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STIMULATION BY ACIDIC PHOSPHOLIPID OF PROTEIN-CATALYZED PHOSPHATIDYLCHOLINE TRANSFER

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Summary

1. The catalyzed transfer of phosphatidylcholine from unilamellar liposomes to mitochondria by phospholipid exchange protein from beef heart or from beef liver is stimulated by the presence of up to 20 mol% acidic phospholipid (phosphatidylinositol or phosphatidic acid) in the liposome. Co-sedimentation of liposomes with mitochondria increases with increasing mol% acidic phospholipid.

2. The catalyzed transfer of phosphatidylcholine from unilamellar liposomes to multilamellar vesicles by beef heart or beef liver exchange proteins is also stimulated by the presence of acidic phospholipid. No co-sedimentation of negatively charged liposomes with multilamellar vesicles is observed.

3. The catalyzed transfer of phosphatidylcholine from multilamellar vesicles to unilamellar liposomes by phospholipid exchange protein from beef heart or beef liver reaches a maximum at 7.5% phosphatidylinositol in the liposomes. Inhibition of phosphatidylcholine transfer was observed at levels of liposome phosphatidylinositol of greater than 15 mol% only in the presence of beef liver exchange protein.

4. Changes in the surface charge of liposomes by the addition of acidic phospholipid were verified by a novel application of polyvinylchloride block electrophoresis that allows the direct measurement of the relative electrophoretic mobility of sonicated vesicles.

Introduction

Phospholipid exchange proteins, which catalyze the transfer of phospholipids between natural and artificial bilayer membranes, have been isolated from many sources [1,2]. It has been suggested that the physiological role of these proteins is to transport phospholipids from their site of synthesis on the

endoplasmic reticulum to other membranes of the cell [3]. These proteins might therefore perform an important function in the maintenance of pre-existing cellular membranes and in the process of membrane biogenesis. The regulation of this intracellular activity has not yet been elucidated. Hellings et al. [4] and Wirtz et al. [5] have suggested that the surface charge of the membrane is important in governing the action of phospholipid exchange proteins in vivo. They based their proposal on kinetic experiments with beef liver exchange protein in two assay systems. The incorporation of negatively charged phospholipids into liposomes inhibited the catalyzed transfer of phosphatidylcholine between vesicles [4] or between vesicles and mitochondria [5].

Zilversmit and Hughes [2] however, observed a slight stimulation in phosphatidylcholine exchange when dicetylphosphate, cardiolipin or phosphatidic acid (10 mol%) was added to liposomes. The contrary results might be due to the use of two different phospholipid exchange proteins or to differences in the assay system. In this paper we have compared the effect of surface charge on the action of exchange proteins from beef heart and beef liver under identical assay conditions.

Materials and Methods

Lipids. Egg phosphatidylcholine (grade I) and bovine brain phosphatidylinositol of highest purity were obtained from Lipid Products, Inc. (England) and Avanti Biochemicals (Birmingham, Ala.), respectively, and used without further purification. Phosphatidic acid was prepared from egg phosphatidylcholine by the action of phospholipase D according to the procedure of Yang [6] and purified on silica gel H with the solvent system chloroform/methanol/acetic acid/water (80 : 13 : 8 : 0.3, v/v). [^{32}P]Phosphatidylcholine was prepared from rat liver as previously described [7]. All phospholipids exhibited a single spot by thin-layer chromatography on silica gel H with the solvent system chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, v/v), and were stored under N_2 at −20°C. Tri[9,10-^{3}H]oleoylglycerol ([^{3}H]triolein) (397 Ci/mol) was obtained from Amersham/Searle (Arlington Heights, Ill.) and purified by silica gel H thin-layer chromatography with hexane/diethyl ether/acetic acid (60 : 40 : 1, v/v). The triolein band was eluted with chloroform and stored at −20°C. Lipid phosphorus was determined by the method of Bartlett [8].

Phospholipid exchange proteins. Phospholipid exchange protein from beef heart cytosol was purified and assayed according to Johnson and Zilversmit [9]. Beef liver phospholipid exchange protein was isolated according to Kamp et al. [10] and assayed according to Johnson and Zilversmit [9]. One unit of phospholipid exchange protein activity is defined as 1 nmol phosphatidylcholine transferred/min at 37°C [2].

Mitochondria. Beef heart mitochondria were prepared as described previously [11] and stored at −20°C. Upon thawing they were heated for 20 min at 80°C to destroy lipolytic activity [2], and washed with incubation buffer before use. Mitochondrial protein concentration was determined by the biuret reaction [12].

Lipid vesicles. Multilamellar vesicles of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (70 : 25 : 5, mol%) were prepared as described pre-

viously [13] by a slightly modified procedure of Bangham et al. [14]. Liposomes, sonicated unilamellar vesicles, containing appropriate phospholipids, a trace of [^{3}H]triolein as non-transferable marker, and butylated hydroxy toluene (1.0 mol%) were prepared as described previously [9].

Exchange incubations. Liposomes as donors: Liposomes composed of [^{32}P]-phosphatidylcholine (2.5 μg phosphatidylcholine phosphorus/ml) and varying amounts of phosphatidylinositol or phosphatidic acid were incubated for 20 or 40 min at 37°C with beef heart or beef liver exchange protein (0.5—1.5 units/ml) and beef heart mitochondria (6.25 mg protein/ml) in sucrose/Tris · HCl (pH 7.4)/EDTA (250 mM/50 mM/1 mM) in a total volume of 0.5 ml. The reaction was terminated by centrifugation for 2 min at 8000 × *g* in an Eppendorf 3200 centrifuge. An aliquot of the supernatant, which contained the liposomes, was counted in the scintillation medium of Gordon and Wolfe [15]. The [^{3}H]-triolein trace in the liposomes served as a non-exchangeable reference marker so that the decrease in the ^{32}P/^{3}H ratio during incubation measured the transfer of [^{32}P]phosphatidylcholine. The small amount (less than 2%) of transfer in the absence of exchange protein was subtracted as background. Linearity was established in incubations of 40 min with aliquots removed every 10 min. The catalyzed exchange was found to be linear with time to 25% transfer of label. Initial velocities were measured in every case from determinations at 20 and 40 min.

When multilamellar vesicles were employed as acceptor particles, the assay system was similar, however, mitochondria were replaced by phosphatidylcholine/phosphatidylethanolamine/cardiolipin (70 : 25 : 5, mol%) multilamellar vesicles (83 μg phospholipid phosphorus/ml). This amount represents a 4-fold excess of available phosphatidylcholine [13]. The reaction was halted after 20 or 40 min by centrifuging at 40000 × *g* for 15 min at 4°C in a Sorvall RC-2B centrifuge. The incubations were carried out in Tris · HCl (pH 7.4)/EDTA (50 mM/5 mM). Sucrose was omitted to allow sedimentation of multilamellar vesicles.

Multilamellar vesicles as donors: Multilamellar vesicles of [^{32}P]phosphatidylcholine/phosphatidylethanolamine/cardiolipin (70 : 25 : 5, mol%) (20 μg phospholipid phosphorus/ml) and a trace of [^{3}H]triolein were incubated at 37°C for 20 and 40 min with a 5-fold excess of available phosphatidylcholine in the form of unlabeled liposomes of varying composition (14 μg phosphatidylcholine phosphorus/ml) and appropriate amounts of beef heart or beef liver exchange protein in Tris · HCl (pH 7.4)/EDTA (50 mM/5 mM). The reaction was halted by centrifuging at 40000 × *g* for 15 min at 4°C in a Sorvall RC-2B centrifuge. Radioactivity in an aliquot of the supernatant was determined as above. The amount of phosphatidylcholine transferred to acceptor liposomes was determined from the ^{32}P in the supernatant corrected for non-sedimentation of the multilamellar vesicles by use of the ^{3}H in the supernatant and the original ^{32}P/^{3}H ratio of the multilamellar vesicles. The correction was less than 2%.

Electrophoresis of liposomes. Geon-Pevikon block electrophoresis, based on the method of Barth et al. [16] as modified by Mahley and Weisgraber [17], was employed to obtain a direct measure of relative electrophoretic mobilities of liposomes. The Geon-Pevikon block (Geon 427 (Goodrich Chemical Co.,

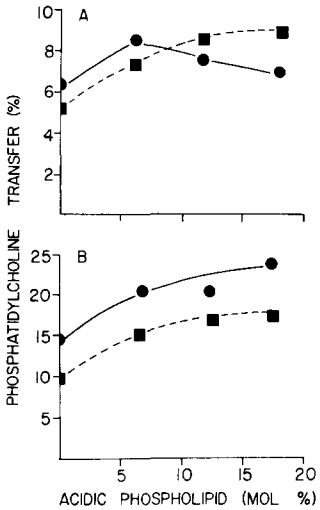
Cleveland, Ohio) -Pevikon C-870 (Mercer Consolidated Corp., Yonker, N.Y.) (1 : 1, w/w)) was set up exactly as described by Mahley and Weisgraber but in Tris · HCl (pH 7.4)/EDTA (50 mM/5 mM). Liposomes with radioactive labels were prepared in this buffer. Liposomes of phosphatidylcholine only were labeled with [^{32}P]phosphatidylcholine; liposomes of phospahtidylcholine/phosphatidylinositol were prepared with [^{3}H]triolein. Aliquots of phosphatidylcholine liposomes were individually mixed with each type of phosphatidylcholine/phosphatidylinositol liposomes and applied to parellel running lanes. A separate aliquot of each mixture was stained with Sudan black B according to Mahley and Weisgraber in order to visualize the progress of the liposomes. The samples were electrophoresed at room temperature for 16 h at a constant current of 50 mA. At the end of the run, excess buffer was allowed to drain through the wicks into towels. Each lane of the block was then cut into 1 cm lengths with a spatula. The blocks of resin were transferred to sintered glass filters and eluted with 15 ml of buffer. The eluates were centrifuged at 2000 × *g* for 20 min to remove small amounts of Geon-Pevikon particles from solution. An aliquot of the supernatant was counted in the scintillation medium of Gordon and Wolfe [15]. With several washes of the resin, recovery of liposomes is quantitative.

Results

The transfer of radioactive phosphatidylcholine from liposomes to mitochondria has been employed as a measure of phospholipid exchange protein activity for several years [18]. In the present experiments the surface charge of the liposome (donor particle) was altered by the addition of acidic phospholipid, phosphatidylinositol or phosphatidic acid. These liposomes of varying composition were incubated with beef heart mitochondria in the presence or absence of beef heart or beef liver exchange protein. The initial rate of [^{32}P]-phosphatidylcholine transfer from liposome to mitochondria was determined. As shown in Fig. 1A the addition of up to 20 mol% phosphatidylinositol to phosphatidylcholine liposomes has little effect on the transfer of phosphatidylcholine by the beef heart protein although a 30% stimulation at 7 mol% phosphatidylinositol is observed. A 60% stimulation of beef liver activity at 18 mol% phosphatidylinositol is observed in similar experiments (Fig. 1A). When phosphatidic acid is added to phosphatidylcholine liposomes to alter the donor particle charge, both the beef liver and the beef heart exchange protein activities are stimulated by the presence of up to 18 mol% acidic phospholipid (Fig. 1B).

Co-sedimentation of the liposomes with the mitochondria, as determined by the recovery of the non-exchangeable marker [^{3}H]triolein, increases with increasing surface charge of the vesicle (Table I). The poor recovery of liposomes in the supernatant after removal of mitochondria may be improved by the addition of bovine serum albumin (5 mg/ml) to the incubation medium. The activity curves of exchange protein vs. surface charge obtained in the presence of bovine serum albumin were essentially the same as those obtained in its absence.

A second assay system for exchange protein activity has recently been



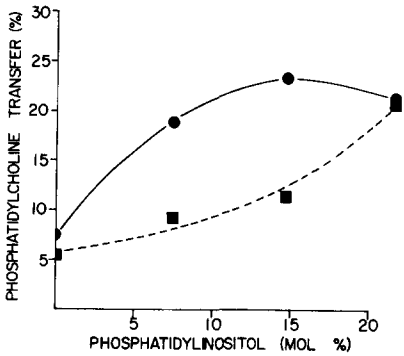


Fig. 1. Transfer of [^{32}P]phosphatidylcholine from liposomes to mitochondria (expressed as percent of [^{32}P]phosphatidylcholine present in liposomes at time zero). Liposomes of [^{32}P]phosphatidylcholine and varying amounts of phosphatidylinositol (A) or phosphatidic acid (B) were incubated with an excess of beef heart mitochondria and either beef heart (●) (0.5 unit/ml in A and 1.0 unit/ml in B) or beef liver (■) (0.75 unit/ml in A and 1.5 units/ml in B) phospholipid exchange protein. Conditions are described in Materials and Methods. Each data point represents duplicate incubations which agreed to within 5% of the value. All values are initial rates with correction for the small amount of non-catalyzed transfer.

Fig. 2. Transfer of [^{32}P]phosphatidylcholine from liposomes to multilamellar vesicles (expressed as percent of [^{32}P]phosphatidylcholine present in liposomes at time zero). Liposomes of [^{32}P]phosphatidylcholine and varying amounts of phosphatidylinositol were incubated with an excess of multilamellar vesicles of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (70 : 25 : 5, mol%) and either beef heart (●) or beef liver (■) phospholipid exchange protein (1.0 unit/ml). Conditions are described in Materials and Methods. Each data point represents duplicate incubations which agreed to within 5% of the value. All values are initial rates with correction for the small amount of non-catalyzed transfer.

developed [13]. This system involves the use of multilamellar vesicles as acceptor particles in place of mitochondria. One of the advantages of this system is the lack of any charge-dependent co-sedimentation of liposomes with the

TABLE I

RECOVERY OF LIPOSOMES AFTER INCUBATION WITH MITOCHONDRIA OR MULTILAMELLAR VESICLES

Incubations of liposomes with mitochondria or multilamellar vesicles of phosphatidylcholine : phosphatidylethanolamine : cardiolipin (70 : 25 : 5, mol%) for 40 min at 37°C. Liposomes contained [^{3}H]triolein as nontransferrable marker. Conditions are described under Methods. Results are expressed as percent [^{3}H]triolein in the supernatant.

mol % phosphatidylinositol	Acceptor particle		
	Mitochondria	Mitochondria + bovine serum albumin *	Multilamellar vesicles
0	86	98	95
7	75	96	99
14	55	89	97
20	42	67	92

* In the presence of 5 mg/ml of crystalline bovine serum albumin.

multilamellar vesicles (Table I). The excellent recovery removes the possibility of liposome heterogeneity causing biased results [13]. Liposomes composed of labeled phosphatidylcholine and varying amounts of phosphatidylinositol were incubated with multilamellar vesicles of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (70 : 25 : 5, mol%) and beef heart or beef liver exchange protein. With either beef liver or beef heart protein the initial rate of catalyzed transfer of [^{32}P]phosphatidylcholine from liposome to multilamellar vesicle was stimulated markedly by the presence of acidic phospholipid in the liposome (Fig. 2).

Multilamellar vesicles (phosphatidylcholine/phosphatidylethanolamine/cardiolipin, 70 : 25 : 5, mol%) may be prepared with [^{32}P]phosphatidylcholine and used as donor particles in an assay system which is analogous in terms of direction of transfer and choice of acceptor particle to that of Wirtz et al. [5] in which [^{14}C]phosphatidylcholine-labeled mitochondria were employed as donor particles. In our experiments multilamellar vesicles were incubated with an excess of unlabeled liposomes of varying composition. The liposome to protein ratio in this system is considerably higher than in the liposome donor system. As shown in Fig. 3, the initial rate of catalyzed transfer of [^{32}P]phosphatidylcholine from multilamellar vesicles to liposomes reaches a maximum for both proteins at 7.5 mol% phosphatidylinositol in phosphatidylcholine liposomes. The beef heart protein activity does not fall below the value for an uncharged surface; whereas, the beef liver activity is inhibited at greater than 15 mol% phosphatidylinositol. Although the phosphatidylcholine transferred, expressed as a percentage of that present in the multilamellar vesicles, is much smaller than that observed in the previous experiments, the accuracy of the measurements with the multilamellar vesicles is excellent. This is due, in part, to the fact that the amount of ^{32}P transferred is measured in the supernatant

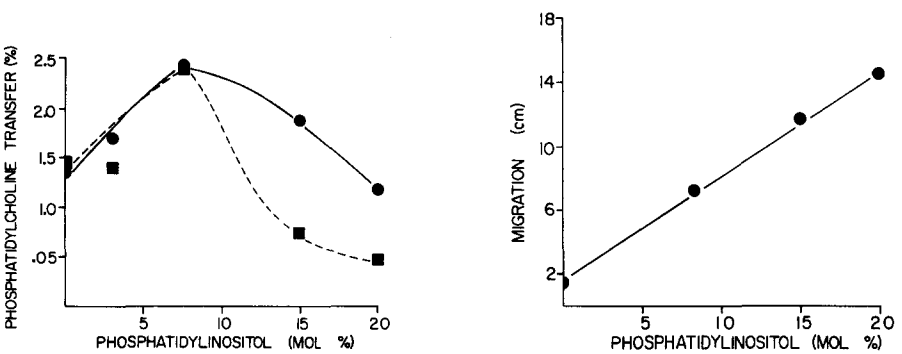


Fig. 3. Transfer of [^{32}P]phosphatidylcholine from multilamellar vesicles to liposomes (expressed as percent of [^{32}P]phosphatidylcholine present in multilamellar vesicles at time zero). Multilamellar vesicles of [^{32}P]phospahtidylcholine/phosphatidylethanolamine/cardiolipin (70 : 25 : 5, mol%) were incubated with an excess of liposomes composed of phosphatidylcholine and varying amounts of phosphatidylinositol in the presence of beef heart (●) or beef liver (■) exchange protein (0.8 unit/ml). Conditions are decribed in Materials and Methods. All values are initial rates with correction for the small amount of non-catalyzed transfer.

Fig. 4. Migration of phosphatidylcholine/phosphatidylinositol liposomes in polyvinylchloride (Geon-Pevikon) block electrophoresis. Conditions are described in Materials and Methods. Electrophoretic mobilities for each type of liposome have been calculated, and are given in the text.

which contains no ^{32}P at time zero. Blank exchange without exchange protein in this system is less than 0.1%.

The change in surface charge of the liposome by addition of acidic phospholipid was measured directly by electrophoresis of the vesicles in a polyvinylchloride (Geon-Pevikon) resin [16,17]. The vesicles were radioactively labeled with either [^{3}H]triolein or [^{32}P]phosphatidylcholine. The migration of the mixed phospholipid vesicles was found to increase linearly with phosphatidylinositol content (Fig. 4). The electrophoresis is carried out for 16 h at a potential gradient of 10 V/cm. Assuming that the migration is linear with time, one can calculate the electrophoretic mobility of these particles. The calculated values are $2.6 \cdot 10^{-6}$, $13.0 \cdot 10^{-6}$, $20.8 \cdot 10^{-6}$ and $26.0 \cdot 10^{-6}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$ for 0, 7.5, 15 and 20 mol% phosphatidylinositol. respectively.

Discussion

Many proteins which act at interfaces, such as phospholipase B from *Penicillium notatum* and phospholipase C from *Clostridium perfringens* [19], exhibit a dependency of activity on the surface charge of the substrate. Phospholipid exchange proteins interact directly with interfaces since they catalyze the transfer of phospholipids between membrane bilayers or monolayers. Hellings et al. [4] observed an inhibition of beef liver exchange protein activity in a liposome-liposome assay system when increasing amounts of acidic phospholipids were included in the sonicated vesicles. Van den Besselaar et al. [20] derived a theoretical rate equation to fit the liposome-liposome exchange data and determined that the apparent dissociation constant of the exchange protein-liposome complex decreases with an increasing acidic phospholipid content of the liposome. Similarly, Wirtz et al. [5] observed an inhibition in the rate of beef liver exchange protein-catalyzed transfer of phosphatidylcholine from mitochondria to liposomes when negatively charged phospholipids were incorporated into the phosphatidylcholine-containing vesicles.

The dependence of the beef heart exchange protein activity on surface charge of the membrane had not been systematically studied, though preliminary observations of a stimulatory effect by negative charge were reported by Zilversmit and Hughes [2]. These contradictory data might be explained by a difference in the source of the protein, heart vs. liver, or by differences in assay conditions. In the present study we compared beef heart exchange protein to beef liver exchange protein under identical conditions in three different assay systems. The liposome-liposome system of Hellings et al. [4] was not employed for two reasons: (1) the recovery of the acceptor particle varies greatly and can be as low as 40% and (2) the separation of the two populations by anion-exchange chromatography requires at least 7 mol% acidic phospholipid; therefore, one is not able to study the effect of exchange protein in the absence of potentially inhibitory molecules. The transfer of phosphatidylcholine from rat liver mitochondria to liposomes, as described by Wirtz et al. [5], was found to be unsuitable due to the large amount of solubilization of mitochondrial phospholipid and protein during the incubation, and the large effect of charge on phosphatidylcholine transfer in the absence of exchange protein.

Three different assay systems were chosen to test the effect of negative

charge on phospholipid exchange. Transfer of phosphatidylcholine from liposomes to mitochondria or from liposomes to multilamellar vesicles of phosphatidylcholine/phosphatidylethanolamine/cardiolipin was monitored in the presence of beef liver and beef heart exchange proteins. The liposome surface was given a negative charge by the incorporation of phosphatidylinositol or phosphatidic acid. Neither protein was inhibited by the surface charge regardless of the acceptor particle. In fact, an apparent stimulation of catalyzed transfer with increasing acidic phospholipid was observed.

Catalyzed transfer from multilamellar vesicles to liposomes exhibits a maximum at approx. 7.5 mol% phosphatidylinositol. This system is analagous to that employed by Wirtz et al. [5] in which inhibition was observed. The data reported by these authors, however, may not be inconsistent with our own, the optimum may have been missed due to the acidic phospholipid concentrations they chose. It is also possible that the small ratios of donor to acceptor phospholipid used in our assays could have minimized, or even reversed, the charge effects observed by Wirtz et al. [5]. In the study by van den Besselaar et al. [20] the degree of exchange inhibition by negative surface charge clearly depended on this ratio and varied from no inhibition to inhibition of 70% when the donor to acceptor ratio was changed from 0.2 to 1.2.

The reason for the maximum activity value in the multilamellar vesicle donor system and not in the other ones studied is not clear. It is most likely not a question of adequate lipid dispersion. The phosphatidylcholine liposomes employed in these studies have been shown by extensive exchange experiments similar to those of Johnson et al. [21] and by Sepharose 4B chromatography to be nearly completely single-walled vesicles with 65—70% available phospholipid. Addition of acidic phospholipid to phosphatidylcholine liposomes reportedly causes an asymmetric distribution of lipids across the bilayer [22]; however, at these concentrations of acidic phospholipid the phosphatidylcholine available pool size would not be expected to change by more than 10%.

Apparently other factors than substrate surface charge may play a role in the regulation of exchange protein activity. We conclude from these results that the effect of membrane surface charge on exchange protein activity is highly dependent on the system under study.

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