 mM phosphate buffer, pH 7.4, and 1.5% β-D-octylglucoside) was diluted 1000-fold in 1 ml of the various solutions containing the corresponding substances. Enzyme activity was measured as outlined in Fig. 1. If the enzyme was diluted in 100 mM phosphate buffer, pH 7.4, the remaining activity was less than 0.02%.

Phospholipids and related substances	mol ratio	Relative enzyme activity (%)	
		A	B
Erythrocyte membrane phospholipids		95	100
Total erythrocyte membrane lipids		91	94
Phosphatidylcholine			103
Phosphatidylserine		15	12
Phosphatidylserine + cholesterol	(1 : 1)	17	
Phosphatidylserine + phosphatidylcholine	(1 : 1)		58
Phosphatidylserine + phosphatidylethanolamine	(1 : 1)	65	
Phosphatidylcholine + phosphatidylethanolamine	(1 : 1)		94
rac-Glycero-1-phosphorylcholine		0.03	
Phosphorylcholine		0.02	
Oleic acid or stearic acid		<0.02	
rac-Glycero-1-phosphorylcholine + oleic acid	(1 : 2)	<0.02	

with phosphatidylcholine containing the incorporated enzyme [12] were treated with phospholipiase C. As shown in Fig. 2A, the acetylcholinesterase activity decreased with time. The loss of activity correlated with the hydrolysis of lecithin. On the other hand, phospholipase A_2 treatment had no effect on acetylcholinesterase activity (Fig. 2B). This result is consistent with the former observation that lysophosphatidylcholine can fully sustain the catalytic activity [3].

To verify whether acetylcholinesterase becomes lipid bound when full enzyme activity is preserved, sucrose density gradient centrifugation was carried out. As shown in Fig. 3A all catalytic activity floated on top of the gradient when the 6.5 S form of the enzyme was mixed with liposomes made from total erythrocyte membrane phospholipids or phosphatidylcholine. Similar results were obtained if acetylcholinesterase dimers were co-reconstituted with phosphatidylserine according to the method of Brunner et al. [11]. However, aggregated acetylcholinesterase ($s_{20,w} > 10$ S) mixed with these lipids sedimented towards the bottom of the gradient and thus was not lipid associated (Fig. 3B).

The binding forces responsible for the enzyme-lipid interaction were examined in the following experiment. Dimeric enzyme was added to preformed lecithin liposomes of increasing lipid concentration as described in Fig. 1. Aliquots of every sample were diluted 10-fold in 0.1 M phosphate buffer, pH 7.4, containing 0.9 M NaCl. Consequently, the phospholipid concentration was reduced 10-times. Under these conditions, no significant loss of activity occurred.

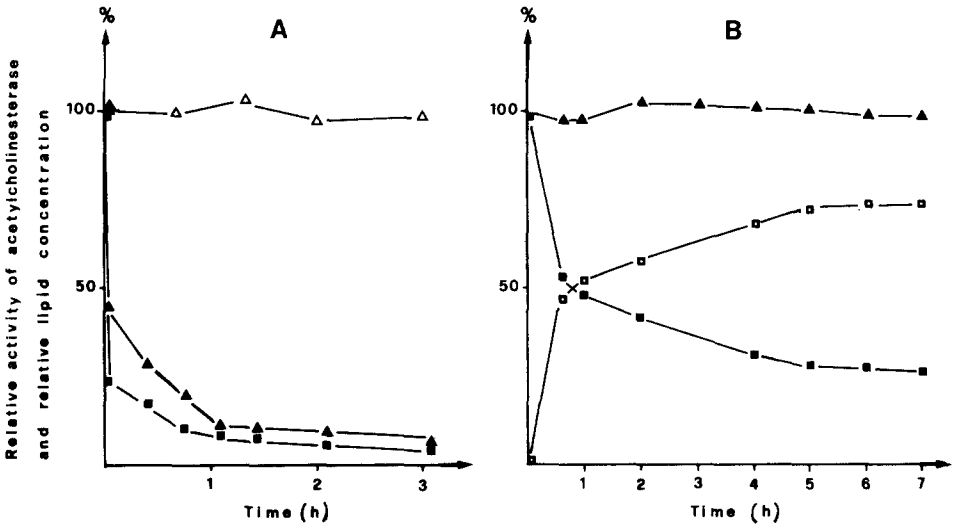

Fig. 2. Effect of phospholipase C and phospholipase A_2 treatment on reconstituted acetylcholinesterase. A sample of liposomes (1 mg lipid/ml) containing 15 I.U./ml acetylcholinesterase was incubated at room temperature with (A) 5 I.U./ml phospholipase C of *Bacillus cereus*, a gift of Dr. P. Ott, or (B) phospholipase A_2 of *Naja naja*, a gift of Dr. Zwaal, in 100 mM Tris-HCl buffer, pH 7.4, containing 10 mM $CaCl_2$. The phosphatidylcholine degradation was checked as follows. Aliquots were extracted with $CHCl_3/CH_3OH$. Spots from thin-layer chromatography were scraped off and phosphorus assayed according to the method of Rouser [14]. The activity was measured as outlined in Fig. 1. Activity of control (△———△) and of phospholipase-treated acetylcholinesterase (▲———▲); concentration of lecithin (■———■) and of lysolecithin (□———□).

The results presented in this paper confirm the finding that the activity of dimeric human erythrocyte membrane acetylcholinesterase can be stabilized by lipids. Since this enzyme form becomes rapidly and irreversibly inactivated in the absence of amphiphilic molecules [3], the ability of lipids to prevent this loss of activity was examined and the nature of the enzyme-lipid interaction investigated. Fig. 1 and Table I demonstrate that preformed zwitterionic phospholipid liposomes are able to sustain the enzyme activity. The concentration dependency (Fig. 1) might reflect a competition between the process of denaturation and the probability of an enzyme molecule meeting a stabilizing lipid vesicle.

All different phospholipids tested sustained the catalytic activity if the liposomes were formed by detergent depletion of solutions containing both enzyme-detergent and lipid-detergent micelles. Hence, this co-reconstitution experiment shows that there is no requirement for a specific head group moiety to maintain the enzyme activity. However, if acetylcholinesterase dimers were added to preformed liposomes prepared with negatively charged phosphatidylserine, a fast and irreversible loss of activity occurred. In this case, an electrostatic repulsion between the negatively charged enzyme (isoelectric point 4.5—5.2; Ref. 2) and the phosphatidylserine vesicles must have prevented the stabilizing protein-lipid interaction. Mixed liposomes prepared with phosphatidylcholine or phosphatidylethanolamine partially retained the activity, indicating that the decrease of the surface charge density [17] lowers this electrostatic repulsion.

The finding that the association of the dimeric enzyme with phosphatidylcholine could not be disrupted in a buffer of high ionic strength excludes an electrostatic interaction as a major force for the binding of the enzyme to liposomes. The interaction appears to be predominantly hydrophobic. This hypothesis is confirmed by the observation that aggregated enzyme, the hydrophobic regions of which are masked [3], does not interact with liposomes (Fig. 3).

To characterize further the nature of the lipid-protein interaction, the influence of chemically defined portions of the phospholipid molecules on the dimeric form of acetylcholinesterase was checked. Diacylglycerides (Fig. 2), glycerophosphorylcholine, phosphorylcholine and free fatty acids (Table I) had no stabilizing effect. From this result, it must be concluded that phospholipids are able to maintain the catalytic active conformation of the enzyme only, if they contain both the polar head groups and the hydrophobic portion of the molecule.

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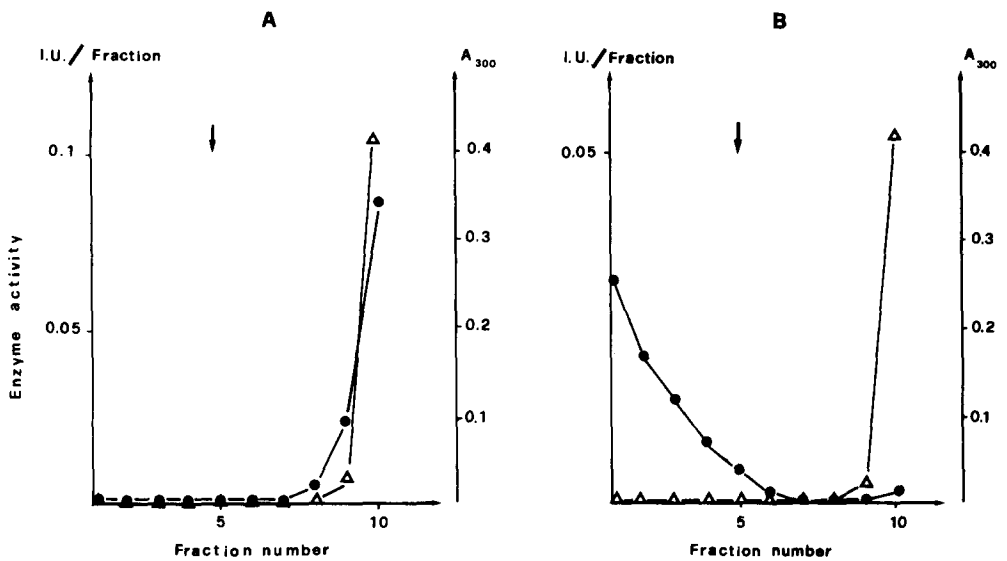

Fig. 3. Association of acetylcholinesterase with phospholipids. Sucrose density gradient centrifugation profiles from incubation mixtures of liposomes with acetylcholinesterase dimers (A) and aggregated enzyme (B) are shown. 7 µl of isolated acetylcholinesterase dimers (0.22 I.U.) were added to 700 µl of buffer, containing liposomes from erythrocyte phospholipids or egg lecithin, respectively, at a concentration of 3.9 mM lipid phosphorus. The same amount of aggregated enzyme was added to lipid suspensions. 500 µl of the enzyme/lipid mixtures were layered on top of a continuous (5—30%) sucrose density gradient. Centrifugation was performed at 4°C and 300 000 × g_{av} for 15 h. Fractions of 1.1 ml were collected. Absorbance at 300 nm served as a measure for the presence of liposomes (△———△). The values were corrected for sucrose absorbance. Catalase served as a marker protein with a sedimentation coefficient of 11.4 S (indicated by arrows). Its activity was measured according to the method of Aebi [16]. Acetylcholinesterase activity was measured as outlined in Fig. 1 (●———●). In the case of phosphatidylserine a discontinuous gradient was used. The liposomes (in 40% sucrose) were layered on a cusion of 60% sucrose. After centrifugation the acetylcholinesterase activity floated at the interphase between 25 and 0% sucrose.

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