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PHOSPHATIDYLINOSITOL TRANSFER PROTEIN FROM BOVINE BRAIN

SUBSTRATE SPECIFICITY AND MEMBRANE BINDING PROPERTIES

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A recently developed fluorimetric transfer assay (Somerharju, P., Brockerhoff, H. and Wirtz, K.W.A. (1981) Biochim. Biophys. Acta 649, 521–528) has been applied to study the substrate specificity and membrane binding of the phosphatidylinositol-transfer protein from bovine brain. The substrate specificity was investigated by measuring the rate of transfer, either directly or indirectly, for a series of phosphatidylinositol analogues which included phosphatidic acid, phosphatidylglycerol as well as three lipids obtained from yeast phosphatidylinositol by partial periodate oxidation and subsequent borohydride reduction. Phosphatidylglycerol and the oxidation products of phosphatidylinositol were transferred at about one tenth of the rate observed for phosphatidylinositol while phosphatidic acid was not transferred. It is concluded that an intact inositol moiety favours the formation of the putative transfer protein-phosphatidylinositol complex. In addition to phosphatidylinositol, the transfer protein also transfers phosphatidylcholine. In order to obtain information on the possible occurrence of two sites of interaction, vesicles consisting of either pure 1-acyl-2-parinaroylphosphatidylinositol or 1-acyl-2-parinaroylphosphatidylcholine were titrated with the protein. Binding of labeled phospholipid to the protein was represented by an increase of lipid fluorescence and found to be much more efficient for phosphatidylinositol than for phosphatidylcholine. This is interpreted to indicate that the protein contains an endogenous phosphatidylinositol molecule which can be easily replaced by exogenous phosphatidylinositol but not by phosphatidylcholine, a lipid with a lower affinity for this protein. Thus the binding sites for the two phospholipids are mutually exclusive, i.e. phosphatidylinositol and phosphatidylcholine cannot be bound to the protein simultaneously. Finally, the effect of acidic phospholipids on the transfer protein activity was studied either by varying the content of phosphatidic acid in the acceptor vesicles or by adding vesicles of pure acidic phospholipids to the normal assay system. The latter vesicles consisted of either phosphatidic acid, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol or cardiolipin. In both instances the transfer protein activity was inhibited, obviously through the enhanced association of the protein with the negatively charged vesicles. These findings strongly suggest that relatively nonspecific ionic forces rather than specific protein-phospholipid headgroup interactions contribute to the association of the phosphatidylinositol-transfer protein with membranes.

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Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid.

Introduction

Phospholipid-transfer proteins are soluble, cytoplasmic proteins capable of stimulating transfer

of phospholipids between various natural and artificial membranes and lipoprotein particles (for a recent review, see Ref. 1). So far, three such proteins have been purified to homogeneity and have been shown to differ in substrate specificity and other properties like molecular weight, isoelectric point and amino acid composition. Perhaps the most interesting of these proteins is the phosphatidylinositol-transfer protein, not only because of its possible involvement in the so-called phosphatidylinositol cycle [2], but also because of its striking specificity pattern: it transfers both PI and PC while other phospholipids studied so far are poor substrates [3–5]. There is clear evidence that the phosphatidylinositol-transfer protein acts as a phospholipid carrier [4,6,7]. The carrier mechanism includes formation of a specific, freely diffusing protein-phospholipid complex. In view of the differences in structure of PI and PC it has been proposed that the protein has two distinct, highly specific binding sites for the inositol and choline moiety [5].

Interaction with membranes presumably precedes the formation of the protein-phospholipid complex. This interaction which involves a hypothetical membrane recognition site on the protein, seems to be sensitive to such membrane properties as fluidity of the membrane core [8] and phospholipid composition [6,9,10]. Moreover, it has been reported that the phosphatidylinositol-transfer protein binds strongly to vesicles of pure PI, but not to vesicles of pure PS or PG [10]. This suggests that the interaction of the protein with membrane interfaces is highly specific rather than being governed by nonspecific ionic or hydrophobic forces.

Recently, protein-catalyzed phospholipid transfer has been investigated with fluorescent analogues of PC and PI containing a *cis*-parinaroyl moiety at the *sn*-2-position [11,12]. In the present study the substrate specificity as well as the membrane binding properties of the phosphatidylinositol-transfer protein from bovine brain have been further elucidated by making use of these fluorescently labeled phospholipids.

Materials and Methods

Phospholipids. Egg yolk PC was obtained from Makor Chemicals (Israel). It was converted into lysophosphatidylcholine by phospholipase A_2-catalyzed hydrolysis [13], and into PG and PA by phospholipase D (Brussels sprouts) catalyzed transphosphatidylation or hydrolysis, respectively [14]. PI and cardiolipin were prepared from autolyzed yeast [15] and purified as described previously [12]. 2-Parinaroyl-phosphatidylcholine was synthesized from lysophosphatidylcholine and *cis*-parinaric acid (Molecular Probes, Plano, Texas, U.S.A.) as described elsewhere [11]. 2-Parinaroylphosphatidylethanolamine, 2-parinaroylphosphatidylglycerol and 2-parinaroylphosphatidic acid were obtained from 2-parinaroylphosphatidylcholine by treatment with phospholipase D [14]. 2-Parinaroylphosphatidylinositol was synthesized by a method recently described [12]. Briefly, this method involved the following steps: (1) acetylation of the free hydroxyl groups on the inositol moiety; (2) cleavage of the *sn*-2-fatty acyl residue by phospholipase A_2; (3) reacylation with parinaroyl anhydride and (4) removal of the acetyl groups by mild alkaline hydrolysis.

Preparation of phosphatidylinositol derivatives. A series of PI derivatives were prepared from yeast PI by partial periodate oxidation and subsequent borohydride reduction. PI (100 μmol) dissolved in 63 ml of chloroform/methanol (1 : 2, v/v) was mixed with 12 ml of aqueous solution of sodium periodate (2 mmol) and the mixture was incubated for 20 h at 20°C. Next 57 ml of chloroform and 18 ml of 0.9% NaCl solution were added, and after vigorous mixing, the phases were separated by low-speed centrifugation. The lower phase was washed once with half a volume of methanol/water (1 : 1, v/v) and subsequently mixed with 5 ml of methanol and 1 mmol of sodium borohydride dissolved in 1 ml of 50 mM $NaHCO_3$. After 4 h of incubation at 20°C, 7.5 ml of 0.9% NaCl solution and 2.5 ml of methanol were added and, after mixing, the phases were separated as above and the lower phase washed once with half a volume of methanol/water (1 : 1, v/v). Finally, the lower phase was taken to dryness, the lipids dissolved in chloroform and purified by preparative thin-layer chromatography using Merck Kieselgel 60 plates developed with chloroform/methanol/25% ammonia/water (90 : 54 : 5.5 : 5.5, v/v). The three major products obtained were partially characterized by infrared spectroscopy and analytical

thin-layer chromatography on normal and borate impregnated Kieselgel 60 plates using the aforementioned solvent system. The borate impregnated plates were prepared by spraying the ready-made plates with 0.4 M boric acid solution until wet followed by reactivation at 120°C for 3 h. The R_F values and the infrared spectroscopy data of the purified derivatives designated PI-D_1, PI-D_2 and PI-D_3, are given in Table I. The R_F values on normal silica gel plates indicate that the molecular weights decrease in the order PI-D_3 > PI-D_2 and PI-D_1. PI-D_1 runs slightly faster than PG both on normal and borate-impregnated plates. This strongly suggests that PI-D_1 has the structure of phosphatidyl-*sn*-2-glycerol, the smallest product theoretically obtainable from PI by periodate oxidation and borohydride reduction. The very low R_F value of PI-D_3 on borate-impregnated plate relative to that on normal plate, suggests that this lipid can form a complex with two borate residues simultaneously and, consequently, has at least four free hydroxyl groups. The infrared spectroscopy data support the conclusions made on the basis of chromatographic behaviour of the three lipids (Table I). By calculating the ratio of absorbance at the OH stretching region (3250 cm^{-1}) to that at the carbonyl stretching region (1740 cm^{-1}) for each lipid and by comparing these values with those obtained for PI (the standard) the number of free hydroxyl groups in each lipid was calculated. The results indicate that PI-D_1, PI-D_2 and PI-D_3 contain 2, 3 and 4 free hydroxyl groups, respectively.

TABLE I

CHROMATOGRAPHIC AND INFRARED SPECTROSCOPY DATA FOR DERIVATIVES OF PHOSPHATIDYLINOSITOL

Lipid	R_F		$A_{OH}/A_{C=O}$ [a]	Number of hydroxyl groups [b]
	Normal plate	Borate plate		
PI	0.30	0.13	0.62	5
PI-D_1	0.62	0.39	0.27	2.2
PI-D_2	0.56	0.22	0.34	2.7
PI-D_3	0.47	0.07	0.46	3.7
PG	0.57	0.32	-	

[a] Ratio of absorbance at 3250 cm^{-1} (-OH stretching) to absorbance at 1740 cm^{-1} (C=O stretching).

[b] Numbers have been calculated from the $A_{OH}/A_{C=O}$ ratio of PI which contains five free hydroxyl groups. We assume that each hydroxyl group contributes equally to the $A_{OH}/A_{C=O}$ ratio.

Phospholipid-transfer proteins. Phosphatidylinositol-transfer protein from bovine brain and phosphatidylcholine-transfer protein from bovine liver were purified according to established procedures [4,16].

Preparation of vesicles. Vesicles containing only unlabeled phospholipids were prepared by the following procedure: lipids were mixed in chloroform and taken to dryness in a stream of nitrogen. Dry lipids were then dispersed in the appropriate buffer and the suspension sonicated under nitrogen atmosphere for 10 min at 0°C with a Branson sonicator. The suspension was centrifuged for 15 min at 20 000 × *g* to remove titanium particles and any undispersed lipid. Vesicles containing labeled phospholipids were prepared by the ethanol injection procedure [17]. Lipids were mixed in 95% ethanol (Merck, Fluorescence grade) and injected into a buffer solution with a Hamilton syringe. Final ethanol concentration was always below 1%.

Fluorimetric measurements. These measurements were carried out essentially as described previously [11]. Excitation and emission wavelength were 325 nm and 415 nm, respectively, and excitation and emission slits were 1.5 and 40 nm, respectively. In the phospholipid-transfer assay, the donor vesicles containing the fluorescent phospholipids were prepared in the cuvette by injection of the lipids dissolved in ethanol (2–10 μl) into 2 ml of 20 mM Tris/5 mM EDTA (pH 7.4). After 10 min of equilibration, transfer was initiated by subsequent addition of acceptor vesicles and phospholipid-transfer protein. For further details, see legend to figures. To avoid oxidation of the parinaroyl chromophore 2 mol% of 2,6-di-*tert*-butyl-4-methylphenol (BHT, Fluka) was included in the acceptor and donor vesicles.

Infrared spectroscopy. Lipids were applied on KBr pellets in dry chloroform and after complete removal of the solvent the spectra were recorded. Samples were then vacuum dried for 0.5 h and the spectra recorded again. The spectra obtained before and after vacuum drying were identical indicating that no water (which would contribute to

the absorbance in the OH stretching region) remained in the samples.

Results

Selfquenching of fluorescent phospholipids

In studying the properties of the phosphatidylinositol-transfer protein use has been made of phospholipids containing a *cis*-parinaroyl moiety at the *sn*-2-position. Vesicles prepared of these labeled lipids display a very low fluorescence due to frequent probe-probe interactions. This selfquenching phenomenon is clearly demonstrated in Fig. 1 where the fluorescence of parinaroyl-phosphatidylcholine in vesicles is presented as a function of dilution with unlabeled PC. As one sees fluorescence remains efficiently quenched as long as the vesicles contain less than 60% unlabeled PC. This is a most useful property as it allows considerable modification of the vesicle phospholipid composition without loss of selfquenching (see below). In the assay used here transfer of labeled phospholipid is measured spectroscopically by mixing quenched donor vesicles containing labeled phospholipid and acceptor vesicles consisting of unlabeled phospholipid. In time, fluorescence of such a mixture of vesicles remains low as the labeled phospholipid stays with the donor vesicle. Upon addition of a phospholipid transfer protein, however, a time-dependent increase of fluorescence intensity is observed

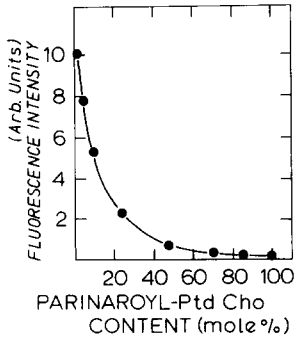

Fig. 1. Fluorescence of parinaroylphosphatidylcholine as a function of dilution with unlabeled PC. A fixed amount (10^{-9} mol) of parinaroylphosphatidylcholine was mixed with varying amounts of egg yolk PC in ethanol and injected into a cuvette containing 2 ml Tris-EDTA buffer. The fluorescence intensity was recorded after 5 min of equilibration.

[11,12]. This increase is due to an incorporation of labeled phospholipid into the unlabeled acceptor vesicles which effectively eliminates selfquenching of the fluorescent probe. The initial fluorescence increase is proportional to the rate of transfer and assumed to be related to the affinity of a phospholipid-transfer protein for the labeled phospholipid.

Substrate specificity

In previous studies it was found that the phosphatidylinositol-transfer protein has a distinct preference for PI but also transfers PC; transfer of PE was negligible [5,18]. Here we have extended these studies by comparing the rates of transfer of parinaroylphosphatidylglycerol and parinaroylphosphatidic acid with that of parinaroylphosphatidylinositol. Donor vesicles were prepared consisting of one of these acidic phospholipids (30 mol%) and parinaroyl- phosphatidylethanolamine (70 mol%). The nontransferable PE was included to avoid an excessive negative charge density on the donor vesicle as this is inhibitory to the transfer protein [6,7]. Labeled PE was used instead of unlabeled one to assure an efficient fluorescence quenching of the donor vesicle. The unlabeled acceptor vesicles consisted of egg yolk PC (98 mol%) and PA (2 mol%). As shown in Fig. 2, addition of phosphatidylinositol-transfer protein to the donor-acceptor vesicle mixtures resulted in a rapid transfer of PI (curve A), a distinct but much slower transfer of PG (curve B), and no transfer of PA (curve C). The latter curve was indistinguishable from the blank, even if the concentration of the transfer protein was increased four-fold. When donor vesicles consisting of unlabeled PI or PG (30 mol%) and parinaroylphosphatidylethanolamine (70% mol%) were used, addition of the transfer protein had no effect on the rate of fluorescence increased (curve D); this confirms the absence of PE transfer.

Substrate specificity is most directly determined when the phospholipid of interest contains a fluorescent probe. In case the phospholipid to be tested is nonfluorescent the affinity of the phosphatidylinositol-transfer protein for this phospholipid can be assessed by an indirect method. This method is based on the data shown in Fig. 3. In this experiment the donor vesicles consisted of

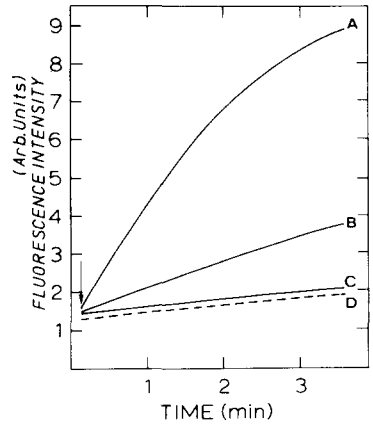

Fig. 2. Transfer of labeled PI, PG and PA by phosphatidylinositol-transfer protein. Donor vesicles consisted of 70 mol% of parinaroylphosphatidylethanolamine and 30 mol% of either parinaroylphosphatidylinositol (curve A), parinaroylphosphatidylglycerol (curve B), parinaroylphosphatidic acid (curve C) or unlabeled PI or PG (curve D). Acceptor vesicles consisted of egg yolk PC/PA (92:2, mol%). Acceptor vesicles (125 nmol lipid phosphorus) were added to the donor vesicles (7.5 nmol lipid phosphorus) in 2 ml Tris-EDTA/200 mM NaCl buffer; transfer was initiated by addition of 3 μg transfer protein. The curves were not corrected for the blank which was identical to curve D.

labeled PC for which the transfer protein has a relatively low affinity, and various amounts of unlabeled PI which is the preferred substrate. Upon addition of transfer protein and unlabeled acceptor vesicles the fluorescence increased with time due to transfer of labeled PC (Fig. 3A). Interestingly, the regular hyperbolic progress curve obtained for donor vesicles with 7 mol% PI (Fig. 3A, curve A) became increasingly sigmoidal when donor vesicles were used containing 17 and 26 mol% PI (Fig. 3A, curves B and C, respectively). As shown in Fig. 3A the initial rate of PC transfer decreased with an increasing PI concentration in the donor vesicles. This agrees with previous observations that the transfer protein is inhibited by increasing the negative surface charge on the vesicles [6,7]. In time, the rate of PC transfer increased progressively giving rise to a sigmoidal curve (Fig. 3A, curves B and C). This increase is due to the fact that the transfer protein preferentially transfers PI to the acceptor vesicles. In this process donor PI is replaced for acceptor PC resulting in a decrease of the donor vesicle surface charge and, consequently, in an increase of labeled PC transfer. In support of a charge effect governing the activity of the transfer protein, the distinctly sigmoidal character of the progress curve was much less prominent in the presence of 0.2 M NaCl (Fig. 3B, curve C).

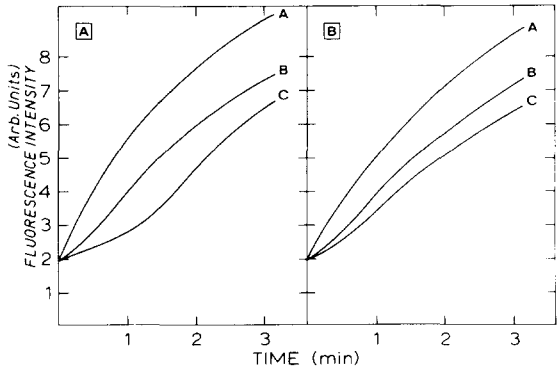

Fig. 3. Effect of PI concentration in the donor vesicles on the transfer of PC. Donor vesicles consisted of parinaroylphosphatidylcholine (3.2 nmol) and 9 mol% (curve A), 17 mol% (curve B) and 26 mol% (curve C) of unlabeled PI. Transfer was initiated by subsequent additions of acceptor vesicles (250 nmol lipid phosphorus) and transfer protein (3 μg) as in the legend to Fig. 2. Transfer reaction proceeded in 2 ml of Tris-EDTA (A) or Tris-EDTA/200 mM NaCl (B).

The effect of negatively charged phospholipids on the rate of transfer of labeled PC was further tested by incorporating either PI, PG or cardiolipin in the donor vesicles (Fig. 4). Incorporation of PG (25 mol%) instead of PI considerably reduced the extent of sigmoidality of the progress curve (curve B). This reflects the fact that PG is less efficiently transferred by the transfer protein than PI (see Fig. 2). Incorporation of cardiolipin (25 mol% of phosphorus) resulted in a relatively low rate of transfer of PC which was constant with time (curve C). The absence of sigmoidality indicates that transfer of cardiolipin is very slow or absent. No sigmoidality in the progress curves was observed when the phosphatidylcholine-transfer protein from bovine liver was added to any of the above donor vesicles (data not shown). This agrees with the absolute specificity of this protein for PC [19]. In conclusion it is evident that (1) sigmoidality is observed only if the negatively charged, i.e. affinity controlling phospholipid of the donor vesicles, is readily transferred by the transfer protein studied, and (2) the extent of sigmoidality

correlates positively with the rate of transfer of the acidic phospholipid.

As the phosphatidylinositol-transfer protein stimulated the transfer of PG, albeit less efficiently than that of PI, it was of interest to study the transfer of phospholipids with structures intermediate to those of PG and PI. Such structural intermediates were prepared from PI by periodate oxidation and subsequent borohydride reduction (see Materials and Methods). This preparation rendered the analogues PI-D_1, PI-D_2 and PI-D_3 which probably contain two, three and four free hydroxyl groups, respectively. Inherent to the method of preparation, labeled PI could not be used as the starting material. We, therefore, tested transfer of the unlabeled analogues PI-D_1, PI-D_2 and PI-D_3 by the indirect method. As shown in Fig. 5 all three analogues gave slightly sigmoidal curves for labeled PC transfer which closely resembled those obtained for PG (see Fig. 4). This indicates that their rate of transfer is of the same order as that of PG and thus about an order of magnitude lower than the rate of PI transfer.

Binding of PI and PC to transfer protein

The relationship between PI and PC binding sites in the phosphatidylinositol-transfer protein is an important but unanswered question [5,7]. We approached this problem by titrating vesicles prepared of parinaroylphosphatidylinositol or parinaroylphosphatidylcholine with the transfer protein. Binding of a labeled phospholipid molecule to the protein will result in an increase of the lipid fluorescence intensity due to elimination of probe-probe interactions prevalent in the vesicle membrane. As shown in Fig. 6, titration of both kinds of vesicles (0.5 nmol lipid phosphorus) gave an increase of fluorescence intensity, indicating that the transfer protein can bind both PI and PC. However, with PI vesicles the fluorescence steadily increased up to addition of 0.45 nmol transfer protein, whereas with PC vesicles the fluorescence increase leveled off at 0.1 nmol transfer protein. This is taken to indicate that the transfer protein prefers to bind PI over PC (see Discussion).

Effect of membrane surface charge

It has been reported that the binding of the phosphatidylinositol-transfer protein to phospholipid vesicles containing PI is strongly enhanced by specific interactions with PI present in the interface rather than by nonspecific ionic forces [10]. We decided to reinvestigate this question because cardiolipin seemed to inhibit the transfer of PC presumably by increasing the affinity of the transfer protein for the membrane (see Fig. 4). Two different approaches were used. First, we

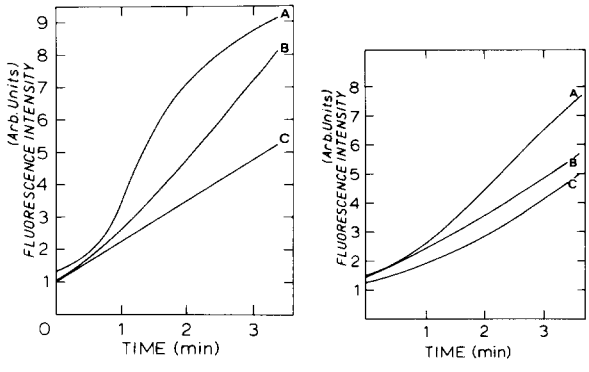

Fig. 4. Effect of acidic phospholipids in the donor vesicles on the transfer of PC. Donor vesicles consisted of parinaroylphosphatidylcholine (3.2 nmol) and 25 mol% of either unlabeled PI (curve A), PG (curve B) or cardiolipin (curve C) in 2 ml of Tris-EDTA buffer. Further conditions of transfer as in legend to Fig. 3.

Fig. 5. Effect of derivatives of PI in the donor vesicles on the transfer of PC. Donor vesicles consisted of parinaroylphosphatidylcholine (3.2 nmol) and 25 mol% of either unlabeled PI-D_1 (curve A), PI-D_2 (curve B) or PI-D_3 (curve C). Further conditions of transfer as in legend to Fig. 3.

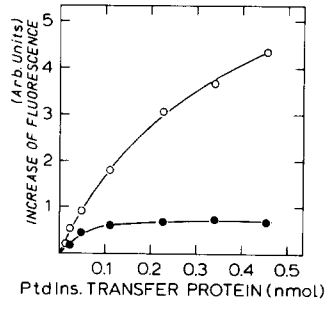

Fig. 6. Binding of labeled PI and PC to phosphatidylinositol-transfer protein. Vesicles (0.5 nmol lipid phosphorus) consisting of either parinaroylphosphatidylinositol (○———○) or parinaroylphosphatidylcholine (●———●) in 2 ml of Tris-EDTA/200 mM NaCl buffer were titrated with transfer protein and the increase of fluorescence plotted as a function of the protein concentration.

studied how the concentration of PA, a nontransferable phospholipid in the acceptor membrane affected the rate of PC transfer by the transfer protein. The acceptor vesicles used contained egg yolk PC and 8 and 17 mol% PA, respectively. As indicated in Fig. 7, rates of transfer were determined under conditions where the acceptor phospholipid concentration was variable and the donor phospholipid concentration constant. The results show that, while PC transfer was only slightly affected by the PA content at low acceptor concentrations, the rates became strongly dependent on this parameter at higher vesicle concentrations, the more charged vesicles being more inhibitory. This behaviour is most probably due to a decrease in the dissociation constant of the protein-acceptor vesicle complex when the acceptor PA content increases [6]. Thus, ionic interactions seem to contribute strongly to the binding of the phosphatidylinositol-transfer protein to phospholipid vesicles.

As a second approach, the effect of vesicles consisting of pure acidic phospholipid on the transfer of PC between normal donor and acceptor vesicles by the transfer protein was studied. The rationale of this type of experiment, first introduced by Machida and Ohnishi [20], is that if the transfer protein binds to acidic phospholipid vesicles, the rate of PC transfer is inhibited. A

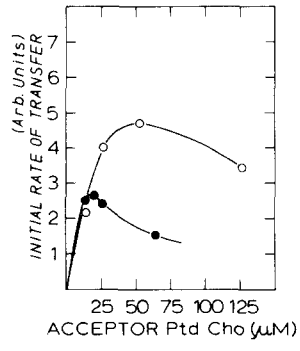

Fig. 7. Effect of phosphatidic acid (PA) concentration in the acceptor vesicles on the rate of PC transfer. The donor vesicles consisted of parinaroylphosphatidylcholine and PA (10 mol%) and the acceptor vesicles of PC and 8 mol% (○——○) or 17 mol% (●——●) PA. Increasing amounts of acceptor vesicles were added to the donor vesicles (5.5 nmol lipid phosphorus) in 2 ml of Tris-EDTA buffer and the initial rate of transfer measured upon addition of 0.4 μg of phosphatidylinositol-transfer protein.

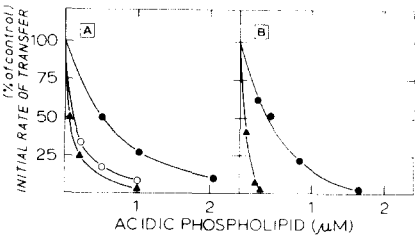

Fig. 8. Effect of vesicles consisting of pure acidic phospholipids on the rate of PC transfer. Donor vesicles consisted of parinaroylphosphatidylcholine and PA (10 mol%) and the acceptor vesicles of PC and PA (2 mol%). After mixing the donor (5.5 nmol lipid phosphorus) and acceptor vesicles (250 nmol lipid phosphorus) increasing amounts of vesicles consisting of either pure PI (●——●), PG (○——○) or PA (▲——▲) were added. Transfer was initiated by addition of 0.4 μg of phosphatidylinositol-transfer protein. Transfer reactions proceeded in 2 ml of Tris-EDTA/200 mM NaCl (A) or Tris-EDTA buffer (B).

marked inhibition of PC transfer was observed when vesicles consisting of either PA, PG and PI were added to the assay system (Fig. 8A). Vesicles consisting of PA were the most efficient inhibitors; PG vesicles were slightly less and PI vesicles considerably less inhibitory at equal concentrations. Similar experiments carried out with cardiolipin and PS showed that cardiolipin behaved very much like PA (at equimolar phosphate concentrations) and PS like PG (data not shown). All these experiments were performed in a buffer containing 0.2 M NaCl; omission of salt somewhat enhanced the inhibition by the acid phospholipid vesicles (Fig. 8B). Altogether, these results support the conclusion that nonspecific ionic interactions strongly influence the association of the phosphatidylinositol-transfer protein with phospholipid vesicles.

Discussion

It has been reported earlier that the phosphatidylinositol-transfer protein from bovine brain can transfer both PI and PC, the rate of PI transfer being about 10-fold higher than that of PC [3,4,21]. No transfer of PE, PS or PA could be detected [7,22]. For the identical protein from bovine heart the relative rates of transfer of PI, PC, sphingomyelin and PE were 100, 35, 5 and 0.1, respectively [5]. Although the relative rates in these studies cannot be compared directly because of the

different assay systems used, it is evident that the phosphatidylinositol-transfer protein shows marked specificity towards PI and PC, structurally two quite different phospholipids. In the present study we have further investigated the substrate specificity of the transfer protein. By measuring directly the transfer of fluorescent phospholipid derivatives it was observed that the protein can also transfer PG, but in accordance with previous reports [5,7] does not transfer PE or PA (Fig. 2). Periodate oxidation of PI and subsequent reduction rendered three derivatives which supposedly contain two, three and four hydroxyl groups in the polar head moiety. By an indirect method evidence was obtained that these derivatives are transferred (Fig. 5). However, their rate of transfer as well as that of PG was an order of magnitude lower than that of PI. Thus it can be concluded that the intact inositol ring is a prerequisite for the phospholipid to be preferentially transferred by the transfer protein.

In an independent study transfer of PG by the transfer protein has been shown in the monolayer-vesicle assay [7]. By this approach evidence has also been obtained that the transfer protein can transfer analogues of PC in which the 3-phosphocholine headgroup has been replaced for 3-phospho-(*N*,*N*-dimethyl)ethanolamine or 3-phospho-(*N*,*N*,*N*-trimethylamino)hexanolamine [23]. Similar analogues are not transferred by the phosphatidylcholine-transfer protein from bovine liver [19]. The phosphatidylinositol-transfer protein is also less discriminative towards modifications in the glycerol-hydrocarbon bonding region of PC than the phosphatidylcholine-transfer protein [7,11]. These results indicate that the phosphatidylinositol-transfer protein is much more sensitive for alterations in the phosphorylinositol than in the phosphorylcholine headgroup. This, in fact, may be the reason why this protein preferably transfers PI.

In studies with acceptor vesicles that lack PI it has been shown that the phosphatidylinositol-transfer protein can transfer PI to these membranes [4,21]. Insertion of PI is compensated for by a transfer of PC in the opposite direction, so that we are dealing with a mechanism of true molecular exchange. Thus, despite the distinct preference for PI, the transfer protein releases PI in a deficient membrane. This raises questions about the affinity of the binding site(s) for PI and PC [4].

In the present study binding was conveniently assayed by titrating vesicles of parinaroylphosphatidylinositol or parinaroylphosphatidylcholine with the transfer protein (Fig. 6). Binding of the labeled lipid to the protein results in an enhanced fluorescence due to the elimination of quenching by probe-probe interactions. This binding is linked to the release of endogenous phospholipid present on the protein [7]. The extent of binding obviously depends on the relative binding affinities of the two phospholipids. In agreement with previous studies [4] binding of PC to the transfer protein was found to be much less extensive than binding of PI (Fig. 6). This difference in binding is most easily explained by assuming that upon isolation the phosphatidylinositol-transfer protein contains an endogenous PI molecule and, secondly, the PI and PC binding sites are mutually exclusive. This endogenous PI molecule could be readily exchanged by a vesicle PI molecule. In contrast, exchange of endogenous PI for vesicle PC stops at a molar protein to vesicle PC ratio of 0.2 (see Fig. 6). This apparent limit to the exchangeability of vesicle PC reflects the fact that, in the course of titration, the PC vesicles become enriched in PI. Then the high affinity of the transfer protein for PI prevents additional exchange of vesicle PC. If the phosphatidylinositol-transfer protein would contain PC as the endogenous phospholipid, one would expect this PC to be readily displaced by both PI and PC giving similar maximal fluorescence intensities. A comparable result is expected if the PI and PC binding sites would be independent. In conclusion, it appears that the binding of PI to the protein prevents the binding of PC. Whether this is due to a physical overlap of the two binding sites in the hydrocarbon region [5] or results from conformational effects, remains to be clarified.

Recently experiments have been performed in which the transfer protein loaded with either [^{14}C]PI or [^{14}C]PC were delivered under monolayers of nonlabeled phospholipids [7]. In support of our conclusions it was calculated from the appearance of radioactivity in the monolayer that [^{14}C]PI on the protein is exchanged for unlabeled

PI and [^{14}C]PC on the protein for unlabeled PC at almost identical rates. Moreover it was shown that [^{14}C]PC on the protein is 20-times more readily exchanged for PI than [^{14}C]PI on the protein for PC.

The efficient binding of parinaroyl phospholipids by the transfer proteins opens up many interesting perspectives for further studies. It becomes feasible to investigate the fast kinetics of both binding and release of phospholipids by these proteins with the use of a rapid-flow apparatus equipped with a fluorescence detector. Changes in fluorescence intensity or polarization are useful parameters for such studies. Another interesting application of the parinaroyl phospholipids is to study the spatial relationship of the PI and PC binding sites in the phosphatidylinositol-transfer protein. In this case use could be made of the energy transfer between protein tryptophane and parinaroyl phospholipids; if the PI and PC sites have considerable overlap in the hydrocarbon binding region, the efficiency of energy transfer should be independent of the parinaroyl lipid bound to the protein. Other properties of parinaroyl lipids such as the sensitivity of fluorescence lifetimes and optical activity to the environment should be also useful in these studies [24,25].

Recently Helmkamp [10] reported that the phosphatidylinositol-transfer protein binds specifically to phospholipid vesicles that contain PI. Binding was not observed when PI was replaced for other acidic phospholipids. This conclusion was based on two findings. First, incorporation of increasing amounts of PA, PS or PG into the acceptor vesicles did not inhibit transfer of PI and PC from the donor microsomal membranes; according to a previous study [26] similar concentrations of PI were strongly inhibitory. Secondly, addition of vesicles consisting of pure PG or PS to the assay mixture had no effect on the phospholipid transfer between microsomes and acceptor vesicles while vesicles or pure PI were inhibitory. The present results are in contradiction with these findings. Addition of PA to the acceptor vesicles clearly decreased the rate of lipid transfer at higher acceptor concentrations (Fig. 7). Moreover, all acidic phospholipids, when added as separate vesicles to the assay solution, effectively inhibited phospholipid transfer between donor and acceptor membranes (Fig. 8). The latter inhibition was salt concentration-dependent. Kinetic analyses have shown that inhibition of transfer is related to a decrease of the apparent dissociation constant for the protein-membrane complex [6]. Thus it seems likely that pure charge-charge interactions strongly contribute to the formation of the protein-membrane complex. The nonspecific inhibition of transfer by acidic phospholipids has been confirmed with the monolayer-vesicles system [7].

Vesicles of PA or cardiolipin inhibited the phosphatidylinositol-transfer protein more efficiently than PI vesicles (Fig. 8). This is probably due to a difference in charge shielding. The negatively charged phosphate moieties on the vesicles of PA or cardiolipin are obviously readily available for interactions with positively charged amino acid residues of the transfer protein while on the PI vesicles the bulky and strongly hydrated inositol headgroups [27] may interfere with this interaction. However, it cannot be excluded that differences in vesicle size and, consequently, in surface curvature are also involved. As shown by Machida and Ohnishi [28], binding of the bovine phosphatidylcholine-transfer protein to PS vesicles was strongly dependent on the vesicle curvature, the smallest vesicles having the highest affinity for the protein.

Although the present discussion has strongly emphasized the involvement of ionic interactions in the association of the transfer protein with vesicles, other factors also play an important part. So it has been demonstrated that the transfer activity is inhibited both by the incorporation of sphingomyelin into PC acceptor vesicles and by addition of pure sphingomyelin vesicles to the donor-acceptor membrane system [9,10,23]. The importance of phospholipid packing is accentuated by recent studies which indicated that transfer activity was strongly influenced by changes in membrane fluidity [10,21].

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