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EFFECT OF BILAYER MEMBRANE CURVATURE ON ACTIVITY OF PHOSPHATIDYLCHOLINE EXCHANGE PROTEIN

KOICHI MACHIDA and SHUN-ICHI OHNISHI

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606 (Japan)

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Summary

Effect of bilayer membrane curvature of substrate phosphatidylcholine and inhibitor phosphatidylserine on the activity of phosphatidylcholine exchange protein has been studied by measuring transfer of spin-labeled phosphatidylcholine between vesicles, vesicles and liposomes, and between liposomes. The transfer rate between vesicles was more than 100 times larger than that between vesicles and liposomes. The transfer rate between liposomes was still smaller than that between vesicles and liposomes and nearly the same as that in the absence of exchange protein. The markedly enhanced exchange with vesicles was ascribed to the asymmetric packing of phospholipid molecules in the outer layer of the highly curved bilayer membrane. The inhibitory effect of phosphatidylserine was also greatly dependent on the membrane curvature. The vesicles with diameter of 17 nm showed more than 20 times larger inhibitory activity than those with diameter of 22 nm. The inhibitory effect of liposomes was very small. The size dependence was ascribed to stronger binding of the exchange protein to membranes with higher curvatures. The protein-mediated transfer from vesicles to spiculated erythrocyte ghosts was about four times faster than that to cup-shaped ghosts. This was ascribed to enhanced transfer to the highly curved spiculated membrane sites rather than greater mobility of phosphatidylcholine in the spiculated ghost membrane.

Introduction

Small, single-bilayer vesicles and large, multilamellar liposomes have largely different curvatures and different physical properties as observed in phase transition [1,2], lateral diffusion [3], and NMR linewidth [4,5]. Such dif-

ference may be reflected in protein-lipid interactions in biological membranes and cause differences in biochemical reactivity and biological activity. Some recent investigations have shown this to be the case in the actions of phospholipase A_2 [6] and phospholipase D (unpublished observation), fusion of cytochrome oxidase vesicles [7], and incorporation of cytochrome oxidase into vesicles [8]. The activity of phospholipid exchange proteins may also be influenced by the curvature of substrate and inhibitor phospholipid vesicles. In our previous study, we have presented some evidence that the inhibition of phosphatidylcholine exchange protein by. phosphatidylserine vesicles was dependent on the size or molecular packing in the vesicles [9]. In the present investigation we show marked dependence of the exchange activity on the curvature of substrate phosphatidylcholine membranes. We have extended the study to erythrocyte ghosts with cup-shaped and spiculated membranes and found an enhanced exchange with the spiculated membrane.

Materials and Methods

Exchange protein, spin labels, and phospholipids. Phosphatidylcholine exchange protein from bovine liver was purified according to Kamp and Wirtz [10]. The protein was stored at −20° C in 50% glycerol and dialyzed against Tris buffer (20 mM Tris-HCl/2 mM EDTA/0.02% NaN_3, pH 7.3) before use. For some experiments, bovine serum albumin was added to the protein solution at a concentration of 0.2 mg/ml to prevent loss of activity [11]. Phosphatidylcholine from egg yolk, phosphatidylserine from beef brain, and spin-labeled phosphatidylcholine with 12-nitroxide stearate at 2-position were prepared as described previously [9]. Phosphatidyltempocholine, containing a nitroxide probe on the choline group, was synthesized by condensation of phosphatidic acid, derived from egg yolk phosphatidylcholine, with tempocholine [12]. Total lipid was extracted from erythrocyte ghosts by the method of Bligh and Dyer [13]. Phosphorus determination was done by the method of Bartlett [14].

Preparation of lipid dispersions. Liposomes: Benzene solution of phospholipid was evaporated under a stream of nitrogen and then placed under vacuum. Residual lipid was dispersed by Vortex mixer in Tris buffer.

Sonicated vesicles: Liposomes were sonicated intermittently for 12—15 min effective time with 20 kHz tip-type sonifier (Kaijo Denki) under nitrogen in a water or ice/water bath. The sonicated dispersion was centrifuged at $10^5 \times g$ for 60 min and the supernatant was used. Fractionation of vesicles was performed in some experiments by gel filtration on Sepharose 6B [9].

Cholate vesicles: Phosphatidylserine liposomes were solubilized with cholate and passed through Sephadex G-50 column as described by Brunner et al. [15]. Single-bilayer vesicles were collected by filtration through Sepharose 6B.

Human erythrocyte ghosts. Ghosts were prepared by hypotonic hemolysis at 0° C for 30 min in 10 mM Tris-buffer, pH 7.4, containing either 2 mM each of ATP and $MgSO_4$ or 2 mM $CaCl_2$. After centrifugation, the packed ghosts were added with 9 vols. of the hemolysis solution and isotonicity was restored by adding 1 vol. of 1.4 M KCl, 0.2 M NaCl, and 100 mM Tris-HCl, pH 7.4. The ghost suspension was incubated at 37° C for 1 h for resealing and shape changes

[16,17]. The shape of ghost was observed under light microscope with phase contrast optics after glutaraldehyde fixation.

Assay of exchange activity. This was done by following the peak height increase in ESR spectrum when spin-labeled phosphatidylcholine vesicles were incubated with unlabeled membranes as described previously [9]. Transfer rate was determined from the amount of transferred spin-labeled phosphatidylcholine divided by incubation time in the presence or absence of a fixed amount of exchange protein. When the acceptor was erythrocyte ghost membrane, the reaction was started by addition of exchange protein and continued for 5 min at 37° C. The mixture was then rapidly cooled, centrifuged, and washed with isotonic buffer. The final pellet was used for ESR measurement. ESR spectra were recorded at 23° C or 0° C on a commercial X-band spectrometer (JEOLCO ME-2X). Integration and subtraction of spectra were done with an EC-100 computer when necessary.

Results

Effect of curvature of substrate membrane

Small, sonicated vesicles of phosphatidylcholine were compared with large liposomes as acceptor particles of exchange protein. The phospholipid exchange rate with vesicles was markedly larger than that with liposomes as shown in Fig. 1. Note that the concentration of phosphatidylcholine in the abscissa was plotted in one order of magnitude difference between vesicles and liposomes. This was done for correction of difference in the external surface which was reported as 60—70% of total for sonicated vesicles [18] and 5—8% for multilamellar liposomes [19,20].

The exchange reaction was satisfactory analyzed by the following kinetic equation:

$$V = kL_1L_2P/(L_1 + L_2)[1 + K(L_1 + L_2)]$$

where V is the protein-mediated transfer rate from spin-labeled phosphatidylcholine vesicles to phosphatidylcholine vesicles, K the binding constant of exchange protein to vesicles, L_1, L_2, and P the concentration of donor, acceptor, and the protein, respectively [9,21]. The broken line in Fig. 1a was drawn according to the equation using $K = 79\ M^{-1}$ [9]. Experimental data agreed well with the theoretical curve. The apparent decrease after the maximum around 2 mM is due to relative predominance of exchange between non-labeled phosphatidylcholine vesicles that was not detectable by the present assay. The transfer rate from vesicles to vesicles was more than 100 times larger than that from vesicles to liposomes when compared at the latter concentration of approx. 2 mM.

The transfer rates in various combinations of vesicles and liposomes were collected in Table I. The rate from liposomes to vesicles was as small as that from vesicles to liposomes. The transfer rate from liposomes to liposomes was still smaller and no significant catalysis by the protein was observed. When liposomes were added to the exchange system between vesicles, the transfer rate was not affected. The ineffectiveness of liposomes in the transfer reac-

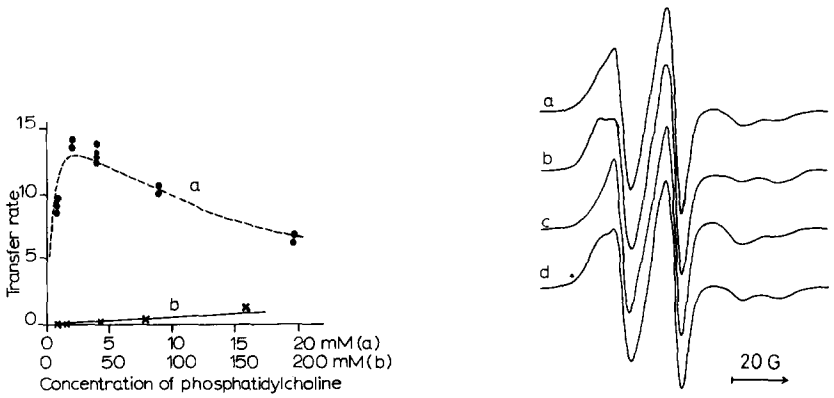

Fig. 1. Transfer rate of spin-labeled phosphatidylcholine from spin-labeled phosphatidylcholine vesicles to phosphatidylcholine vesicles (a) and liposomes (b) as a function of the acceptor concentration. Sonicated spin-labeled phosphatidylcholine vesicles (0.43 mM) and varying concentrations of sonicated phosphatidylcholine vesicles (•) or liposomes (X) were incubated in the presence of exchange protein at 23°C and the transfer rate was measured from the peak height increase in ESR spectrum divided by incubation time. The protein-mediated transfer rate was calculated by subtraction of transfer in the absence of the protein and given in arbitrary units.

Fig. 2. ESR spectrum of phosphatidyltempocholine in vesicles and liposomes at 0°C. Sonicated vesicles consisting of 0.54 μmol of the spin label and 15.7 μmol of egg yolk phosphatidylcholine were mixed with 0.1 ml of Tris buffer (a) or 270 mM sodium ascorbate (pH 6.8) (b), kept for 10 min on ice and passed through Sephadex G-25 coarse column. (c) Subtraction of spectrum (b) from (a) after adjusting the difference in concentrations. (d) Liposomes consisting of the spin label and phosphatidylcholine at 1 : 29 molar ratio.

tions was not therefore due to strong binding of the exchange protein to liposomes.

Differences in the physical state of the outer and inner layers of vesicles and of liposome surfaces were examined by phosphatidyltempocholine incorporated in the membrane. Figs. 2a and b are the ESR spectra from phosphatidylcholine vesicles before and after addition of ascorbate, respectively, and Fig. 2c is a subtraction of them. The spectra 2b and 2c therefore correspond to the labels situated in the inner and outer layers of vesicles, respectively, since the

TABLE I

TRANSFER RATES BETWEEN VESICLES AND VESICLES, VESICLES AND LIPOSOMES, LIPOSOMES AND LIPOSOMES CATALYZED BY EXCHANGE PROTEIN

The rate of transfer of spin-labeled phosphatidyl choline (PC*) molecules between various combinations of membrane systems was determined at 23°C in μM/min. The protein-mediated transfer was calculated as a difference between those in the presence and absence of a fixed amount of the protein.

	Acceptor			
	Vesicles		Liposomes	
Phosphatidylcholine:	6.7 mM	10.0 mM	67 mM	100 mM
Donor PC*				
Vesicles (0.5 mM)		48 ± 3		2.5 ± 0.2
Liposomes (5.0 mM)	2.4 ± 0.5	2.3 ± 0.7	1.3 ± 1.4	1.9 ± 1.3

reducing agent will not permeate the membrane at 0°C. The results suggest more mobile packing of the phospholipid head group in the outer layer and a relative predominance of immobilized fractions in the inner layer of vesicles, in agreement with Kornberg and McConnell [12]. On the other hand, ascorbate addition to liposomes only reduced the signal intensity without changing the spectral shape. The ESR spectrum (Fig. 2d) was somewhat intermediate between those of outer and inner layers of vesicles. The molecular packing in the outer and inner layers of liposome was the same and was more tight than that in the outer layer of vesicles.

Effect of curvature of inhibitor membrane

Anionic phospholipids such as phosphatidylserine and phosphatidic acid inhibited catalytic activity of phosphatidylcholine exchange protein [9,22]. Dependence of the inhibitory effect on the membrane curvature was studied in more detail than in our previous study [9]. Sonicated vesicles strongly inhibited exchange protein at very low concentrations when liposomes showed no inhibition (Fig. 3a and b). To achieve a similar extent of inhibition, several hundred times larger concentrations of liposomes were required. When sonicated vesicles were freeze-thawed, the inhibitory effect was markedly reduced and became similar to that of liposomes (Fig. 3c). It was reported that freeze-thawing caused fusion of vesicles to multilamellar liposomes [1,2].

Sonicated preparation was not yet homogeneous and fractionated by gel filtration on Sepharose 6B. The elution pattern and the inhibitory effect of the fractions are shown in Fig. 4. Three peaks were obtained. Peak I at the void volume contained liposomes and/or larger vesicles (greater than or equal to 50 nm in diameter). Peaks II and III consisted of vesicles with daimeters of 22 nm and 17 nm, respectively. The latter was similar to that obtained by sonication in the absence of added salts [23]. The inhibition curve (Fig. 4b) clearly indicates much stronger inhibitory activity of the smaller vesicles at peak III than the larger vesicles at peak II. The inhibition curve Fig. 4c was obtained using much larger concentration of phosphatidylserine, where the inhibition by peak III vesicles was saturated. This curve indicates capability

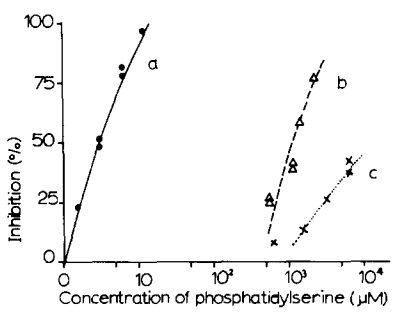

Fig. 3. Inhibition of phosphatidylcholine exchange activity by phosphatidylserine vesicles (a), liposomes (b), and freeze-thawed vesicles (c). The exchange activity was assayed at 23°C in a system consisting of spin-labeled phosphatidylcholine vesicles (0.43 mM), phosphatidylcholine vesicles (10.5 mM), phosphatidylcholine exchange protein, and varying concentrations of phoshatidylserine. Freeze-thawing of vesicles was done five times. The transfer rate in the absence of phosphatidylserine was 12.3 μM/min.

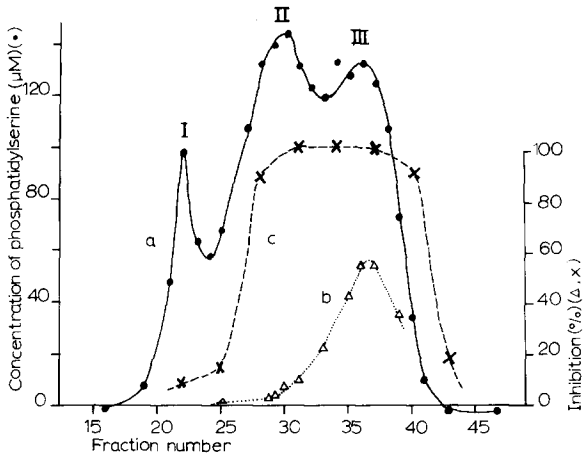

Fig. 4. Elution profile of sonicated phosphatidylserine dispersion from Sepharose 6B and inhibition of phosphatidylcholine exchange activity by the fractions. The eluate from the column (1.7 × 80 cm) at 6°C was collected in 3-ml fractions. (a) Concentration of phosphatidylserine. (b and c) Inhibition by the fractions diluted to 1/225 (b) and 1/5 (c) was assayed in a similar manner to that described in Fig. 3 and given in percent.

of peak II vesicles of inhibition at larger concentrations. The cholate vesicles were eluted near peak III and showed almost the same inhibitory activity as that obtained by sonication. The inhibitory effect was therefore dependent on the vesicular size but not on the method of preparation.

The inhibitory activity of various preparations of phosphatidylserine membranes was compared quantitatively in Table II by the concentration to give 50% inhibition. The smaller vesicles had more than 20 times larger inhibitory activity than the larger ones.

Effect of shape of erythrocyte membrane

The marked dependency of phospholipid exchange activity on the substrate bilayer curvature suggested us to examine enhanced exchange at the spiculated membrane sites of erythrocytes. The cup-shaped and spiculated ghosts were

TABLE II

INHIBITORY ACTIVITY OF PHOSPHATIDYLSERINE VESICLES AND LIPOSOMES

The concentration of phosphatidylserine which inhibited 50% of the exchange activity of the protein is given in μM. Vesicular size was determined by the elution position from the Sepharose 6B column with reference to standard samples: Qβ phage, thyroglobulin, γ-globulin, myoglobulin and serine [28].

	Liposomes	Large vesicles peak II	Smaller vesicles peak III
Vesicular size:		22 nm	17 nm
Sonication		13.3	0.58
Cholate vesicles			0.47
Sonication at higher temperature		19.6	
Sonication in 100 mM NaCl		13.1	
Dispersion by vortex mixer	$1.4 \cdot 10^3$		

TABLE III

TRANSFER RATE OF SPIN-LABELED PHOSPHATIDYLCHOLINE MOLECULES FROM SPIN-LABELED PHOSPHATIDYLCHOLINE VESICLES TO CUP-SHAPED AND SPICULATED GHOST MEMBRANES

Cup-shaped or spiculated ghosts were prepared in the medium containing 2 mM MgATP or 2 mM $CaCl_2$ as described in Materials and Methods and incubated with spin-labeled phosphatidylcholine (PC*) vesicles (81 μM) in the presence of exchange protein at 37°C for 5 min. Additives in the assay medium (2 mM $CaCl_2$ or 2 mM MgATP) were added just before the start of incubation. Transfer of spin-labeled phosphatidylcholine molecules was measured by the central peak height of ESR spectrum and expressed in arbitrary units per mg of ghost phospholipid.

Shape of ghosts	Additives in		Exchange protein	Transferred PC*	
	Lysis solution	Assay medium		Overall	Protein-mediated
Cup	MgATP	—	—	0.7	
	—	—	+	0.9	0.2
		$CaCl_2$	—	0.8	
		$CaCl_2$	+	1.8	1.0
Spiculated	$CaCl_2$	—	—	0.9	
	—	—	+	5.3	4.4
		MgATP	—	0.9	
		MgATP	+	5.2	4.3

prepared by incubation in the presence of MgATP and Ca^{2+}, respectively [16,17]. The transfer from spin-labeled phosphatidylcholine vesicles to the ghost membranes with different shapes was assayed at 37°C for 5 min. The results showed that the protein-mediated transfer to the spiculated membrane was more than four times faster than that to the cup-shaped membrane (Table III).

ESR spectra of spin-labeled phosphatidylcholine transferred to the spiculated and cup-shaped ghost membranes were measured and compared with each other. The overall splitting value for the spiculated membrane (51.3 G) was smaller than that for the cup-shaped membrane (53.1 G), in qualitative agreement with Tanaka and Ohnishi [24]. The result indicates more fluid environments of spin-labeled phosphatidylcholine in the spiculated than in the cup-shaped membranes.

Discussion

The activity of exchange protein was markedly dependent on the membrane curvature of substrate phosphatidylcholine and of inhibitor phosphatidylserine. The protein rapidly exchanged phosphatidylcholine with vesicles but very slowly with liposomes. The exchange reaction may be conveniently divided into three processes; binding of the protein to the membrane, exchange of the protein-bound phospholipid with the membrane phospholipid, and detachment of the protein. Some mobility of phospholipid molecules in the membrane is necessary for the process(es) as indicated by ESR spectrum of phosphatidyltempocholine. Our preliminary observation of increased exchange between spin-labeled phosphatidylcholine vesicles and dimyristoylphos-

phatidylcholine vesicles above the latter phase transition temperature also supports this view. However, the enormous difference in the exchanges with vesicles and with liposomes cannot be explained by the mobility alone in view of rather small difference in the head group mobility in the outer layer of vesicles and in the liposomal membrane. The unusual asymmetric packing of phospolipids in the outer layer of highly curved membranes may be responsible for the fascilitated exchange. The average area per phosphatidylcholine head group on the outer surface of sonicated vesicles was estimated as 74 $Å^2$, while the area at the monolayer interface was 46 $Å^2$ [18]. Of the three elementary processes mentioned above, we think that the exchange of the protein-bound phospholipid with the membrane phospholipid is the process most enhanced. The binding of exchange protein to phosphatidylcholine vesicles was inherently weak [9]. The faster exchange with the spiculated ghost than that with the cup-shaped one may be probably due to facilitated exchange at the highly curved spiculated membranes sites rather than more mobility of phosphatidylcholine in that membrane.

The inhibition of phosphatidylcholine exchange protein by phosphatidylserine vesicles was ascribed to binding of the protein to the vesicles and inexchangeability of the protein-bound phospholipid with the membrane phosphatidylserine [9]. The marked differences in the inhibitory effect between smaller and larger vesicles and liposomes may therefore be largely due to differences in the binding strength. It is remarkable that the 5 nm difference in the diameter of vesicles resulted in more than 20-fold difference in the inhibitory activity. This is consistent with elution of the exchange protein together with peak III of phosphatidylserine vesicles [9], which indicates stronger binding of the protein to the smaller vesicles.

Wirtz et al. [22] observed only small difference in the protein-mediated transfers from mitochondria to sonicated vesicles and to hand-shaken liposomes. This is expected since mitochondrial membranes had small curvatures and can be ineffective as liposomes. The observation by DiCorleto and Zilversmit [25] of negligibly small transfer from liposomes to vesicles of phosphatidylcholine is not inconsistent with the present result. Very recently, Wirtz et al. [26] have studied the protein-mediated exchange of phosphatidylcholine between vesicles and liposomes consisting of phosphatidylcholine and phosphatidic acid (9 : 1 molar ratio). According to their kinetic analysis, both the formation and disruption of the protein-membrane complex was 50—100 times higher for the vesicles than for liposomes. Since the formation and disruption in the analysis included all the three elementary processes mentioned above, their results may not be inconsistent with the present proposal of the enhanced exchange of the protein-bound phospholipid with that in the highly curved bilayer membranes.

Cell surfaces are heterogeneous in various aspects. The present study points out possible biological significance of heterogeneity in the membrane curvature. Proteins located in highly curved sites may have some characteristic activities. Or protein locations may be dependent on the membrane curvature. It is interesting to note in this connection specific localization of a large intrinsic membrane protein to the incisures and margins of rod outer segment disk [27].

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