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The effect of polyphosphoinositides and phosphatidic acid on the phosphatidylinositol transfer protein from bovine brain: a kinetic study

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The phosphatidylinositol transfer protein from bovine brain (PI-TP) has lipid transfer characteristics which make it well suited to maintain phosphatidylinositol (PI) levels in intracellular membranes (Van Paridon, P.A., Gadella, Jr., T.W.J., Somerharju, P.J. and Wirtz, K.W.A. (1987) Biochim. Biophys. Acta 903, 68–77). Using a continuous fluorimetric transfer assay we have investigated in what way phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA) affect the transfer activity of this protein in model systems. The effects were analysed by application of a kinetic model which yielded the association constant (K) and dissociation rate constant (k_-) for the PI-TP/vesicle complex. Incorporation of PA, PIP and PIP_2 into the phosphatidylcholine-containing vesicles increased the association constant solely by diminishing the dissociation rate constant. This effect could be completely accounted for by changes in the membrane surface charge density. In contrast to the inhibitory effect of PA, the inhibition caused by PIP_2 was completely abolished by the addition of neomycin, in agreement with the observed preferential binding of this polyamine antibiotic to PIP_2. A rise in pH from 5.5 to 8 drastically reduced the association constant for vesicles containing 16 mol% PA (e.g., from 38 to 2 mM^{-1}), without affecting the V_{max}. This effect could be mainly attributed to an increase in the negative charge on PI-TP (isoelectric point 5.5), resulting in an enhanced repulsion. Increasing the negative membrane surface charge at pH 7.4 had the opposite effect. This is interpreted to indicate that the membrane interaction site on PI-TP must be positively charged, overcoming the repulsive forces between PI-TP and the vesicle. Addition of PIP_2 micelles as a third component in the transfer assay strongly inhibited PI-TP transfer activity. The extent of inhibition suggests a very high affinity of PI-TP for this lipid.

Introduction

The phosphatidylinositol-transfer protein (PI-TP) is a soluble cytosolic protein capable of transferring PI between various membranes, PI-TP has been isolated from a large number of sources, such as bovine brain [1], bovine heart [2], rat liver [3], human platelets [4] and yeast [5]. Apart from the preferred substrate PI, PI-TP is also able to bind and transfer PC [6,7], and has a very low affinity

Abbreviations: PI-TP, (bovine brain) phosphatidylinositol transfer protein; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; 1-Pal,2-Pyr(10)PC, 1-palmitoyl-2-pyrenedecanoylphosphatidylcholine; TNP-PE, trinitrophenyl-phosphatidylethanolamine.

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towards phosphatidylglycerol and sphingomyelin [8]. In phospholipid monolayer experiments, however, a somewhat different specificity pattern was observed, since in this case phosphatidylglycerol was also efficiently transferred [9]. The difference in specificity may be related to the extremely low affinity of the transfer protein for the monolayer. This remarkable specificity pattern with a distinct affinity for phospholipids that have completely different polar headgroups, has attracted a great deal of attention. In a recent paper [10] we have presented evidence to indicate that the dual specificity for PI and PC enables PI-TP to mediate a net transfer of PI towards a membrane deficient in PI, in return for a PC molecule. Several studies have shown that PI synthesis is specifically localized in the endoplasmic reticulum [11–15], and is spatially separated from the degradative pathways involved in the stimulus-induced phosphoinositide metabolism [16]. PI-TP may then function as a fine-tuning mechanism in the maintenance of membrane PI levels [17]. On the other hand, studies with a pituitary tumour cell line provide evidence that in this case PI synthesis may occur in the endoplasmic reticulum as well as in the plasma membrane [18]. This observation however could not be confirmed in other cell lines [19].

The dual specificity of PI-TP has considerably complicated the kinetic analysis of the lipid-transfer process. Helmkamp et al. [20] have shown that the kinetics can be analyzed in terms of a 'ping-pong bi-bi' model. However, due to the complexity of the mechanism, these authors have preferred to describe the properties of PI-TP in terms of Michaelis-Menten kinetics, which fails to provide detailed in sight in the individual steps of the transfer process [6,9,20–23]. Despite this limitation, the latter approach has provided valuable information on the effects of membrane surface charge [6,9,21], membrane fluidity [22], pH and ionic strength [23], and local anaesthetics [24] on the activity of PI-TP. It was shown that exchange of the endogenous phospholipid molecule for a PI or PC molecule from the interface is determined by the relative affinity of PI-TP for PI and PC, and by the relative abundance of these lipids in the membrane [10]. Membrane surface charge, a parameter which has a large influence on the lipid transfer rates [6,9], did not affect the specificity of the transfer protein. Hence, it appears that the interaction of PI-TP with a membrane surface, and the exchange of the endogenous lipid for a lipid molecule from the interface, are two separate processes.

In the present study we have investigated the effect of PA, PIP and PIP_2 on the interaction of PI-TP with a membrane surface. The data were analyzed according to the kinetic model proposed by Van den Besselaar et al. [25], yielding detailed information on the individual kinetic constants that describe the transfer process. To avoid complications caused by the dual specificity of the transfer protein, transfer of PC was measured between membranes devoid of PI. The kinetic analysis in this study was based on a highly accurate, continuous spectrofluorimetric transfer assay in which the transfer of pyrene-labeled PC was measured between phospholipid vesicles [10]. This study shows that PI-TP is very sensitive towards factors that influence the electrostatic interaction between this protein and the membrane interface. The extremely high affinity of PI-TP for phosphorylated PI species was especially noted.

Materials and Methods

Materials

Egg-yolk, PC, as well as PA and PG prepared from egg-yolk PC, was purchased from Sigma. PS, PIP and PIP_2 were isolated from bovine brain [26–28]. [^{3}H]Cholesteryl oleate (50 Ci/mmol) and [^{14}C]methyl iodide (58.8 Ci/mol) were obtained from Amersham. [^{14}C]PC was prepared by demethylation of egg-yolk PC and remethylation with [^{14}C]methyl iodide [29]. 1-Pal,2-Pyr(10)PC was synthesized from 1-palmitoyl-*sn*-glycero-3-phosphocholine and pyrenedecanoic acid [30–32]. TNP-PE was prepared from egg-yolk-PE and TNBS [33]. PI-TP was purified from bovine brain as described before [34]. Sepharose 4B was from Pharmacia.

Methods

Fluorimetric measurements. The measurements were performed using an SLM-Aminco SPF 500C spectrofluorimeter equipped with a thermostatically controlled cuvette holder and a magnetic

stirring device. Excitation and emission were at 346 nm and 378 nm, respectively. Excitation and emission slits were 10 nm.

Preparation of vesicles. Vesicles consisting of 1-Pal,2-Pyr(10)PC (i.e., donor vesicles) were prepared by injection of an ethanolic solution of the fluorescent lipid (0.1 mM in Merck Uvasol ethanol, 10 μl) into the cuvette containing 2 ml 20 mM Tris-HCl/5 mM EDTA/100 mM NaCl (pH 7.4) ('Tris buffer') [35]. The buffer was routinely filtered (Milipore, 0.45 μm). Prior to use, the donor vesicles were allowed a 2 min equilibration period.

Vesicles consisting of egg-yolk PC and varying amounts of PA, PIP or PIP_2 (i.e., acceptor vesicles) were prepared by drying down the lipid mixture from chloroform (or from chloroform/methanol/water, 80:20:1 (v/v) in the case of PIP and PIP_2), followed by suspension in the Tris buffer (1–10 mM phospholipid) and ultrasonication for 10 min at 0°C with a Branson sonicator under a nitrogen atmosphere. The vesicle preparation was centrifuged for 15 min at $20000 \times g$, to remove titanium particles and undispersed lipid. The lipid dispersion was subsequently applied to a Sepharose 4B column (0.9×30 cm), and eluted with Tris buffer (9.8 ml/h, 0.5 ml fractions). Routinely, the peak fractions that eluted between 18 and 20 ml were combined and used in the experiments. Sizing of the vesicles was necessary to avoid effects of vesicle size on PI-TP activity (unpublished observation, see also Ref. 36).

Vesicles consisting of pure negatively charged lipids (PI, PA, PG, PS, PIP and PIP_2) (inhibitory vesicles) were prepared by ultrasonication of the respective lipid emulsions (0.1 mM) in Tris buffer as described above.

Phospholipid transfer assay. The fluorescence transfer assay was carried out essentially as described before [10,31,37]. Briefly, acceptor vesicles (0.25–120 μM) were added to the donor vesicles (0.5 μM) in the cuvette. The spontaneous transfer of 1-Pal,2-Pyr(10)PC from donor to acceptor vesicles was recorded by following the increase in the pyrene monomer fluorescence intensity. Subsequentiy, PI-TP (1–2 μg) was added to initiate the protein-mediated lipid transfer reaction. The transfer rates were corrected for the spontaneous transfer, which in all cases was found to be less than 5% of the protein-mediated transfer. The pyrene monomer fluorescence was calibrated with a standard vesicle preparation consisting of 1-Pal,2-Pyr(10)PC diluted with egg-yolk PC (1:1000 mol/mol), so that the transfer rates could be expressed in pmol PyrPC transferred per min. The transfer assays were performed at 37°C, under continuous stirring, in a final volume of 2 ml.

The concentration of the fluorescent lipid dissolved in ethanol was estimated by measuring the absorbance at 346 nm on a Hitachi U-3200 spectrophotometer ($\varepsilon = 42000\ M^{-1} \cdot cm^{-1}$ [32]). Lipid phosphorus was determined as described by Rouser et al. [38].

The lipid concentration of the acceptor vesicles after chromatography on Sepharose 4B, as well as the concentration of the inhibitory vesicles after ultrasonication, were estimated by phosphorus determination [34].

Kinetic treatment. The kinetic model for protein-mediated lipid transfer as originally described by Van den Besselaar et al. [25] for the transfer of PC by the PC-transfer protein, was applied to the transfer of PC by PI-TP. In this model, the transfer rate between donor vesicles (L_1) and acceptor vesicles (L_2) is given by the following rate equation:

$$V_0 = \frac{k_1[L_1]k_2[L_2][P]}{(k_1[L_1]+k_2[L_2])(1+K_1[L_1]+K_2[L_2])} \quad (1)$$

where k_1 and k_2 are the rate constants for the association of the transfer protein with the donor and acceptor vesicle, respectively, K_1 and K_2 are the respective association constants and P represents the transfer protein. Since under our experimental conditions the donor vesicle concentration was very low, we take the term $K_1[L_1]$ to be $\ll 1$. Then the rate equation becomes equal to:

$$V_0 = \frac{k_1[L_1]k_2[L_2][P]}{(k_1[L_1]+k_2[L_2])(1+K_2[L_2])} \quad (2)$$

Estimation of k_1, k_2 and K_2 would require a cumbersome fitting procedure [25,39]. This can be avoided by rearranging Eqn. 2 into a polynom from which the kinetic constants can be calculated by a simple linear least-squares optimization pro-

cedure. The following polynom is derived from Eqn. 2:

$$\frac{[L_2]}{V_0} = \frac{1}{k_2[P]} + \left(\frac{1}{k_1[L_1][P]} + \frac{K_2}{k_2[P]}\right)\cdot[L_2] + \left(\frac{K_2}{k_1[L_1][P]}\right)\cdot[L_2]^2 \quad (3)$$

When the donor vesicle concentration $[L_1]$ and the protein concentration [P] are fixed, this polynom is of the form:

$$\frac{[L_2]}{V_0} = A + B[L_2] + C[L_2]^2 \quad (4)$$

In which A, B and C are constants. From the least-squares optimization procedure (polynom fit) A, B and C were calculated, thereby yielding k_1, k_2 and K_2. The dissociation rate constant k_{-2} follows from $k_{-2} = k_2/K_2$.

In Eqn. 2 the term $K_2[L_2]$ represents the substrate inhibition that occurs at high $[L_2]$ (see Fig. 1). To estimate V_{max}, the substrate inhibition is neglected (i.e., $K_2[L_2] = 0$) and $[L_2]$ is taken to be infinite (i.e., $k_2[L_2] \gg k_1[L_1]$); then Eqn. 2 becomes $V_{max} = k_1[L_1][P]$.

Results

Kinetic treatment

We have applied the kinetic model proposed by Van den Besselaar et al. [25] to analyse the transfer kinetics of PI-TP. Conditions were chosen under which the protein transfers only PC. In our fluorimetric assay system the concentration of the donor vesicle consisting of 1-Pal,2-Pyr(10)PC is very low relative to the acceptor vesicle concentration. The importance of electrostatic interactions in the functioning of PI-TP was investigated by varying the acceptor vesicle concentration under different assay conditions (e.g., pH, ionic strength, lipid composition). The transfer activity as a function of the acceptor vesicle lipid concentration is shown in Fig. 1. Substitution of the experimental data points into Eqn. 3 yields the parameters k_1, k_2 and K_2 (for values see legend to Fig. 1). The curve dictated by these parameters is in excellent agreement with the experimental data. This confirms that the kinetic model proposed by Van den Besselaar et al. [25] describes the mode of action of PI-TP. Characteristic for the transfer mechanism, the transfer rate as a function of the acceptor vesicle concentration goes through an optimum (Fig. 1). The extent of the subsequent decrease in activity is a measure for the affinity of PI-TP for the acceptor vesicles (i.e., K_2). This inhibition reflects that PI-TP increasingly interacts with acceptor vesicles, thereby deminishing the chance that PI-TP interacts with a donor vesicle. As K_2 represents the affinity of PI-TP for the acceptor vesicles, we have preferred to relate the electrostatic effects on the activity of PI-TP to this constant.

Effect of membrane charge

The effect of membrane charge on the transfer activity of PI-TP was studied by using acceptor vesicles consisting of PC and varying amounts of PA, PIP or PIP_2. It had been shown [8,40] that PA and PIP are no substrate for PI-TP. This is probably equally true for PIP_2. From the transfer rates, the association constant, K_2, was derived. In Fig. 2A the association constant, K_2, is presented as a function of the negatively charged phospholipid

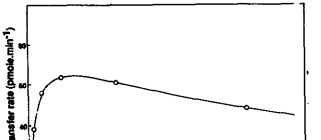

Fig. 1. Kinetic analysis of PC transfer by PI-TP. 1-Pal,2-Pyr(10)PC transfer was measured as a function of the acceptor lipid concentration. The conditions were: donor vesicles, 0.5 μM PyrPC; acceptor vesicles, PC-PA (92:8, mol%); PI-TP, 1 μg/ml; total volume, 2 ml 20 mM Tris-HCl/5 mM EDTA/100 mM NaCl (pH 7.4). The initial transfer rates are represented by the open circles. Kinetic analysis as described in Materials and Methods yielded: $k_1 = 82.77 \pm 0.52$ nmol·min^{-1}·μg^{-1}·mM^{-1}, $k_2 = 12.79 \pm 0.42$ nmol·min^{-1}·μg^{-1}·mM^{-1}, $K_2 = 5.883 \pm 0.037$ mM^{-1}. Substitution of these constants into Eqn. 2 resulted in the curve connecting the experimental data points.

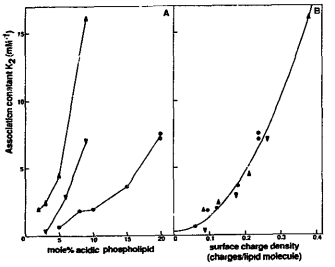

Fig. 2. Effect of membrane charge on the association constant, K_2. Acceptor vesicles consisting of PC and varying amounts of PA (●), PIP (▼) or PIP_2 (▲), were prepared and sized on a Sepharose 4B column. The transfer assay conditions were identical to the assay conditions described in the legend of Fig. 1. The association constant, K_2, was calculated, and is represented as a function of the mol% of acidic lipid in the acceptor vesicles (A), or as a function of the number of negative charges per acceptor lipid molecule (B). The number of negative charges per acceptor lipid molecule was calculated from the acidic lipid content, and the negative charge (Q) of the acidic lipids at pH 7.4 as calculated from the pK_a values of the lipids [28,41].

content. It is evident that an increasing amount of acidic phospholipids leads to higher association constants. Furthermore, it appears that introduction of PIP or PIP_2 into the acceptor membrane gives rise to a more pronounced increase in the K_2 value than that observed with PA. However, if one considers the molecular charge (Q) of the acidic phospholipids at pH 7.4 (Q(PA) = −1.20, Q(PIP) = −2.94 and $Q(PIP_2)$ = −4.20 [28,41]) a nonlinear relationship between the calculated average surface charge density and the association constant is apparent, independent of the type of acidic phospholipid used (see Fig. 2B).

Effect of neomycin

The polyamine antibiotic neomycin has been shown to bind negatively charged lipids, with a preference for the very acidic PIP and PIP_2 [42,43]. We have investigated the effect of neomycin on the transfer activity of PI-TP with PC acceptor vesicles containing PIP_2 (5 mol%) as compared to vesicles containing PA (20 mol%). As is shown in Fig. 3A, an increase of the concentration of PIP_2-containing vesicles results in a decrease of the rate of PC transfer. This inhibitory effect is completely eliminated in the presence of neomycin. Under these conditions the association constant, K_2, decreased from 4.46 mM^{-1} to 0.273 mM^{-1}. In agreement with the reported preference for PIP_2, neomycin had much less effect on the transfer when the acceptor vesicles contained PA (Fig. 3B). In this instance, the K_2 decreased from 7.43 to 4.91 mM^{-1}). From these results we conclude that by interacting with PIP_2, neomycin shields off the negative charges, thereby decreasing the affinity of PI-TP for the membranes and thus eliminating the inhibitory effect of the acceptor vesicles at high concentrations.

Effect of pH

Increasing the pH from 5.5 to 8 lowers the association constant, K_2, of PI-TP for the acceptor vesicles (PC-PA; 84:16 mol%) from approx. 40 mM^{-1} to 2 mM^{-1}. Apparently, pH affects the interaction of the transfer protein with the vesicle surface. On the other hand, the intrinsic transfer activity of the protein, as illustrated by the maximal transfer velocity, was hardly affected (Fig.

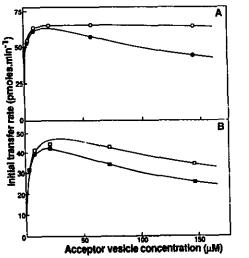

Fig. 3. Effect of neomycin on PI-TP. 1-Pal,2-Pyr(10)PC transfer was measured as a function of the acceptor lipid concentration using vesicles consisting of PC-PIP_2 (95:5 mol%; ○, ●), or of PC-PA (80:20 mol%; □, ■). Transfer was measured either in the presence (○, □) or absence (●, ■) of neomycine, added in a molar ratio of 1.4:1 to the total acceptor liquid concentration. Kinetic analysis, represented by the drawn curves, yielded the association constants K_2 given in Results.

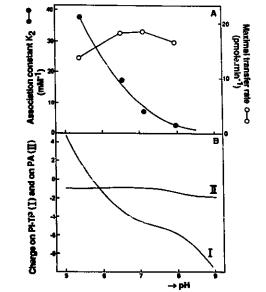

Fig. 4. Effect of pH on the transfer activity of PI-TP. (A) The assay conditions were: donor vesicles 1-Pal,2-Pyr(10)PC (0.5 μM); acceptor vesicles PC-BA (84:16, mol%; 0–120 μM); PI-TP, 0.5 μg/ml in 2 ml of 20 mM Tris-maleate/5 mM EDTA/100 mM NaCl. The association constant, K_2 (●), was determined as a function of the pH as described in the Materials and Methods. The maximal transfer rates (○) were calculated from the kinetic constants by optimizing Eqn. 3 with respect to the acceptor concentration, and subsequent substitution of this concentration together with the kinetic constants into Eqn. 2. (B) The charge on PI-TP (curve I) and PA (present in the acceptor membrane; curve II) as a function of pH. The charge on PA was calculated using the pK values (pK_1 = 3.0 and pK_2 = 8.0 [37]). The approximate charge on PI-transfer protein was estimated from the pK_a values for the amino acids and the amino-acid composition [57]. The unknown glutamine/glutamic acid, and asparagine/aspartic acid ratios could be estimated from the known isoelectric points of PI-TP carrying a PC molecule (i.e., p = 5.7), and of PI-TP carrying a PI molecule (i.e., when the protein has an overal charge of +1, compensating for the negative charge on the PI molecule; at pH 5.5).

4A). The decrease in the K_2 value cannot be explained in terms of a change in the vesicle surface charge, as in the pH range of 5.5 to 8 the charge on PA hardly alters (Fig. 3B, curve II), while in this pH range the charge on PI-TP changes from approx. +2 to approx. −6. This implies that the decrease in the K_2 value with increasing pH results from the electrostatic repulsion between the transfer protein and the vesicle surface. Similar results have been obtained with acceptor vesicles consisting of PC-PIP$_2$ (95:5 mol%; data not shown).

The decrease in K_2 observed with increasing pH (Fig. 4) was further analyzed in terms of association and dissociation rate constants (k_2 and k_{-2}, respectively). As is shown in Table I, changing the pH from 6.1 to 8.1 using acceptor vesicles containing 8 mol% PA, increased the dissociation rate constant 16-fold, whereas the association rate constant increased only 2-fold. An increase of the acidic phospholipid (PIP) content of the vesicles, on the other hand, leads to a 25-fold decrease in the dissociation rate constant, again without much effect on the association rate constant (see Table I). In this instance, the value of k_{-2} increases despite the overall enhanced electrostatic repulsion between vesicles and protein. This indicates that an increase of the overall negative charge of the protein has an effect on the interaction with the vesicle surface which differs from that observed when the negative charge on the interface itself increases (see Discussion). Apparently, the enhanced electrostatic repulsion at higher pH mainly affects the dissociation of the transfer protein/vesicle-membrane complex.

Inhibition by pure negatively charged vesicles/micelles

The effect of negatively charged phospholipids on the transfer activity of PI-TP was further studied by adding a small amount of vesicles consisting of a single negatively charged lipid (inhibitory vesicles) to the standard assay. as has

TABLE I

EFFECT OF ACCEPTOR MEMBRANE CHARGE AND pH ON THE KINETIC PARAMETERS DESCRIBING PC-TRANSFER BY PI-TP

Acidic lipid	Molar ratio (%)	pH	k_2 [a]	k_{-2} [b]	K_2 [c]
PA	8	6.1	14.0±4.7	2.0±0.7	7.0 ±0.6
PA	8	7.1	17.6±0.6	3.0±0.1	5.88 ±0.04
PA	8	7.5	18.6±1.6	10.0±0.8	1.86 ±0.06
PA	8	8.1	32.0±1.3	33.0±1.3	0.974±0.003
PIP	3	7.4	10.2±2.0	32 ±6	0.313±0.007
PIP	6	7.4	12.6±5.5	4.4±1.9	2.87 ±0.11
PIP	9	7.4	9.4±3.6	1.3±0.5	7.0 ±0.4

[a] Association rate constant (nmol·min^{-1}·μg^{-1}·mM^{-1}).
[b] Dissociation rate constant (nmol·min^{-1}·μg^{-1}).
[c] Association constant (mM^{-1}).

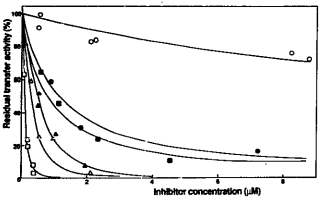

Fig. 5. Inhibition of PI-TP by pure acidic phospholipids. The transfer assay was performed as described in the legend of Fig. 1, with acceptor vesicles consisting of PC-PA (85:15, mol%). The sonicated acidic phospholipids, PI (○), PG (●), PS (■), PA (▲), PIP (△) and PIP_2 (□) were added before the assay was initiated by the addition of PI-TP. The residual transfer activity is represented as a function of the acidic lipid concentration.

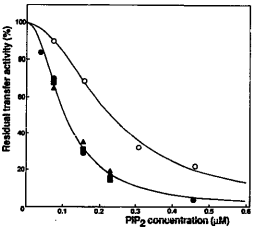

Fig. 6. Effect of pH and Mg^{2+} on the transfer inhibition by PIP_2. The inhibition of PI-TP transfer activity by PIP_2 micelles was measured as described in the legend of Fig. 5, at pH 6.0 (■), 7.2 (●), 8.5 (▲), and 7.2 in the presence of 1 mM Mg^{2+} (○).

been described before [6,44]. The transfer activity as a function of the inhibitory vesicle concentration is presented in Fig. 5. It is evident that very small amounts of these inhibitory vesicles are sufficient to completely abolish the transfer activity. The inhibitory activity of these vesicles increased in the order of PI > PG > PS > PA > PIP > PIP_2. It appears that PI behaves anomalously, most likely due to the fact that PI-TP can also bind PI. The acidic lipid concentration at 50% inhibition of transfer, decreases by a factor of 10 going from PG to PIP_2 (Table II). It is striking that even at a 1400-fold excess of acceptor vesicles (PC-PA 85:15 mol%; 142 μM phospholipid), PIP_2 (0.1 μM) is able to inhibit PI-TP by 50%. Another

TABLE II

INHIBITION OF PI-TP BY PURE NEGATIVELY CHARGED LIPIDS

Acidic lipid	EC_{50} [a] (μM)	Inhibitor/PI-TP [b] at the EC_{50}
PG	1.1	80
PS	0.8	50
PA	0.61	42
PIP	0.33	23
PIP_2	0.10	7

[a] Concentration of inhibitor at 50% inhibition (μM).

[b] Molar ratio of inhibitor to PI-TP at 50% inhibition; Assay conditions were: donor, 0.5 μM 1-Pal,2-Pyr(10)PC; acceptor, 142 μM PC-PA (85:15, mol%); PI-TP, 0.5 μg/ml.

expression for the efficiency of the inhibition is the molar ratio of inhibitory phospholipid (EC_{50}) to PI-TP. This ratio decreases from 80 for PG, to 7 for PIP_2, indicating an extremely efficient inhibition by the latter lipid. The fact that PIP_2, and possibly PIP are present as micelles [45], whereas the other lipids form bilayers, may to some extent explain the extremely efficient inhibition as compared to the other lipids.

The inhibitory effect of PIP_2 micelles on the activity of PI-TP was further investigated by varying the pH of the assay medium. As seen from Fig. 6, the inhibition of transfer by PIP_2 was not affected by variation of the pH between 6 and 8.5, despite the charge shift on the PIP_2 molecule from −3.9 to −4.9 [28]. On the other hand, addition of 1 mM Mg^{2+} diminished the inhibitory effect of PIP_2 approx. 2-fold.

Discussion

Phospholipid transfer between membranes catalyzed by lipid transfer proteins is a complex process, involving the interaction of the protein with two different membranes, exchange of the protein-bound lipid molecule for a lipid molecule present in the membranes, and the diffusion of the lipid-transfer protein complex between the membranes [8,46]. To understand the role of these proteins in the cell, it is necessary to study the factors that may regulate their transfer activity.

For the PC-specific transfer protein from bovine liver, a number of kinetic studies have appeared that describe the influence of factors such as membrane charge, fluidity, lipid composition and curvature, pH and divalent cations (for a review see Refs. 46, 47). For PI-TP form bovine brain, however, the kinetics are considerably more complicated because of the dual specificity for PI and PC. Since a complete kinetic description of PI-TP involving the transfer of both PC and PI is very complicated, we have simplified our assay system by measuring the transfer of PC between membranes that lack PI. Under these conditions, the kinetic behaviour of PI-TP agrees perfectly with the model proposed by Van den Besselaar et al. [25] (see Fig. 1). In principle, this model can also be applied to PI-TP mediated transfer of PI between membranes that lack PC. However, in practice this is not possible, as the ensuing membranes would inhibit the transfer protein due to an extreme negative charge. Another possibility would be the use of the nontransferrable PE as a matrix lipid. We have preferred the former approach as PE has major effects on both membrane lipid organization [49] and PI-TP transfer activity [21]. We believe that the effects of surface charge, ionic composition of the medium and other factors on the transfer activity as analyzed by the kinetic model in the present study are also valid for those situations in which both PI and PC are present. This presumes that the interaction of PI-TP with the membrane interface and the subsequent binding of either PI or PC are independent steps in the transfer process.

Previously, contradictory results have been reported on the effects of negatively charged lipids on PI-TP transfer activity. Incorporation of PA (15–17 mol%) into the acceptor vesicles was found to inhibit PI transfer from a monolayer [9], and PC transfer from donor vesicles [6]. In a microsome-vesicle assay, however, no decrease in activity was observed when PA was incorporated into the vesicles, while PI was found to be strongly inhibitory [21]. From studies using vesicle-vesicle assay systems [6,9,23], and from monolayer experiments [9] it was concluded that relatively nonspecific ionic interactions must be responsible for the inhibition of PI-TP by PI and other acidic phospholipids. In the present study we were able to show that an increased content of acidic phospholipids in the acceptor membrane causes an increase in the affinity (K_2) of PI-TP for this membrane (Fig. 2A). Under our assay conditions, i.e., in the presence of a large excess of acceptor vesicles, a high K_2 is reflected in an inhibition of the lipid transfer. Experiments with PA, PIP and PIP_2, strongly suggest that the overall negative surface charge rather than specific protein–phospholipid headgroup interactions, determine the affinity (K_2) of PI-TP for a membrane (Fig. 2B). This is in agreement with earlier observations [6,9].

Since the positively charged polyamine antibiotic neomycin binds acidic phospholipids [41,42], we were interested to see whether neomycin had an effect on the association constant of PI-TP for negatively charged membranes. It was found that neomycin reduced the affinity of PI-TP for vesicles containing 5 mol% PIP_2 (Fig. 3A) much more efficiently than for vesicles containing 20 mol% PA (Fig. 3B). This reduction in the affinity is most likely caused by neomycin shielding the negative surface charge, thereby eliminating acceptor vesicle substrate inhibition. These observations are in agreement with the reported strong affinity of neomycin for PIP_2 [43]. From the estimated K_2 values, the surface charge reduction caused by neomycin could be estimated (Fig. 2B). For the vesicles containing 20 mol% PA, the charge is only slightly reduced, to a level comparable with a vesicle PA content of 17%. However, for the vesicles containing 5 mol% PIP_2 (comparable to approx. 17% PA) a complete charge neutralization occurred upon the addition of neomycin.

In previous studies it was reported that tertiary amine local anaesthetics preferentially inhibit the PI-transfer activity of PI-TP, and not the PC-transfer activity [24,50]. This discrimination was taken to indicate the formation of a specific complex between PI and the anaesthetics. Under our assay conditions, the effect of neomycin on the transfer activity is due only to the interaction with PIP_2. However, it remains to be determined whether neomycin could also have a specific effect on the PI-TP mediated transfer of PI.

Recently it was reported that pH has an effect on the activity of PI-TP [23]. Here we have shown that the association of PI-TP with acceptor vesicles (PC–PA, 84:16 mol%) is greatly dependent on

pH (Fig. 3A). This effect cannot be ascribed to changes in the intrinsic transfer capability of PI-TP, since the maximal transfer velocity was hardly affected. The charge vs. pH profiles for the acceptor vesicles and for the transfer protein (Fig. 3B) indicate that the decrease in K_2 with increasing pH is primarily due to the large increase in the negative charge of PI-TP resulting in an enhanced repulsion. Below the isoelectric point of the transfer protein (pH 5.5), the transfer activity completely disappeared, probably as a result of extremely strong binding of the overall positively charged protein to the negatively charged vesicle surface. It is of note that, at pH 7.4, the association constant of PI-TP increases for vesicles containing increasing amounts of acidic lipids (see Fig. 2). Since at this pH PI-TP has an overall negative charge, the increased affinity points to a membrane interaction site on PI-TP, which is positively charged. This would imply that under this condition the attraction between the interaction site and the vesicles prevails over the repulsive forces. The combination of the attractive and repulsive forces which govern the interaction between the acceptor vesicles and PI-TP will affect the equilibrium.

From the kinetic analyses it became evident that the effects of surface charge and pH on the association constant K_2 are almost completely due to changes in the dissociation rate constant k_{-2}. Apparently, pH and surface charge affect mainly the disruption of the PI-TP/vesicle complex. The fact that the association rate constant (k_2) was hardly affected suggests that this constant is controlled mainly by the collision frequency of PI-TP and the acceptor vesicles. Since the K_2 can be estimated much more accurately than the k_{-2}, and since K_2 is independent of the absolute transfer velocities measured, this parameter provides reliable and reproducible information about the interaction of PI-TP with the vesicle interface. In a previous study Van den Besselaar et al. [25] found that an increase in membrane surface charge also caused an increase in the association constant (K_2) of the PC-transfer protein/vesicle complex. In an extension of this study [36,39] it was demonstrated that the association rate constant was hardly affected by an increase of the vesicle PA content, in contrast to the dissociation constant which increased considerably. In this respect the transfer kinetics of PI-TP closely resemble those of PC-transfer protein.

An extreme case of charge inhibition is observed when vesicles consisting of only negatively charged phospholipids are added as a third component in the transfer assay (see Fig. 5). This phenomenon has been studied before in a fluorimetric transfer assay using 2-parinaroylPC [6]. The transfer is completely blocked at very low concentrations of acidic phospholipid relative to PI-TP (see Table II). This efficient inhibition of transfer is most likely caused by a very strong binding of the putative positively charged membrane interaction site on the protein with the highly negatively charged membrane surface. The inhibition cannot be explained by the denaturation of PI-TP at the highly charged surface, since the addition of Mg^{2+} to vesicles consisting of PA, PS and PG restores virtually all transfer activity (data not shown). In the case of PIP_2, only approx. 50% of the original activity could be restored by Mg^{2+}. This suggests that Mg^{2+} is not able to completely disrupt the PI-TP/PIP_2 complex. Apart from PI, there appears to be a correlation between the negative charge on the acidic phospholipid used, which increases in the order of PG, PS > PA > PIP > PIP_2 (i.e., negative charge/molecule of 1.0; 1.0; 1.2; 2.9; 4.2), and the extent of transfer inhibition. The anomalous behaviour of pure PI vesicles could be explained by the uptake of one PI molecule into the lipid-binding site of PI-TP. This one additional negative charge in the lipid-binding site may result in an increased repulsion between the inhibitory vesicles and PI-TP. It is to be noted that 50% transfer inhibition by PIP and PIP_2 was achieved at very low lipid-to-protein ratios (i.e., 23 and 7 mol lipid/mol PI-TP, respectively). Since PIP_2 forms small micelles consisting of 82 molecules [45], this ratio indicates that at 50% inhibition approx. 6 PI-TP molecules are bound per micelle (30 Å radius). Small-angle neutron diffraction measurements on PI-TP have indicated that the protein has approximately the same size as the PIP_2 micelles (radius ≈ 32 Å; unpublished observation). Taken together, this suggests that at 50% inhibition, the PIP_2 micelle may be surrounded by six closely packed PI-TP molecules. On the other hand, it remains to be established

whether the micelle preserves its integrity upon binding of the PI-TP molecules.

The inhibition by PIP_2 was found to be independent of the pH in the range of pH 6.0 to 8.5, while in this range the charge on PIP_2 increases from −3.9 to −4.9. Apparently, above a certain surface charge density, no further increase in the inhibition can occur. Mg^{2+} (1 mM), however, was able to reduce the inhibition by PIP_2, presumably by a partial neutralization of the negative surface charge.

From the model experiments described above, it is clear that a number of factors influence the activity of PI-TP. Membrane characteristics such as membrane curvature, composition and surface charge, and factors such as pH and the presence of divalent cations and polyamines have been shown to regulate the transfer activity of PI-TP. Although these conclusions were drawn from model experiments, they may also apply to the situation inside the intact cell. As for the effects of membrane surface charge, the occurrence of various PI and PIP_2 pools present in the cell, possibly in the vicinity of membrane receptors involved in the PI-cycle [51,52], are of interest. In addition, the membrane surface charge can be neutralized by divalent cations and polyamines, both implicated as important regulators of cell functioning [53,54]. Local variations in the intracellular pH may be another factor in regulating the activity of PI-TP [55].

Since the proposed role for PI-TP in the stimulus-induced PI turnover, involves the transfer of PI from intracellular sites towards the plasma membrane, it remains to be determined whether changes induced by the stimulus, could invoke changes in PI-TP activity. Three well-characterized stimulus-induced responses are the hydrolysis of PIP_2, the rise in the intracellular Ca^{2+} concentration, and the rise in the intracellular pH [56–58]. Each of these responses would result in an increased dissociation rate constant, thereby contributing to an enhanced transfer activity of PI-TP.

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