Regulation of the Activity of Phosphatidylcholine Transfer Protein by Vesicle Phosphatidic Acid and Membrane Curvature: A Fluorescence Study Using 2-Parinaroylphosphatidylcholine†

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ABSTRACT: The kinetic parameters which govern the interaction between the phosphatidylcholine transfer protein (PC-TP) and unilamellar vesicles have been investigated. Effects of vesicle charge and curvature on the rates of transfer of 1-acyl-2-parinaroyl-*sn*-glycero-3-phosphocholine (PnA-PC) have been determined by using a recently developed fluorometric assay [Somerharju, P., Brockerhoff, H., & Wirtz, K. W. A. (1981) *Biochim. Biophys. Acta 649*, 521–528]. An increase of the phosphatidic acid (PA) content of PC vesicles resulted in an increase of the apparent association constant (K) of complex formation between PC-TP and these vesicles. A concomitant increase of the association rate constant indicated that PA stimulated the activity of PC-TP. Unilamellar vesicles composed of PC/PA (80:20 mol %) were prepared either by controlled detergent dialysis or by ultrasonication and sized by molecular sieve chromatography on Sepharose 4B. The diameter of the vesicles as determined by photon counting spectroscopy ranged from 22 to 50 nm. Kinetic analysis of the protein-mediated transfer of PnA-PC to these sized vesicles demonstrated that the association constant K increased greatly with decreasing vesicle size. K was smaller than 2 mM^{-1} for large-size vesicles (30-nm diameter and larger) and up to 57 mM^{-1} for the smaller size vesicles (22 nm). Determination of the phospholipid composition of vesicle outer and inner monolayers by ^{31}P nuclear magnetic resonance spectroscopy has ruled out variations in this composition to be the cause of the difference in K values. Hence, this difference most likely reflects the effect of high curvature on the interaction of PC-TP with the vesicle interface. The fluorescence assay was found to be very suited in demonstrating net transfer of PnA-PC by PC-TP to vesicles composed of either pure PA or phosphatidylethanolamine/PA (80:20 mol %). About 10% of the PnA-PC in the donor vesicles was shown to be available for net transfer.

The phosphatidylcholine transfer protein (PC-TP)[1] from bovine liver catalyzes in vitro the transfer of PC between membrane interfaces. It acts as a carrier by forming a one to one molar complex with PC. The mode of action of PC-TP has been extensively studied [for a recent review, see Wirtz (1982)]. Under conditions where PC-TP catalyzes an exchange of PC between a donor and an acceptor membrane, the kinetics of this process have been described involving both single bilayer vesicles (van den Besselaar et al., 1975; Machida & Ohnishi, 1978) and multilamellar liposomes (Wirtz et al., 1979; Bozatto & Tinker, 1982). In these studies, the rate of PC transfer was highly influenced by the amount of negatively charged phospholipids in the membrane, the type of vesicles used, and structural surface defects. Furthermore, PC-TP was found to be very sensitive to whether the phospholipid bilayer existed in the gel or liquid-crystalline state (Kasper & Helmkamp, 1981; Bozatto & Tinker, 1982).

Recently, a fluorometric transfer assay was developed on the basis of the relief of self-quenching when PnA-PC is transferred from quenched donor vesicles consisting of pure PnA-PC to unquenched acceptor vesicles (Somerharju et al., 1981). Other continuous spectroscopic assays are based on the resonance energy transfer between different chromophores (Nichols & Pagano, 1983) or changes in the electron spin resonance spectrum (Machida & Ohnishi, 1978). Application of PnA-PC is attractive, as *cis*-parinaric acid, localized at the *sn*-2 position, is a naturally occurring fatty acid giving minimal disturbance of the membrane bilayer (Sklar et al., 1975; Schroeder et al., 1976) or of lipid/protein complexes (Sklar et al., 1977; Berkhout et al., 1984).

In the present study, the PnA-PC transfer assay has been used to elucidate the dependency of the kinetic parameters of PC-TP on the vesicle content of acidic phospholipids (i.e., PA) and vesicle curvature. In addition, the net transfer of PC by PC-TP, as observed in previous studies (Wirtz et al., 1980; Nichols & Pagano, 1983), was further explored by using this assay.

Materials and Methods

Materials. Egg yolk phosphatidylcholine (PC), type III-E, was obtained from Sigma, and *cis*-parinaric acid was purchased from Molecular Probes, Plano, TX.

Phosphatidic acid (PA) was prepared from PC by treatment with phospholipase D from savoy cabbage (Davidson & Long, 1958) and converted into the sodium salt (van Dijck et al., 1978). 2-Parinaroylphosphatidylcholine (PnA-PC) was synthesized as described by Somerharju et al. (1981). 2-Parinaroylphosphatidic acid (PnA-PA) and 2-parinaroylphosphatidylethanolamine (PnA-PE) were generous gifts from P. Somerharju [for preparation, see Somerharju et al. (1983)]. The PnA-phospholipids (1–5 mM) were dissolved in ethanol and stored at −20 °C in the dark, in the presence of 1 mol % butylated hydroxytoluene (BHT). [*methyl*-^{14}C]PC (specific activity 58 Ci/mol) was prepared by the method of Stoffel

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid disodium salt; Tris, tris(hydroxymethyl)aminomethane; BHT, butylated hydroxytoluene; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PnA-PC, 1-acyl-2-parinaroyl-*sn*-glycero-3-phosphocholine; PnA-PA, 1-acyl-2-parinaroyl-*sn*-glycero-3-phosphoric acid; PnA-PE, 1-acyl-2-parinaroyl-*sn*-glycero-3-phosphoethanolamine; PC-TP, phosphatidylcholine transfer protein; PCS, photon counting spectroscopy; NMR, nuclear magnetic resonance.

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(1975). Phosphatidylethanolamine (PE) from egg yolk was a generous gift from P. van Hoogevest. The phosphatidylcholine transfer protein (PC-TP) was isolated from bovine liver according to established procedures (Westerman et al., 1983). The protein (0.125 mg/mL) was stored at −20 °C in 50% glycerol containing 10 mM sodium citrate/20 mM disodium hydrogen phosphate, pH 5.0. Sepharose 4B was from Pharmacia, and BHT was obtained from Aldrich.

Preparation of Vesicles. Vesicles consisting of PnA-phospholipids were prepared by the ethanol injection technique (Batzri & Korn, 1973). PnA-phospholipids in ethanol (2–10 nmol) were directly injected into the cuvette containing 2 mL of TES buffer (20 mM Tris-HCl/5 mM EDTA/100 mM NaCl, pH 7.4). The buffer was degassed under vacuum and saturated with argon for 30 min before use. The amount of ethanol did not exceed 0.5% (v/v). After injection, the vesicles were equilibrated for 5–10 min before measurements. Vesicles of nonfluorescent phospholipids (20–50 mM) were prepared by sonication in TES buffer for 20 min at 0 °C with a Branson sonifier as described by Somerharju et al. (1983). The vesicles were centrifuged at 45 000 rpm for 60 min in a Beckman rotor (R50) to remove titanium particles, dust, and multilamellar structures. Unilamellar vesicles were also prepared by controlled dialysis using a Minilipoprep apparatus (Diachema, Switzerland), as described by Zumbuehl & Weder (1981). Micelles were prepared by vortexing a mixture of 33 μmol of sodium cholate, 20 μmol of PC, and variable amounts of PA in 1 mL of TES buffer followed by dialysis at 14 rotations/min against 1 L of TES buffer at 20 °C for 24 h. To determine the lipid concentration in the different stages of handling, the vesicles (sonicated or Lipoprep) contained a trace amount of radioactive PC (specific activity 2×10^{-3} Ci/mol).

The vesicles were subjected to molecular sieve chromatography on a Sepharose 4B column (43 × 1.5 cm) at 4 °C, as described by Huang (1969). Prior to use, the column had been saturated with PC vesicles to eliminate adsorptive effects. The column was eluted with TES buffer at a rate of 6.5 mL/h, and fractions of 1.6 mL were collected. The phospholipid concentration in the fractions was measured by determining the radioactivity. Vesicles prepared for NMR experiments were separated on a larger Sepharose 4B column (70 × 2.5 cm) and eluted at a rate of 16 mL/h. Since the vesicle concentration in the eluent was too low to study by ^{31}P NMR, fractions (4.6 mL) were pooled and concentrated to a total volume of 1.5 mL by using an Amicon ultrafiltration apparatus containing an XM100A filter. The combined fractions consisted of a top fraction (V_e between 234 and 244 mL) and a side fraction (V_e between 257 and 276 mL). After the concentration step, the recovery of the vesicles was higher than 80% as determined by measuring the radioactivity.

Fluorometric Measurements. The measurements were carried out essentially as described before (Somerharju, 1981), using a Perkin-Elmer MFP-3 fluorometer. Excitation and emission were at 324 and 420 nm, respectively. The emisson slit was 2 nm and the excitation slit 40 nm. During the measurements, the content of the cuvette was continuously stirred at a temperature of 25 °C. In the phospholipid transfer assay, the moles of PnA-PC transferred were derived from the increase of fluorescence intensity. For this calculation, standard curves were used, based on the fluorescence intensity of acceptor vesicles containing unquenched PnA-PC. The initial rates of transfer were monitored on a chart recorder. Progress curves were measured at time intervals to prevent degradation of PnA-PC by the incoming light. In the kinetic experiments, the fluorescence intensity was corrected for changes in the volume after addition of the sonicated acceptor vesicles. No corrections for inner and outer filtering effects were made, as the optical density at the wavelengths of excitation and emission was below 0.1 in the kinetic measurements.

Kinetic Treatment. The rate at which PC-TP catalyzes PC exchange between donor vesicles (L_1) and acceptor vesicles (L_2) is expressed by the following theoretical rate equation, as derived by van den Besselaar et al. (1975):

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

k_1 and k_2 are the rate constants of association of PC-TP for donor and acceptor vesicles, respectively; K_1 and K_2 are the apparent association constants of the complex between PC-TP and the donor and the acceptor vesicle, respectively, and $[L_1]$ and $[L_2]$ are the concentrations of donor and acceptor phospholipid, respectively, involved in the exchange process, i.e., the outer monolayer of the vesicles which contains about 70% of the vesicle PC. In the present study, a large excess of acceptor over donor vesicles was used. Under these conditions, the rate equation (eq 1) is approximated by the following expression:

$$V_0 = \frac{k_1[\text{PnA-PC}][\text{PC-TP}]}{1 + K_2[L_2]} \quad (2)$$

[PnA-PC] is the concentration of the fluorescent donor PC (i.e., L_1). K_2 and k_1 are obtained from the intercepts on the abscissa and the ordinate by plotting V_0^{-1} against $[L_2]$ at constant [PnA-PC] [see also Machida & Ohnishi (1978)].

Photon Counting Spectroscopy (PCS). The size of the vesicles obtained after fractionation by Sepharose 4B column chromatography was determined from the diffusion coefficient measured by photon counting spectroscopy. For the measurements, an argon ion laser was used (Spectra Physics Model 165-09) at a wavelength of 488 nm. The scattered light was detected with an EMI 9558 photomultiplier at angles ranging from 60° to 135°, amplified, and analyzed by using a Malvern K 7025, 64-points correlator. For a detailed description of the procedure, see Kops-Werkhoven & Fynaut (1981) and Kremer et al. (1977). The values of the hydrodynamic radius obtained for the vesicles in the different column fractions were used to calibrate the Sepharose 4B column.

Nuclear Magnetic Resonance (NMR). ^{31}P NMR spectra of the phospholipid vesicles were recorded at 25 °C on a Bruker WH-90 spectrometer operating at 36.4 MHz on ^{31}P NMR as described by de Kruijff et al. (1976) and Koter et al. (1978). The PC concentrations of the vesicles were 13 mM (top fraction) and 19 mM (side fraction), respectively, in TES buffer containing 25% ^2H_2O. A total of 1000 transients were recorded with a 1.7-s interpulse time, using 90° pulses under conditions of broad-band decoupling.

The phospholipid composition of the vesicles was determined from the integrated areas of the ^{31}P resonances of PC and PA. The phospholipid composition of the inner monolayer was determined by addition of $CoCl_2$ (10 mM), which broadens the signal from the outer monolayer beyond detection (Berden et al., 1974). By comparison of the intensity of the remaining signal with that for the signal in the absence of Co^{2+}, the transbilayer distribution of the phospholipids could be measured.

From the line width ($\Delta\nu$) of the ^{31}P NMR resonance of phospholipids in vesicles, the vesicle size can be estimated according to $\Delta\nu = ca^3 + d$, where a is the vesicle radius and c and d are constants, characteristic of chemical shift an-

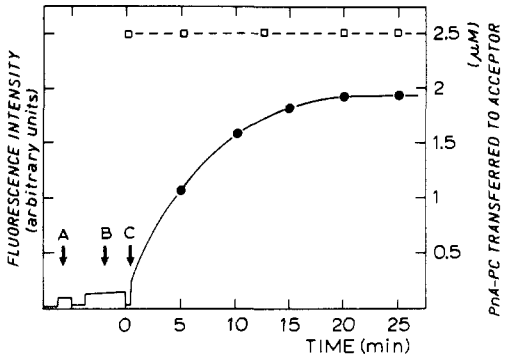

FIGURE 1: Progress curve for PC-TP-catalyzed PnA-PC transfer from quenched donor to unquenched acceptor vesicles. Donor vesicles were prepared in the cuvette by injection of an ethanolic solution containing PnA-PC (5 nmol) and PA (0.25 nmol) into 2 mL of TES buffer (20 mM Tris-HCl/5 mM EDTA/100 mM NaCl, pH 7.4) (arrow A). After 10 min of equilibration, sonicated acceptor vesicles (500 nmol of phospholipid) consisting of egg PC/PA (80:20 mol %) were added (arrow B). After addition of 0.5 μg of PC-TP (arrow C), the fluorescence intensity was recorded continuously for 1 min in order to measure the initial rate of PnA-PC transfer. Further increase in fluorescence intensity was measured at distinct time intervals (●). The maximal fluorescence level (□) was obtained by mixing of donor PnA-PC with acceptor phospholipid, prior to injection into the cuvette. For further details, see Materials and Methods.

isotropy and dipolar interactions by the phosphate phosphorus. It is estimated that $c = (2.9 \pm 0.2) \times 10^{-6}$ s^{-1}·Å^{-3} at 36.4 MHz and 25 °C and $d = 1$ Hz (de Kruijff et al., 1976).

Results

Transfer of PnA-PC by PC-TP. Vesicles containing more than 50% PnA-PC are practically nonfluorescent due to self-quenching of the probe whereas vesicles containing less than 1% PnA-PC are fully fluorescent. This property of PnA-PC forms the basis of the spectrofluorometric transfer assay developed by Somerharju et al. (1981). Figure 1 shows the fluorescence intensity upon subsequent addition of PnA-PC donor vesicles (point A), a 100-fold excess of unlabeled acceptor vesicles (point B), and PC-TP (point C) to the cuvette. From the progress curve, it is evident that PnA-PC is effectively transferred only in the presence of PC-TP. The fluorescence intensity levels off at a maximum of 70% of the value obtained by premixing the donor and acceptor phospholipids. This is in agreement with previous observations that only phospholipids of the outer vesicle monolayer are available for transfer (Johnson et al., 1975; Rothman & Dawidowicz, 1975; de Kruijff & Wirtz, 1977). As indicated in Figure 1, the fluorescence intensity is directly related to the amount of PnA-PC transferred to the acceptor vesicles. Hence, this assay enabled us to express initial rates of transfer in terms of absolute amounts of PnA-PC per minute.

Effect of Acidic Phospholipids. The effect of acidic phospholipids on the PC-TP-mediated transfer was studied by incorporating different amounts of PA in the donor and acceptor vesicles. Initial rates of transfer as a function of the amount of acceptor PC at constant donor PnA-PC concentration are shown in Figure 2 where in one experiment both donor and acceptor contained 5 mol % PA as compared to 20 mol % in the other experiment. In agreement with a previous study (Somerharju et al., 1981), the 20% PA vesicles yielded higher rates of transfer than the 5% PA vesicles at relatively low concentrations and lower rates of transfer at relatively high concentrations. This may explain the observation by DiCorleto et al. (1977) that under their conditions, acidic phospholipids stimulated the catalyzed transfer of PC. Under the conditions of our experiment, all acceptor concentrations used were in

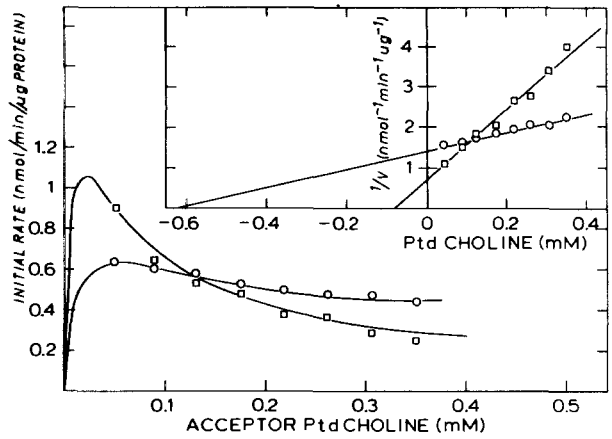

FIGURE 2: Effect of vesicle PA content on the transfer of PnA-PC by PC-TP. The donor vesicles were prepared by injection of an ethanolic solution containing PnA-PC (7 nmol) and either 5 (○) or 20 mol % PA (□) into 2 mL of TES buffer. Acceptor vesicles were prepared by sonication and consisted of egg PC and either 5 (○) or 20 mol % PA (□). Transfer was initiated by addition of 0.3 μg of PC-TP and the initial rate determined as a function of acceptor PC concentration. The insert shows the plot of V_0^{-1} vs. acceptor PC concentration. The PC concentration indicated represents the exchangeable pool of the acceptor vesicles, i.e., the outer monolayer. The drawn curves were calculated from the rate equation of van den Besselaar et al. (1975) by using the derived constants k_1 and K_2. In this calculation, we have assumed that $k_1 = k_2$ and $K_1 = K_2$, because of donor and acceptor vesicles having the same lipid composition.

Table I: Effect of Vesicle Phosphatidic Acid Content on Kinetic Parameters[a]

donor[b] (mol % PA)	acceptor[b] (mol % PA)	N[c]	k_1 (nmol mM^{-1} min^{-1} μg^{-1})	K_2 (mM^{-1})	k_{-1}[d] (nmol min^{-1} μg^{-1})
2	2	5	240 ± 30	1.6 ± 0.4	150 ± 56
10	10	3	300 ± 30	3.6 ± 1.4	83 ± 41
20	20	4	440 ± 130	19 ± 5	23 ± 13
5	20	2	250 ± 20	24 ± 9	

[a] Values ± standard deviations. [b] Vesicles are composed of PC and the indicated amounts of PA. [c] Number of reciprocal plots used for the calculation of the parameters k_1 and K_2. Each plot was based on at least 20 points and was calculated by linear regression analysis. [d] This parameter is calculated from $k_{-1} = k_1/K_2$ (see text).

large excess to the donor concentration, so that inhibition kinetics prevailed. These kinetics are governed by the constants k_1 and K_2 which can be derived from the plot of V_0^{-1} against the acceptor PC concentration (see insert to Figure 2). The results of different experiments are summarized in Table I. The association rate constant, k_1, approximately doubles going from 2% PA to 20% PA vesicles, whereas the apparent association constant, K_2, increases approximately 15-fold. A value of 3 mM^{-1} has been reported for K, involving PC vesicles containing 5% PA (Helmkamp, 1980). In the instance where donor and acceptor vesicles have the same PA/PC content, the kinetic parameters should be similar for both vesicles (i.e., $k_1 = k_2$ and $K_1 = K_2$). This implies that the dissociation rate constant, k_{-1}, directly follows from these constants (i.e., $k_{-1} = k_1/K_2$). As shown in Table I, k_{-1} has a 5-fold lower value for the 20% than for the 2% PA vesicles. In summary, it appears that an increase of the vesicle PA content enhances the association and lowers the dissociation of PC-TP from the vesicle interface, leading to the characteristic effects on the rates of intervesicle transfer as exemplified in Figure 2. This kinetic analysis is in virtual agreement with that of previous studies (van den Besselaar et al., 1975; Wirtz et al., 1979).

Effect of Vesicle Size. Multilamellar liposomes as compared to unilamellar vesicles of similar phospholipid composition are

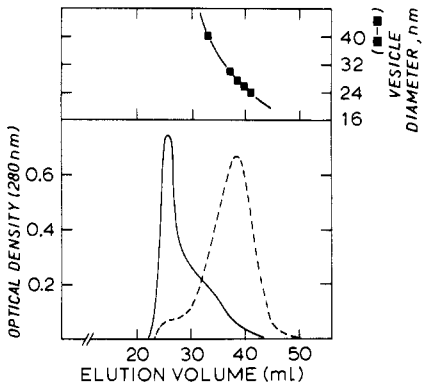

FIGURE 3: Fractionation of vesicles containing PC/PA (80:20 mol %) by molecular sieve chromatography. The vesicles were prepared by controlled detergent dialysis (—) or ultrasonic irradiation (---). The lipid dispersion (20 mM phospholipid in 1 mL of TES buffer) was applied to a Sepharose 4B column (43 × 1.5 cm) and eluted as described under Materials and Methods. The upper panel shows the average diameter of the vesicles in different fractions (1.6 mL) over the peak, as determined by photon counting spectroscopy (■).

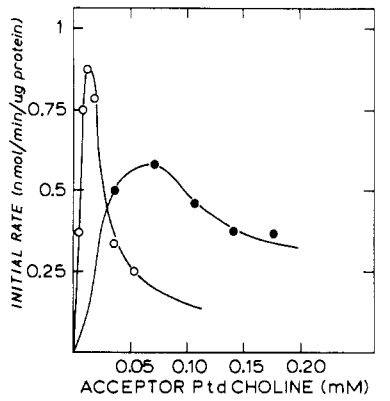

FIGURE 4: Effect of vesicle size on the transfer of PnA-PC. The donor vesicles were prepared by injection of an ethanolic solution containing PnA-PC (5 nmol) and PA (20 mol %) into 2 mL of TES buffer. Acceptor vesicles (80:20 mol % PC:PA) were prepared by sonication and sized on a Sepharose 4B column. Vesicles of 28 (●) or 22 nm (○) in diameter were used in the experiment. Transfer was initiated by addition of 0.6 μg of PC-TP and the initial rate determined as a function of exchangeable acceptor PC concentration.

poor substrates in the PC-TP-catalyzed transfer reaction (DiCorleto & Zilversmit, 1977; Wirtz et al., 1979; Bozatto & Tinker, 1982). Recently, Machida & Ohnishi (1980) provided evidence that the interaction of PC-TP with unilamellar phosphatidylserine vesicles was greatly dependent on the vesicle diameter. To determine whether vesicle size has an effect on the rate of transfer, PC vesicles containing 20 mol % PA were prepared by two different methods. The first method involved controlled dialysis using a Lipoprep apparatus and yielded unilamellar vesicles ranging from 40 to 60 nm in diameter as shown by negative-stain electron microscopy. The second method involved ultrasonic irradiation of a phospholipid suspension. Both vesicle populations were sized on a Sepharose 4B column (Figure 3). About 70% of the Lipoprep vesicles eluted in the void volume (size of 50–60 nm), while the remainder was recovered in fractions containing smaller vesicles. The bulk of the sonicated vesicles eluted in an apparently homogeneous peak after the void volume. The size distribution over the peak was estimated by photon counting spectroscopy of various column fractions, and the results obtained are shown in Figure 3. The average hydrodynamic diameter of the vesicles ranged from 22 to 40 nm; the vesicles in the top fraction have an average diameter of 28 nm. This compares to average diameters ranging from 16 to 26 nm for sonicated vesicles consisting of only egg PC (Brouilette et al., 1982).

The sized Lipoprep vesicles (50–60 nm in diameter) were used as acceptor vesicles in the transfer assay. Transfer by PC-TP was not inhibited by increasing the concentration of these vesicles (cf. Figure 2). This indicates that the vesicle content of 20% PA has no measurable effect on the kinetics of PC-TP when the vesicles have a relatively large size. The effect of vesicle size on the kinetics of PC-TP was further investigated by using sonicated vesicles (80:20 mol % PC:PA) sized by molecular sieve chromatography (Figure 3). The rate curves obtained for very small (22 nm in diameter) and average size (28 nm in diameter) acceptor vesicles are shown in Figure 4. It is evident that the rate of transfer as a function of increasing acceptor concentration is much more sensitive to the 22-nm vesicles than to the 28-nm vesicles. From the inhibition kinetics, the association constant K_2 was determined and found to be 7 times larger for the 22-nm vesicles, as compared to the 28-nm vesicles (Table II, experiment 1). Table II shows the relationship between K_2 and the size of vesicles (80:20 mol % PC:PA) determined for three different vesicle preparations (i.e., experiments 1–3). The effect of PA

Table II: Association Constant in Relation to Vesicle Size[a]

	association constant (mM^{-1})[b]		
diameter (nm)	expt I	expt II	expt III
22	57		
24	15.4		17.9
26	16.4	26.5	
28	7.9	11.6	
30			5.6
32	<2		
38		3.3	
40			<2

[a] The vesicles used consisted of PC/PA (80:20 mol %). After fractionation by molecular sieve chromatography, fractions over the peak were selected for measuring vesicle size and determination of kinetic parameters. [b] The association rate constant, k_1, for the donor vesicles (80:20 mol % PnA-PC:PA) was 508 ± 123 nmol mM^{-1} min^{-1} μg^{-1} (n = 9).

content on K_2 seems to be negligible for vesicles with a diameter greater than 30 nm. Although the absolute values of K_2, determined at a particular average vesicle size, differed for the various preparations, there is clear proof that the association constant of the acceptor vesicle/PC-TP complex increases sharply with decreasing vesicle size. As follows from Tables I and II, the rate constant of association (k_1) for the donor vesicles compares very well independent of whether unsized or sized acceptor vesicles were used (i.e., 440 ± 130 and 508 ± 123 nmol mM^{-1} min^{-1} μg^{-1}, respectively). Similar experiments with sized PC vesicles containing 5 mol % PA did not show large inhibition of transfer activity for the vesicles of very small size (results not shown). This indicates that both PA content and vesicle size are determining factors in the PC-TP-catalyzed PC transfer.

To investigate whether the strong association of PC-TP with the small-sized vesicles (see Table II) could be due to an asymmetric distribution of PA in those vesicles, the localization of the phospholipids was studied by using ^{31}P NMR techniques (Koter et al., 1978). For comparison, the top fractions (28-nm vesicles) and side fractions (22-nm vesicles) were analyzed (for preparation of samples, see Materials and Methods). As shown in Figure 5A, the NMR spectrum of the 22-nm vesicles yields four resonances at chemical shift positions of −4.6, −4.7, −5.8, and −6.9 ppm downfield from external triphenylphosphine in chloroform which represent PC in the outer and inner monolayer and PA in the outer and inner monolayer, respectively. The spectrum obtained after the NMR signal

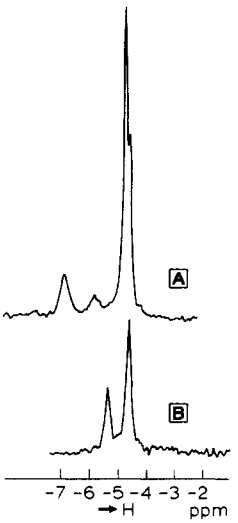

FIGURE 5: 36.4-MHz ^{31}P NMR spectra of sized sonicated vesicles. Vesicles (80:20 mol % PC:PA) of an estimated 22 nm in diameter were used in the analysis (for preparation, see Materials and Methods). PC/PA vesicles (spectrum A); PC/PA vesicles in the presence of 10 mM $CoCl_2$ (spectrum B). The resonance peaks are downfield from external triphenylphosphine in chloroform.

Table III: Phospholipid Composition of Inner and Outer Vesicle Monolayers As Measured by ^{31}P NMR

	28-nm vesicles		22-nm vesicles	
	PC (mol %)	PA (mol %)	PC (mol %)	PA (mol %)
total vesicle[a]	78	22	77	23
inside monolayer[a]	75	25	64	36
outside monolayer[b]	79	21	82	18

[a] The PC/PA composition of the whole vesicles and of the inner monolayer of the vesicles was directly measured from the ratio of the peak areas of the PC and PA resonances before and after addition of $CoCl_2$ (see Materials and Methods). The distribution of lipid over the inner and outer halves of the bilayer was derived from the sum of the peak areas of the PC and PA resonances before and after addition of $CoCl_2$. The inner monolayer of the 28- and 22-nm vesicles contained 29% and 28% of the total lipid, respectively. [b] The PA/PC composition of the outer monolayer was calculated from the lipid composition of the inner monolayer and the total vesicle and the lipid distribution over outer and inner monolayers.

of the outer monolayer is quenched by the addition of $CoCl_2$ is shown in Figure 5B. From these spectra, the phospholipid content and composition of inner and outer vesicle monolayers have been estimated (Table III). The 22-nm vesicles have 28% of the total phospholipid in the inner monolayer as compared to 29% for the 28-nm vesicles. The PA content of the 22-nm vesicles and of their inner and outer monlayers is 23%, 36%, and 18%, respectively, as compared to 22%, 25%, and 21%, respectively, for the 28-nm vesicles. This indicates that PA is asymmetrically distributed in the small-sized vesicles, with an increase of PA in the inner monolayer, and a relatively small decrease of PA in the outer monolayer. Hence, the sharply increased association of PC-TP with the 22-nm vesicles is not due to an anomalous phospholipid composition of the outer monolayer. The size of the vesicles as estimated from the line width of the ^{31}P NMR spectrum is 23 nm for the top fraction and 21 nm for the side fraction. These values agree reasonably well with those estimated by photon counting spectroscopy (see Figure 3).

Net Transfer of PnA-PC. A unidirectional release of PC by PC-TP into membrane interfaces devoid of PC has been shown by electron spin resonance and resonance energy transfer techniques (Wirtz et al., 1980; Nichols & Pagano, 1983). Here, the protein-mediated transfer of PnA-PC to

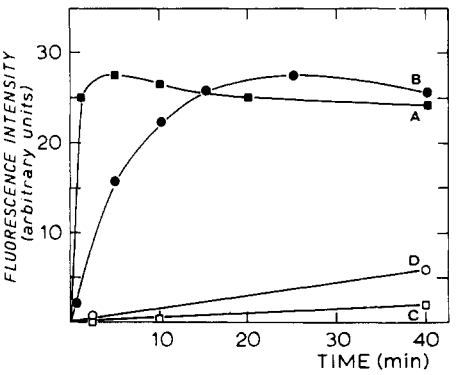

FIGURE 6: Net transfer of PnA-PC to vesicles of pure PA as mediated by PC-TP. Donor vesicles of PnA-containing phospholipids were prepared by injection of an ethanolic solution into 2 mL of TES buffer and acceptor vesicles by sonication. Reactions were initiated by addition of 2.8 μg of PC-TP. Both donor and acceptor vesicles contained 10 nmol of phospholipid. PC-TP-catalyzed transfer of PnA-PC from donor vesicles of PnA-PC/PA (80:20 mol %) to acceptor vesicles of PC/PA (80:20 mol %) (curve A) and to vesicles of pure PA (curve B); transfer of PnA-PC to PA vesicles in the absence of PC-TP (curve C); transfer of PnA-PA from vesicles of pure PnA-PA to vesicles of PC/PA (80:20 mol %) in the presence of PC-TP (curve D).

vesicles of PC/PA (80:20 mol %) was compared with that to vesicles of pure PA (Figure 6). In this experiment, the concentration of the donor vesicles (80:20 mol % PnA-PC:PA) was identical with that of the acceptor vesicles (5 μM). Under the conditions of exchange (curve A), the fluorescence intensity attained a maximum after 5 min, followed by a slow decrease due to self-quenching of the incoming PnA-PC in the PC/PA acceptor vesicles. This self-quenching becomes apparent when the acceptor contains 10 mol % PnA-PC (Somerharju et al., 1983). The protein-mediated transfer of PnA-PC to the PA vesicles proceeded much slower, attaining a maximal fluorescence intensity in 25 min (curve B). This suggests that at least 10% of the donor PnA-PC has been incorporated into the PA vesicles. In the absence of PC-TP, the transfer of PnA-PC to the PA vesicles was negligible (curve C). To investigate the possibility of PC-TP transferring PA in the opposite direction, donor vesicles of pure PnA-PA were incubated with acceptor vesicles of PC/PA (80:20 mol %). A very slow increase of fluorescence intensity was observed which may, in part, reflect a gradual spontaneous redistribution of PnA-PA between the vesicles (curve D). However, the progress curves B and D conclusively show that PC-TP is able to net transfer PnA-PC to the PA vesicles. In some experiments, the PA acceptor vesicles were sized by molecular sieve chromatography before use. Initial rates of PC-TP-mediated net transfer of PnA-PC to the sized PA vesicles (ranging from 22 to 36 nm in diameter) did not significantly differ. This suggests that the vesicle size does not control the rate at which PC-TP transfers PnA-PC to the acceptor vesicles.

Net transfer of PnA-PC was also shown by using acceptor vesicles of PE/PA (80:20 mol %) (curve B, Figure 7). The ratio between donor and acceptor phospholipid was 1:20 so that the nanomoles of PnA-PC transferred was directly related to the fluorescence intensity. The PC-TP-mediated transfer of PnA-PC to the PE/PA vesicles was about 10 times slower than that to the PC/PA vesicles (curve A). Maximal transfer of PnA-PC to the PE/PA vesicles was about 15% of that to the PC/PA vesicles. Spontaneous transfer of PnA-PC to the PE/PA vesicles was negligible (curve C). Back-transfer of PE or PA was investigated by using donor vesicles of PnA-PE/PnA-PA (80:20 mol %) and acceptor vesicles of PC/PA (80:20 mol %). Addition of PC-TP resulted in a transfer equal

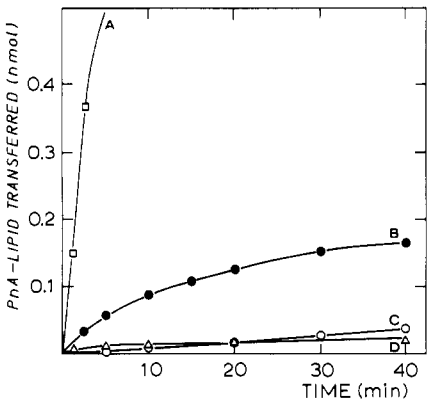

FIGURE 7: Net transfer of PnA-PC to vesicles of PE/PA as mediated by PC-TP. Donor and acceptor vesicles were present in 2 mL of TES (see legend to Figure 6). The donor and acceptor vesicles contained 2.8 and 55 nmol of phospholipid, respectively. Transfer of PnA-PC from vesicles of PnA-PC/PA (80:20 mol %) to vesicles of PC/PA (80:20 mol %) in the presence of 0.8 μg of PC-TP (curve A) and to vesicles of PE/PA (80:20 mol %) in the presence of 3.2 μg of PC-TP (curve B); transfer of PnA-PC to PE/PA vesicles in absence of PC-TP (curve C); transfer of PnA-PE/PnA-PA from vesicles of PnA-PE/PnA-PA (80:20 mol %) to vesicles of PC/PA (80:20 mol %) in the presence of 3.2 μg of PC-TP (curve D).

to the spontaneous transfer (curves D and C), indicating that PC-TP does not transfer PnA-PE or PnA-PA in agreement with previous studies (Kamp et al., 1977; Nichols & Pagano, 1983).

Discussion

In the present study, we have used the PnA-PC transfer assay to investigate the effects of acidic phospholipids (i.e., PA) and vesicle size on the kinetics of the PC-TP-mediated transfer of PC between vesicles. In some previous studies (DiCorleto & Zilversmit, 1977; DiCorleto et al., 1977), acidic phospholipids were shown to enhance the rate of transfer, whereas in other studies (van den Besselaar et al., 1975; Wirtz et al., 1979) these phospholipids gave rise to a diminished rate. Here we have demonstrated that an increase of the vesicle PA content stimulates the PC-TP-mediated transfer of PC at relatively low acceptor concentrations and inhibits at higher concentrations (see Figure 2). This kinetic pattern reflects the competition between donor and acceptor vesicles for the freely diffusing PC carrier under conditions where PC-TP has a higher affinity for the more negatively charged membranes (Somerharju et al., 1981).

As shown in Table I, the apparent association constant, K, increases with increasing vesicle PA content. These values are in good agreement with previous results (Wirtz et al., 1979; Helmkamp, 1980). At constant donor and PC-TP concentration, the rate of transfer is proportional to the association rate constant, k_1 (see eq 2). This implies that PC-TP is stimulated by an increasing vesicle PA content (see Table I). In this context, it is of interest to note that multilamellar liposomes of pure soy PC do not function with PC-TP in the transfer assay but do so if phosphatidylinositol (30 mol %) and cardiolipin (5 mol %) are mixed in with the soy PC (DiCorleto & Zilversmit, 1977). This has been confirmed by Bozatto & Tinker (1982) using liposomes of dipalmitoyl-PC with and without dipalmitoylphosphatidylglycerol (5 mol %). Apparently, the presence of acidic phospholipids in the interface facilitates the removal of PC by PC-TP from that interface. This may be related to the electrostatic repulsion perturbing the packing of PC in the bilayer. That packing is an important parameter in the transfer process was suggested by the finding that unilamellar vesicles were much better substrates than multilamellar liposomes of the same composition. Machida & Ohnishi (1980) reported that the transfer rate of spin-labeled PC to PC vesicles was more than 100 times larger than to PC liposomes. This agreed with the observation that the association and dissociation rate constants of the PC-TP/membrane complex were 50–100 times larger for vesicles (90:10 mol % PC/PA) than for liposomes (Wirtz et al., 1979). That the interaction of PC-TP with the vesicle is greatly affected by its curvature has been shown in the present study using sized vesicles consisting of PC/PA (80:20 mol %). A decrease of the vesicle size was accompanied by a large increase of the association constant (Table II). Vesicles with a diameter larger than 30 nm barely gave rise to inhibition kinetics ($K < 2$ mM^{-1}), whereas vesicles with a diameter of 22 nm bound PC-TP very strongly ($K = 57$ mM^{-1}; see Figure 4). These vesicles and the ones with an intermediate diameter represented different fractions from one peak obtained by molecular sieve chromatography (Figure 3). In agreement with other studies (Wetterau & Jonas, 1982; Brouillette et al., 1982), this one peak is a composite of unilamellar vesicles of different size. That is, the vesicle diameter determined for each peak fraction is an average value. Hence, we should reckon with the possibility that PC-TP interacts strongly only with the smallest size vesicles. This would offer one explanation as to why the value of K decreases gradually going from peak fractions with small size to larger size vesicles. However, it is evident that vesicles of homogeneous size are required to satisfactorily answer this point. Vesicle size as an important parameter was also pointed out by Machida & Ohnishi (1980), who reported that vesicles of pure PS with a diameter of 17 nm strongly inhibited PC-TP in contrast to PS vesicles with a diameter of 22 nm.

The PC vesicles containing 20 mol % PA range from 22 to 40 nm in diameter (see Figure 3). This compared to a range of 15–27 nm for unilamellar vesicles of egg PC only (Brouillette et al., 1982). An increase in vesicle size in relation to PA content has been reported before (Johnson, 1973). As for the smallest size vesicles (22 nm in diameter) used in the transfer assay, it is very likely that the hydrated outer surface area per PC molecule is significantly larger than that for vesicles with a diameter larger than 30 nm. This surface area has been calculated to be 84 Å^2 for PC in vesicles (21 nm in diameter) as compared to 72 Å^2 for a maximally hydrated planar PC molecule (Small, 1967; Cornell et al., 1980). This difference in surface area is reflected in a greater average freedom of motion for the hydrocarbon chains (Lentz et al., 1976; Petersen & Chan, 1977). The present study suggests that the extreme curvature in conjunction with high surface charge favors the formation of a strong complex between PC-TP and the smallest size vesicles. It remains to be determined whether this interaction is accompanied by a penetration of PC-TP into the interface. To date, we have no evidence that under conditions of a low value of K PC-TP needs to penetrate into the interface to exert its action. Recently, increasing bilayer curvature was shown to facilitate the penetration of apolipoprotein A-I into the bilayer (Wetterau & Jonas, 1982). In addition to surface charge and vesicle size, the activity of PC-TP is also sensitive to whether PC is in the gel or liquid-crystalline state (Kasper & Helmkamp, 1981) and to the occurrence of vesicle surface defects (Bozatto & Tinker, 1982). All these various parameters seem to indicate that the activity of PC-TP is to a large extent determined by the facility with which a PC molecule flips from the interface onto the protein.

In agreement with previous studies (Wirtz & Devaux, 1980; Nichols & Pagano, 1983), we have shown that PC-TP can give a net transfer of PnA-PC to vesicles composed of either PA or PE/PA (80:20 mol %). It was estimated that about 10% of the donor PnA-PC could be transferred to the acceptor as compared to 20% using spin-labeled PC (Wirtz & Devaux, 1980). In the above studies, the PC donor vesicles contained 20–25 mol % PA. Interestingly, no transfer of PC was observed to vesicles of either PA, PS, or PE/PA (80:20 mol %) when donor vesicles of pure PC were used (Nichols & Pagano, 1983). This suggests that the negative charge on the donor vesicle generates the proper condition for PC-TP devoid of an endogenous PC molecule to extract a PC molecule from the interface (i.e., net transfer). Whether such conditions occur in the cell remains a matter of speculation. Recent studies by Yaffe & Kennedy (1983) raised some doubt on phospholipid transfer proteins being responsible for the intracellular movement of phospholipids.

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