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A SPIN-LABEL STUDY OF PHOSPHATIDYLCHOLINE EXCHANGE PROTEIN

REGULATION OF THE ACTIVITY BY PHOSPHATIDYLSERINE AND CALCIUM ION

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Summary

Exchange of phosphatidylcholine catalyzed by exchange protein has been studied by a new technique using spin-labeled phosphatidylcholine (PC*). The exchange activity was assayed by the change in electron spin resonance (ESR) spectrum when PC* vesicles were incubated with unlabeled phospholipid vesicles. The method utilizes decrease in the spin-spin exchange interaction and does not require separation of the donor and acceptor vesicles.

(1) The transfer rate, v, from PC* to phosphatidylcholine vesicles was described by

$$v = k[\text{PC}^*][\text{protein}]/(1 + K[\text{PC}])$$

for large excess of acceptor vesicles where k is the rate constant for association of the protein with PC* vesicles and K the binding constant of the protein with phosphatidylcholine vesicles. The transfer rate at 23°C was 3.2 μmol · min^{-1} · (mg protein)$^{-1}$ · (mM PC*)$^{-1}$ at [PC] = 14.7 mM, k = 136 mM^{-1} · min^{-1}, and K = 79 M^{-1}.

(2) The exchange activity was inhibited by addition of phosphatidylserine vesicles and also by mixed phosphatidylserine-phosphatidylcholine vesicles. The inhibition was abolished by Ca^{2+} and Mg^{2+}.

(3) The ESR spectrum of PC* complexed with exchange protein showed strong immobilization of the lipid alkyl chain, suggesting van der Waals-type binding of the acyl chain to the protein interior. The endogeneous PC* molecule was readily exchanged with phosphatidylcholine but practically inexchangeable with phosphatidylserine in the vesicle membranes.

(4) A gel chromatographic analysis indicated weak interaction of the exchange protein with phosphatidylcholine vesicles but strong binding to phosphatidylserine vesicles.

The inhibitory effect of the anionic lipid vesicles can be explained by their binding to the protein and incapability of exchange of the endogenous phosphatidylcholine with phosphatidylserine in the anionic membranes. The restoration by Ca^{2+} and Mg^{2+} may be due to binding of these cations to the anionic lipids, making the protein free from the vesicles. The divalent cations thus act regulatorily for the exchange reaction.

Introduction

Stimulating effect of phospholipid exchange between biological membranes has been found in the supernatant fraction of various tissues and ascribed to a protein denoted phospholipid exchange protein [1—4]. Although its physiological role is not yet clearly understood, the protein is of interest in relation to protein-lipid interaction, dynamic behavior of biological membranes, and membrane biogenesis. Phosphatidylcholine exchange protein has been studied most extensively [5]. It contained one mole of non-covalently bound phosphatidylcholine per mol of protein [6] and catalyzed exchange of phosphatidylcholine with high specificity [7]. The exchange activity was inhibited by liposomes containing anionic lipids [8,9].

We have used a phosphatidylcholine spin label (PC*) to study the exchange mechanism by the exchange protein. The exchange activity was assayed by incubating 'donor' PC* vesicles with unlabeled 'acceptor' vesicles [10]. This method utilizes disappearance of the spin-spin exchange broadening in ESR spectrum when PC* molecules were transferred to and diluted in the unlabeled membrane. It does not require separation of the donor and acceptor vesicles for the assay. It is noted in this connection that the NMR method using paramagnetic probe ions also does not require separation of the vesicles [11].

Materials and Methods

Phospholipid spin labels and phospholipids

PC* was prepared by the reaction of egg lysolecithin with anhydride of 12-nitroxide stearic acid [12]. Spin-labeled phosphatidylethanolamine was synthesized by substitution of the choline group of PC* with ethanolamine under catalytic action of phospholipase D according to Dr. T. Maeda of this laboratory. Phosphatidylcholine from egg yolk and phosphatidylserine from beef brain were isolated and purified according to [13] and [14], respectively. Phosphatidylethanolamine from *Escherichia coli* was a gift of Dr. M. Kito. Purity of phospholipids and spin labels was checked by silica gel thin layer chromatography developed with chloroform/methanol/water (65 : 25 : 4 by vol).

Phospholipid mixtures were suspended in 20 mM Tris · HCl buffer (pH 7.3), sonicated and centrifuged as described previously [10]. The supernatant was used in most experiments and, when more homogeneous single bilayer vesicles were necessary, was fractionated by Sepharose 4B column [15].

Phosphatidylcholine exchange protein

The exchange protein was isolated from beef liver and purified according to

Kamp and Wirtz [16]. For some experiments, the eluate from carboxymethylcellulose column was used without further purification. The protein was used either directly in citrate buffer (50 mM citric acid/0.1 M Na_2HPO_4/10 mM β-mercaptoethanol, pH 5.0), or after dialysis against 20 mM Tris · HCl buffer. Concentration of the protein, when necessary, was accomplished by dialysis against 25% (w/v) polyethylene glycol No. 6000 (M_r 7800—9000, Nakarai Chemicals) in the citrate or Tris buffer, followed by dialysis against Tris.

Binding of the exchange protein to phospholipid vesicles was studied with agarose gel chromatography. The highly active fraction eluted from carboxymethylcellulose column was mixed with the lipid vesicles, incubated for 10 min at 20°C, and, after cooling to 4°C, eluted from Sepharose 6B column (1.7 × 85 cm) with 20 mM Tris buffer [17]. For preparation of PC*-exchange protein complex, an excess amount of PC* vesicles was added to the highly active protein fraction, incubated for 10 min at 20°C, and purified with Sephadex G-50 chromatography [16].

Assay of exchange activity

This was done by measuring transfer of PC* molecules from PC* to unlabeled phospholipid vesicles as described by Maeda and Ohnishi [10]. Donor PC* vesicles (≈50 nmol) were mixed with acceptor vesicles (≈1 μmol) and the exchange protein in a total volume of 60 μl. The ESR spectrum of the mixture was measured at 23°C. The low-field peak height of the three-line component (see arrow in Fig. 1) was measured and plotted against time and the transfer rate was determined from the initial slope. The transfer in the absence of exchange protein was measured and subtracted to obtain the protein-mediated transfer.

ESR spectrum was measured using an X-band spectrometer (JEOL ME-X). Concentration of PC* was determined by comparison with the signal intensity of a known concentration of PC*. Protein and phospholipid concentrations were determined as described in refs. 18 and 19, respectively.

Results

Transfer of PC mediated by exchange protein*

Fig. 1 shows a typical example of change in the ESR spectrum when PC* vesicles were mixed with phosphatidylcholine vesicles and incubated in the presence of exchange protein. A sharp three-line spectrum due to the transferred PC* molecules appeared and grew rapidly with time. The transfer rate, v, was obtained from the initial slope and tabulated in Table I. The rate was proportional to concentrations of the protein and PC* vesicles for a large excess of acceptor vesicles, and can be described by the following equation as proposed by van den Besselaar et al. [20].

$$v = k[\text{PC}^*][\text{protein}]/(1 + K[\text{PC}])$$

where k is the rate constant for association of the protein with the donor vesicles and K the apparent binding constant of the protein to the acceptor vesicles. [PC] is concentration of the acceptor phosphatidylcholine vesicles. K and k were obtained from the intercepts on the abscissa and the ordinate of a plot

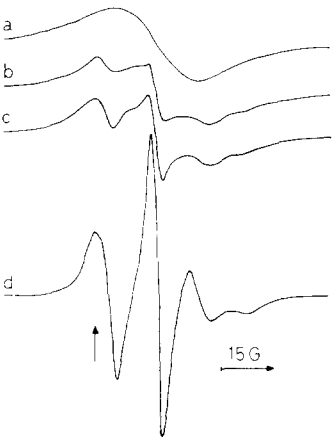

Fig. 1. Change in the ESR spectrum of PC* vesicles on incubation with phosphatidylcholine vesicles in the presence of exchange protein. PC* vesicles (10.3 nmol) were mixed with phosphatidylcholine vesicles (882 nmol) and exchange protein (0.053 μg) in a total volume of 60 μl of 20 mM Tris · HCl buffer (pH 7.3) and incubated at 23°C: (a) before mixing; (b) 7 min after mixing; (c) 18 min after mixing; and (d) 50 min after mixing (1.07 μg of protein for this case). The spectrometer gain in d was one half of that for a, b, and c. Sonicated PC* vesicles were fractionated by Sepharose 4B column and homogeneous single-walled vesicles were used.

of v^{-1} against [PC] as 79 $M^{-1} \cdot min^{-1}$ and 136 $mM^{-1} \cdot min^{-1}$, respectively.

The growth of the three-line component was slowed down after the initial rapid phase (≈10 min) and the amount of the transferred PC* remained at a level of ≈70% of the initially added PC*. This result suggests exchange of PC* molecules located only on the outer layer of vesicles, in agreement with the conclusion drawn previously [21,22].

The exchange protein showed a high specificity for phosphatidylcholine [7,23]. ESR measurements indicated that the protein accelerated transfer of PC* between PC*-phosphatidylcholine-phosphatidylethanolamine (1 : 1 : 1) and phosphatidylcholine-phosphatidylethanolamine (2 : 1) vesicles but did not catalyze transfer of spin-labeled phosphatidylethanolamine between spin-

TABLE I

TRANSFER OF PC* MOLECULE FROM PC* TO PC VESICLES MEDIATED BY EXCHANGE PROTEIN

The transfer rate was determined at 23°C as described in Materials and Methods. The protein-mediated transfer rate was calculated by subtraction of the rate in the absence of the protein.

Exchange protein (μg/ml)	[PC*] (mM)	[PC] (mM)	Transfer rate		Specific activity (μmol/$min^{-1} \cdot mg^{-1}$ protein · mM^{-1} PC*)
			Overall (μM/min)	Protein-mediated (μM/min)	
0	0.171	14.7	0.21	0	—
0.89	0.171	14.7	0.70	0.49	3.22
1.78	0.171	14.7	1.15	0.94	3.10
0	0.68	14.9	1.0	0	—
9.05	0.68	14.9	26.3	25.3	4.12

labeled phosphatidylethanolamine-phosphatidylcholine (1 : 2) and phosphatidylethanolamine-phosphatidylcholine (1 : 2) vesicles.

Inhibitory effect of phosphatidylserine vesicles and restoration by Ca^{2+}

Inhibitory effect of phosphatidylserine was demonstrated using two different acceptor vesicles; mixed phosphatidylcholine-phosphatidylserine vesicles and mixture of phosphatidylcholine and phosphatidylserine vesicles. When donor PC* vesicles were incubated with the mixed vesicles, the transfer rate decreased with increase in phosphatidylserine content of the binary membrane, a half inhibition being attained at 1.5 mol% (see Fig. 2). When the mixture of vesicles was used as acceptor, the transfer rate was reduced much larger, half inhibition being observed at 0.34 mol%. In the latter experiments, the vesicles were mixed immediately before assay so that spontaneous transfer of phosphatidylserine to phosphatidylcholine vesicles was negligibly small during the assay [10]. The inhibitory effect cannot, therefore, be ascribed to modification of phosphatidylcholine vesicles by incorporation of phosphatidylserine. Electrostatic repulsion between negative charges of the anionic lipid and of the protein surface is not also likely to be responsible, since the inhibition occurred likewise in a pH 5.5 solution and the isoelectric point of the protein was reported as 5.8 [5].

The inhibition by phosphatidylserine vesicles was abolished by Ca^{2+} and Mg^{2+}. When PC* vesicles were incubated with a mixture of phosphatidylserine and phosphatidylcholine vesicles, the coexisting anionic lipids inhibited the exchange activity almost completely (Fig. 3a) and the transfer reaction was

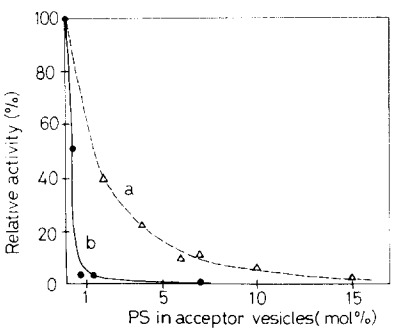

Fig. 2. Inhibition by phosphatidylserine of the activity of phosphatidylcholine exchange protein. The protein-mediated transfer of PC* from PC* vesicles (0.55 mM) to (a) mixed phosphatidylserine-phosphatidylcholine vesicles and (b) mixture of phosphatidylserine and phosphatidylcholine vesicles was plotted against mole fraction of phosphatidylserine in the acceptor system. Concentration of phosphatidylcholine was 15 mM. PS, phosphatidylserine.

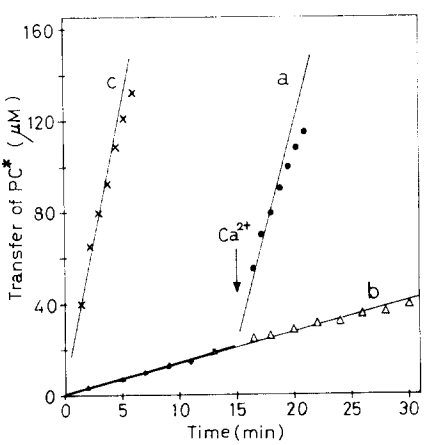

Fig. 3. Restoration by Ca^{2+} of the inhibitory effect of phosphatidylserine on the exchange activity. PC* (37 nmol), phosphatidylcholine (1100 nmol), and phosphatidylserine (1.5 nmol) vesicles were incubated in the presence (a) and absence (b) of exchange protein. After 15 min (indicated by arrow), $CaCl_2$ was added to a final concentration of 16 mM. The trace c is a control, the transfer in the absence of phosphatidylserine vesicles.

nearly the same as that in the absence of protein (Fig. 3b). However, when Ca^{2+} was added to the incubation mixture, the transfer was greatly accelerated and became as fast as that in the absence of phosphatidylserine vesicles (Fig. 3c). A study of concentration dependence of the restoration by Ca^{2+} showed that a half restoration was obtained at 2 mM and almost full recovery at 15 mM. The restoration is not due to modification of the protein by Ca^{2+} since the transfer between PC* and phosphatidylcholine vesicles was not increased but even slightly inhibited by 15 mM Ca^{2+}.

When 15 mM $CaCl_2$ was added to a mixture of phosphatidylserine and phosphatidylcholine vesicles prior to addition of exchange protein, the inhibitory action of the anionic lipid was not observed. The exchange protein was protected by Ca^{2+} from inhibition by phosphatidylserine. The restoration and protection by Ca^{2+} were observed likewise for mixed phosphatidylserine-phosphatidylcholine vesicles as acceptor although less effective.

Mg^{2+} was effective as much as Ca^{2+} in the restoration and protection, while monovalent cations such as Na^+ and K^+ were ineffective.

Binding of exchange protein to phosphatidylserine vesicles

The exchange protein was incubated with phosphatidylserine or phosphatidylcholine vesicles and eluted from Sepharose 6B column (Fig. 4). On incubation with phosphatidylcholine, the exchange activity and protein were eluted in the region of fraction 53, far from phospholipid fractions (Fig. 4A). This result indicates a weak interaction between the exchange protein and phosphatidylcholine vesicles, in agreement with the small binding constant K and also with the result by Kamp et al. [24].

When the exchange protein was incubated with phosphatidylserine vesicles, the elution profile was markedly different (Fig. 4B). Very little activity and protein were recovered from fractions 49—57 corresponding to the free exchange protein. Instead, the main activity shifted to the fractions in the region of 33 where the anionic vesicles eluted, indicating a strong binding of the protein to the vesicles. It is noted that phosphatidylserine eluted in three separated peaks and the exchange protein was eluted with the last peak. Sonicated dispersion of phosphatidylserine also showed three peaks at the same elution position as that in Fig. 4B.

ESR spectrum of PC complexed with exchange protein*

ESR spectrum of the PC*-exchange protein complex gave a very large overall splitting of 63 G, indicating that the PC* molecule, or more specifically its alkyl chain, was firmly bound to the protein (Fig. 5a). When phosphatidylcholine vesicles were added to the complex at a mole ratio of 17 : 1 and incubated, the spectrum changed into that of the labels in fluid bilayer membranes (Fig. 5b). The bound PC* molecules were therefore exchanged with phosphatidylcholine in the vesicles.

When phosphatidylserine vesicles were added to the complex, however, the spectrum was essentially unchanged. Fig. 5c shows the spectrum after incubation with excessive amount of phosphatidylserine (160 : 1). The immobilized component decreased only slightly ($\approx$16%). Even after treatment with a large excess of the anionic lipid (600 : 1), the immobilized component still

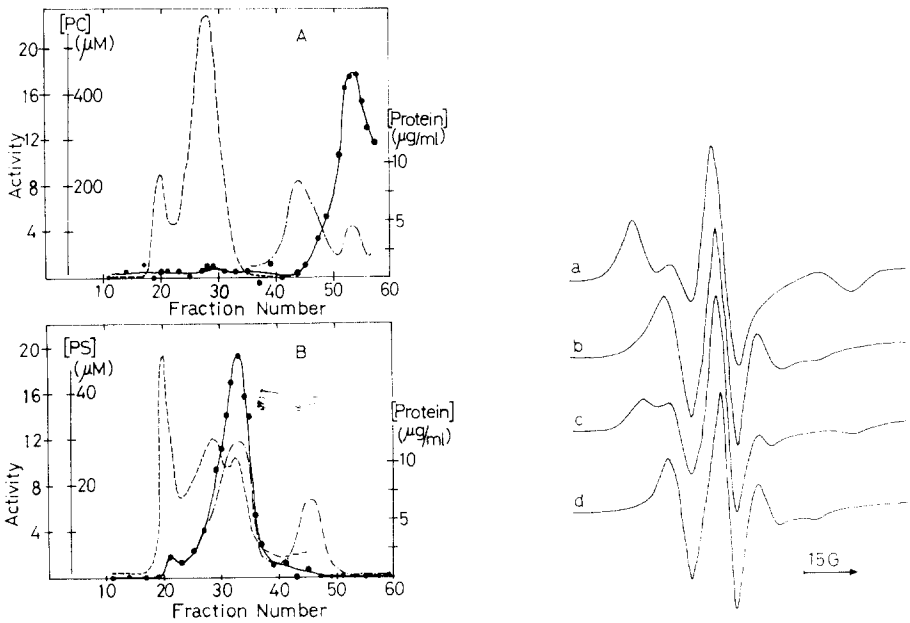

Fig. 4. Elution profile of the mixture of exchange protein with (A) phosphatidylcholine and (B) phosphatidylserine vesicles. Exchange protein (353 μg as total protein) was incubated with phosphatidylcholine (13.8 μmol) or phosphatidylserine (1.17 μmol) vesicles in 20 mM Tris · HCl, eluted from a Sepharose 6B column, and 3-ml fractions were collected. Concentration of phospholipid (- - - - -) and of protein (— · — ·), and the exchange activity in the presence of 15 mM $CaCl_2$ (———) were measured. The activity was expressed in units of μM of transferred PC* for 8 min. The protein fraction used for these experiments contained extraneous proteins which were eluted around fraction 45.

Fig. 5. ESR spectrum of the PC*-exchange protein complex (a). In b the complex was incubated with phosphatidylcholine vesicles at a mole ratio of 1 : 17. (c) The complex was incubated with phosphatidylserine vesicles at a mole ratio of 1 : 160. (d) The mixture in c was incubated with phosphatidylcholine vesicles (protein : phosphatidylserine : phosphatidylcholine = 1 : 160 : 340). The complex was prepared by incubation of the protein (1.83 μg/μl) with excessive amount of PC* vesicles at 20°C for 10 min and purified as described in Materials and Methods.

remained, although the bilayer component became larger. The endogeneous PC* molecule was therefore practically inexchangeable with phosphatidylserine in the membrane. The observed increase of the bilayer component may be partly due to some exchangeable contamination. When phosphatidylcholine vesicles were added to the mixture of the complex and phosphatidylserine vesicles, the immobilized component disappeared and the spectrum became typical of the labels in fluid bilayer membrane (Fig. 5d). This indicates transfer of the endogeneous PC* molecule to the phosphatidylcholine vesicles in spite of the presence of phosphatidylserine vesicles. The rate of phospholipid exchange was still very small, however, in the experimental condition of ESR measurement (mole ratio of phosphatidylserine : phosphatidylcholine : protein = 160 : 340 : 1).

Discussion

The present study has demonstrated that the spin label method provides a powerful, sensitive and widely-applicable technique for the assay of exchange

activity which does not require separation of the donor and acceptor vesicles. The nitroxide moiety in PC* does not largely alter the characteristic properties of phosphatidylcholine. Rousselet et al. [25] have recently observed that the transfer of PC* with 16-nitroxide stearate at the 2-position was only slightly less effective than [^{32}P]phosphatidylcholine of rat liver.

ESR spectrum of the PC*-exchange protein complex showed strong immobilization of the lipid alkyl chain. Similar immobilized spectrum has been observed independently by Devaux et al. [26] by computer subtraction. The strong immobilization suggests importance of van der Waals-type interaction between the lipid acyl chains and the protein interior for the binding. The interaction between the choline head group and the protein must be important for mutual recognition. Kamp et al. [24] have suggested two binding sites in the exchange protein, a hydrophilic and specific recognition site and a hydrophobic non-specific one.

Interaction between the exchange protein and phosphatidylcholine vesicles was weak as judged from the small binding constant and the elution profile. On the other hand, binding of the protein to phosphatidylserine vesicles was strong. Furthermore, the endogeneous phosphatidylcholine was hardly exchanged with phosphatidylserine in vesicles. This is in marked contrast to the ready exchange with phosphatidylcholine. The inhibitory effect of phosphatidylserine is thus ascribed to the firm binding of the protein to the anionic lipid bilayer membranes and the inexchangeability of the endogeneous phosphatidylcholine.

The elution profile of phosphatidylserine consisted of three peaks (Fig. 4B). The first peak was at void volume and composed of multi-layered liposomes. The second one eluted at the same position as the single layered vesicles of phosphatidylcholine (compare with Fig. 4A). The last one contained vesicles of smaller size and may correspond to those of 16 nm diameter obtained in the absence of salt [27]. Since the exchange activity profile coincided with the last peak, interaction of the protein with phosphatidylserine vesicles appears to depend on the size or packing of the phospholipid molecules. This is under investigation.

Wirtz et al. [9] have described inhibitory effect of phosphatidylserine in the exchange of phosphatidylcholine between mitochondria and mixed phosphatidylserine-phosphatidylcholine vesicles. These results are in qualitative agreement with the present results taking the difference in the assay conditions (far fewer donor vesicles in the present system) into account.

Restoration of the exchange activity by Ca^{2+} and Mg^{2+} probably arises from binding of these divalent cations to the anionic membranes so that the bound exchange protein may now be released. In view of significant contents of anionic phospholipids in biological membranes, the exchange protein in vivo may be sometimes bound to the membranes and sometimes released by divalent cations. Regulation of the exchange activity by such mechanism could be physiologically significant.

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