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Spontaneous intervesicular transfer of anionic phospholipids differing in the nature of their polar headgroup

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A set of synthetic negatively charged phospholipids with different polar headgroups was investigated with respect to their spontaneous transferability between 'fluid' small unilamellar vesicles. As donor vesicles dimyristoylphosphatidylcholine mixed with an anionic lipid in a 9:1 molar ratio was used whereas the recipient particles solely consisted of dimyristoylphosphatidylcholine. The progress of the anionic lipid transfer was followed by continuous free-flow electrophoresis. In a first approach, the different anionic phospholipids used were esters of phosphatidic acid and simple alcohols (from ethanol to hexanol), polyalcohols (ethyleneglycol, glycerol, erythritol) and serine. In spite of their different nature, the effect of these anionic lipids on the melting behavior of the various types of mixed donors is limited. At 33°C in 5 mM Tes (pH 7.0), 10 mM potassium chloride, dimyristoylphosphatidic acid transfers with a halftime of 156 min. This transfer rate gradually decreases upon progressive addition of methyl(ene) residues in the polar headgroup. In contrast, all polyalcohol and serine derivatives move at a faster rate. These different transfer rates are correlated with the hydrophobic/hydrophilic character of the polar domains. We also proved that an additional methylene group present in the fatty acyl chains has a stronger reducing effect on the speed of the transfer process than if it is present in the polar moiety. The identical activation energies found for dimyristoylphosphatidic acid and -phosphatidylbutanol further indicate that the butyl chain in the bilayer is not shielded from the aqueous environment. Secondly, also pH affects the transfer of dimyristoylphosphatidic acid: above pH 7.0 transfer occurs at a relatively fast speed whereas below pH 4.5 it is considerably retarded. These observations are discussed in terms of hydrogen bonding, electrostatic interactions, as well as the intrinsic hydration properties of the different dissociation states of the phosphate group.

Introduction

Using a wide variety of physical techniques, it is well established that phospholipids are able to move spontaneously between distinct membrane entities (for review see Ref. 1). Although the molecular details of the transfer phenomenon are not completely defined, on the basis of the available experimental evidence, most investigators as-

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Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; PG, phosphatidylglycerol. $DC_{13:0}PX$, DMPX, $DC_{15:0}PX$ and DPPX, di-fatty acyl form of phospholipid where PX = PC, PA, PS, PG or phosphate linked to another base written in full. L, Lauroyl; $C_{13:0}$, tridecanoyl; M, myristoyl; $C_{15:0}$, pentadecanoyl; P, palmitoyl; Tes, 2-([tris(hydroxymethyl)methyl]amino)ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; T_t, phase transition temperature; $t_{1/2}$, halftime.

sume the so-called 'aqueous transfer model' to explain the transfer sequence [2,3]. This model proposes that the lipid molecule is first desorbed from the donor particle, followed by a convection through the aqueous phase and subsequently it is taken up by the acceptor. Detailed kinetic analyses, performed by McLean and Phillips [4] and Nichols and Pagano [5], further point to the escape-step as being rate-limiting.

The rate of transfer can be affected by the degree of fluidity of the bilayer matrix [2,6] and in the case of charged lipids also by the membrane surface charge [7]. Also the chemical structure of the transferable phospholipid molecule itself is of great importance. In this respect the role played by the fatty acyl side chains is quite well understood [8]. Very recently, Massey et al. [9] were even able to predict the transfer of different phosphatidylcholines on the basis of their retention times in hydrophobic chromatography. In contrast, detailed studies concerning the impact of the polar headgroup composition on the transfer process between membranes are still scarce [10–12].

In the present work, we attempt to evaluate to what extent hydrophobic and hydrophilic groups, present in a phospholipid molecule, determine its transfer properties. To this end, we first synthesized a number of anionic dimyristoylphospholipids with different alkyl or polyhydroxyalkyl residues attached to the phosphate group and investigated their transfer properties. Secondly, we analyzed the transfer characteristics of phospholipids bearing the same amount of methyl(ene) residues but which are differently distributed over the fatty acyl side chains and the polar headgroup. Thirdly, we also determined how changes in the number of charges, present in the polar headgroup, modulate the kinetic behavior. DMPA was chosen for this purpose since it has two dissociable protons.

Materials

DLPC, $DC_{13:0}PC$, DMPC and DMPS were purchased from Avanti (Birmingham, AL). DPPA was obtained from Serdary (London, Canada). Sodium salts of DMPEthanol, DMPPropanol, DMPButanol, DMPPentanol, DMPHexanol, DMPEthyleneglycol, DMPG, DMPErythritol, $DC_{13:0}PEthanol$ and DLPButanol were synthesized in this laboratory from the corresponding phosphatidylcholines by single step transphosphatidylation catalyzed by phospholipase D according to Eibl and Kovatchev [13]. Phospholipase D was obtained as a crude extract from Brussels sprouts. One milligram of the crude enzyme was used per 3 mg of phospholipid, present in the incubation mixture. After the exchange reaction, the material was taken up in chloroform and purified by preparative thin-layer chromatography on silica gel plates (Merck, Darmstadt, F.R.G.) using chloroform/methanol/ammonium hydroxide (25% ammonia by weight) (65 : 30 : 3, by vol.). DMPA was collected as a major by-product during the purification of DMPPentanol and DMPHexanol. $DC_{15:0}PA$ was prepared by hydrolysis of $DC_{15:0}PC$ (Avanti) with phospholipase D according to the above-mentioned procedure. All lipids ran as a single spot on overloaded analytical thin-layer plates. [^{14}C]Cholesteryloleate was an Amersham product.

Methods

Vesicle preparation. Small unilamellar vesicles with a diameter of about 30 nm were prepared by the sonication procedure and were characterized as previously described [3]. [^{14}C]Cholesteryloleate, used as a non-exchangeable marker, was incorporated in the acceptor vesicles at a ratio of 0.05 μCi/mg of vesicle lipid. It was added before the organic solvent evaporation step. Sonication media were made up of 10 mM potassium chloride in the presence of the following buffer components (each in a 5 mM concentration): acetate (pH 4.1, 4.5, 5.1), Mes (pH 6.0), Tes (pH 7.0, 7.2, 7.4), Tris (pH 8.4) and glycine (pH 9.0). At extreme pH values 15 mM hydrogen chloride or 15 mM potassium hydroxide were used. After their generation, the vesicles were immediately used for the kinetic experiments.

Detection of phase transitions. Gel-to-liquid crystal phase transition temperatures of the vesicles (2 mg of phospholipid/ml) were deduced from light scattering measurements at 90° on an Aminco Bowman spectrophotofluorometer. The change in scattering intensity (excitation and emission wavelength set at 400 nm) was recorded continuously as

a function of temperature (Fluke digital thermometer) on a Hewlett Packard *X-Y* recorder.

Free-flow electrophoresis. Continuous free-flow electrophoresis was performed essentially as described [3]. The electrophoresis chamber was filled with the same buffers as used for vesicle sonication. The unbuffered incubation mixtures at pH 2.2 and 11.0, however, were injected in a 5 mM Tes buffer (pH 7.0), 10 mM potassium chloride. The buffers in the electrode system had the same composition as the separation buffer but were 5-times concentrated.

Kinetic treatment. Transfer of anionic phospholipids was followed by measuring time-dependent changes in the electrophoretic migration distance of the vesicles. True first-order rate constants were calculated and from these halftimes were computed [3].

Results

Physical state of the vesicles

Sonicated dispersions of the single component, anionic dimyristoylphospholipids used in this study all demonstrate an ordered $\rightleftharpoons$ disordered phase transition at pH 7.0 (not shown). DMPA, $DC_{15:0}PA$ and DPPA exhibit a distinct inflection point in their light scattering scans at 53, 64 and 73°C, respectively. The T_t of DMPA is drastically reduced upon linking an alcohol base to the phosphatidyl moiety. Within the homologous series of the mono-alcohol derivatives a progressive decrease is observed upon lengthening the alkyl chain. The T_t values equal 23°C for DMPEthanol, 19°C for DMPPropanol, 13°C for DMPButanol, 9°C for DMPPentanol and 8°C for DMPHexanol. Also, the polyalcohol derivatives 'melt' at lower temperatures than DMPA; the T_t of DMPEthyleneglycol and DMPG equals 25 and 24°C, respectively. With DMPErythritol the transition zone is spread evenly starting from 45 to below 0°C. For DMPS a value of 36°C is found. No melting behavior was observed for $DC_{13:0}PEthanol$ and DLPButanol above 0°C. For mixtures of DMPC with these acidic phospholipids (in a molar ratio of 9:1) the T_t values vary between 21 and 28°C (depending on the anionic lipid type), which is in the transition zone of pure DMPC vesicles ($T_t = 24$°C). The transition curves

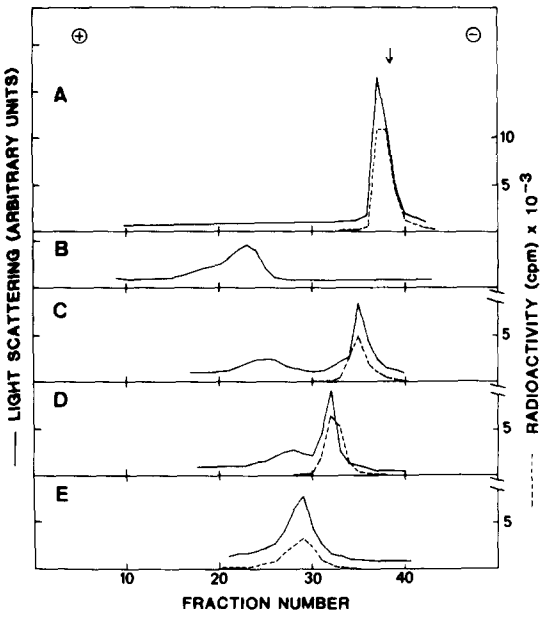


Fig. 1. Measurement of DMPA transfer. Free-flow electrophoresis of (A) DMPC acceptors, (B) DMPC-DMPA (9:1) donors and some representative runs of an equimolar mixture of both as a function of incubation time: (C) after 77 min, (D) after 197 min and (E) after 24 h. Incubation and fractionation were carried out at 33°C in 5 mM Tes, pH 7.0, 10 mM potassium chloride. In these conditions the current equals 150 mA with an electric field set at 60 V/cm. Lipid concentration equals 2 $mg \cdot ml^{-1}$. The arrow indicates that injection occurred above fraction 39.

(not shown) are somewhat broadened but the general form is similar to that of DMPC. This indicates that lipid segregation does not occur [14].

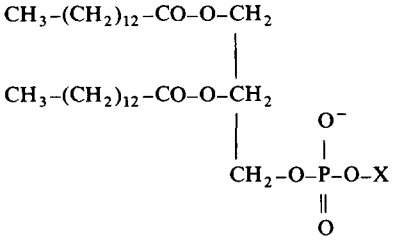
Transfer of anionic dimyristoylphospholipids at neutral pH

In our assay conditions for free-flow electrophoresis, the zwitterionic vesicles are immobile in the electric field. Upon incorporation of 10% of whatever negatively charged dimyristoylphospholipid used in this study, the resulting particles elute at the anodic side and are collected in fraction 22–23 at neutral pH. In equimolar mixtures of these anionic vesicles (donors) with neutral DMPC vesicles (acceptors) the difference in electrophoretic migration distance gradually decreases due to intervesicular transfer of the anionic molecule [15]. This time-dependent process is exemplified in Fig. 1 for the vesicle couple DMPC-

TABLE I

CHEMICAL STRUCTURES AND TRANSFER RATES OF ANIONIC DIMYRISTOYLPHOSPHOLIPIDS AT NEUTRAL pH

Experiments are performed at 33°C in 5 mM Tes (pH 7.0)/10 mM potassium chloride. The donors consist of DMPC-anionic phospholipid mixtures (molar ratio 9:1); the recipient vesicles are built up of DMPC. Donors and acceptors are mixed in equimolar amounts. Lipid concentration equals 2 $mg \cdot ml^{-1}$.



Part	-X	Halftime
A	-H	156 min
B	$-CH_2-CH_3$	270 min
	$-CH_2-CH_2-CH_3$	379 min
	$-CH_2-CH_2-CH_2-CH_3$	758 min
	$-CH_2-CH_2-CH_2-CH_2-CH_3$	> 24 h
	$-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3$	> 24 h
C	$-CH_2-CH_2OH$	71 min
	$-CH_2-CHOH-CH_2OH$	41 min
	$-CH_2-CHOH-CHOH-CH_2OH$	47 min
D	$-CH_2-CH(NH_3^+)-COO^-$	41 min

DMPA (9:1) and DMPC. In all set-ups, mentioned in Table I, we found that the transfer process can be described by first-order mathematics for several halftime periods.

The $t_{1/2}$ values listed in Table I show that the transfer speeds are influenced by the nature of the phospholipid polar headgroup. At pH 7.0, DMPA equilibrates over the two vesicle populations with a $t_{1/2}$ of 156 min (Table IA). The addition of progressively growing alkyl chains to the phosphate group has a strong reducing effect on the transfer properties (Table IB). Contrarily, attaching short polyalcohols to DMPA improves the transfer capacity up to four times but this effect is rather insensitive to the chain length (Table IC). DMPS, bearing two negative and one positive charge at neutral pH [16] also moves faster than DMPA does (Table ID).

Temperature dependence of phospholipid transfer

We also studied the transfer process between 26 and 41°C. Over this temperature range, both donors and acceptors exist in a fluid state. We first investigated the temperature-dependent behavior of DMPA. The effect of introducing hydrophobic parts in the polar headgroup on the partition behavior was also studied as a function of temperature. DMPButanol was chosen as a representative molecule since its transfer kinetics are sufficiently fast to be followed over the entire temperature range. In Fig. 2 the Arrhenius plots [log(rate constant) versus (1/temperature)] are constructed. The activation energies, deduced from their slopes, equal 120 kJ/mol for DMPA and 119 kJ/mol for DMPButanol.

Transfer of phospholipids with the same number of methyl(ene) groups

We also examined the transfer properties of phospholipids containing the same total amount of $-CH_2-/-CH_3$ groups (30, 28 or 26) but which are either entirely located in the fatty acyl side chains or in part also in the polar headgroup. The $t_{1/2}$ values are given in Table II. Within the same series it appears that shifting a methyl(ene) residue from

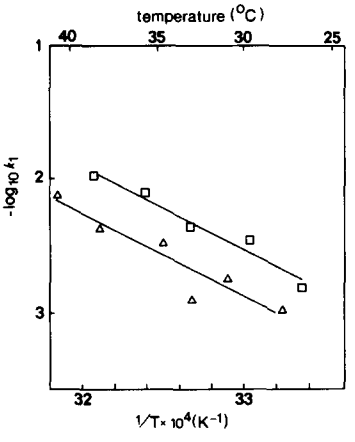


Fig. 2. Arrhenius plots for the transfer of (□) DMPA and (△) DMPButanol at pH 7.0. The incubation mixture contains equimolar amounts of DMPC recipient and DMPC-DMPA (9:1) or DMPC-DMPButanol (9:1) donor membranes. The lines are fitted by linear regression. Other aspects of the assay system are described in Experimental procedures.

TABLE II

KINETIC DATA FOR THE TRANSFER OF NEGATIVELY CHARGED PHOSPHOLIPIDS BEARING THE SAME TOTAL NUMBER OF METHYL(ENE) GROUPS

The experimental conditions are given in the legend to Table I. t_∞ means the time at which equilibrium is reached.

Number of $-CH_2-(-CH_3)$ groups	Transferable phospholipid	Rate
30	DPPA	No transfer observed
	DMPButanol	$t_{1/2}$ = 758 min
28	$DC_{15:0}PA$	No transfer observed
	DMPEthanol	$t_{1/2}$ = 270 min
26	DMPA	$t_{1/2}$ = 156 min
	$DC_{13:0}$PEthanol	t_∞ < 10 min
	DLPButanol	t_∞ < 10 min

the acyl chains to the polar region improves the transfer process.

Transfer of DMPA at different pH values

The polar part of DMPA can also be varied by changing the pH of the medium, which alters the headgroup charge. This effect on the transfer of DMPA is illustrated in Fig. 3. Between pH 4.5 and 7.0 similar $t_{1/2}$ values ($\simeq$ 160 min) are found. Below pH 4.5 the transfer event is strongly retarded, whereas above pH 7.2 transfer occurs too fast to be measured accurately in our experimental conditions; the $t_{1/2}$ values equal at most 10 min.

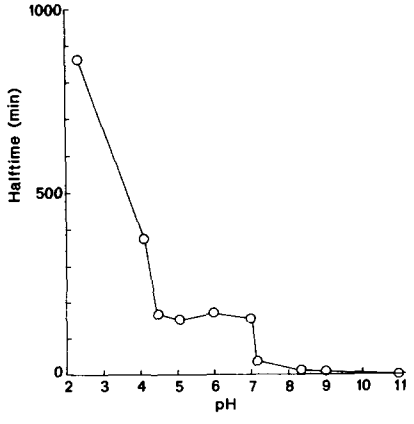


Fig. 3. pH dependency of the transfer rate of DMPA at 33°C. Mixed DMPC-DMPA vesicles act as donors; DMPC vesicles are acceptors. Assay conditions, including the different media for incubation and analysis are described in the Experimental procedures.

Discussion

In this work, we especially focused our attention on the role played by the nature of the polar headgroup region in determining the transfer capacity of a phospholipid molecule. Therefore, we took special care to keep uniform all bulk phase parameters which might influence the transfer process such as the degree of membrane fluidity [2] and the membrane surface charge [7,10]. Indeed, since all the donor vesicle types used in this study contain the same molar amount of anionic lipids and since they migrate at pH 7.0 with a similar velocity in the electric field, we have assumed that electrostatic repulsion forces exerted on the transferred lipid species are identical. The degree of membrane fluidity of the different mixtures is also at a comparable level since all transfer assays are performed at a few degrees above their nearly equal transition temperatures. Consequently, for the anionic dimyristoylphospholipids at neutral pH, we presume that differences in transfer rate mainly reflect the different behavior of the headgroups with respect to their (aqueous) environment.

In view of the aqueous phase transfer hypothesis [2,3], the increase in the $t_{1/2}$ values upon addition of $-CH_2-(-CH_3)$-residues to the phosphate group of DMPA can be explained by a presumed decrease in the critical bilayer concentration of the resulting phospholipids. Interestingly, in this respect Okahata et al. [17] observed lower monomolecular solubilities in water of nonionic amphiphiles with increasing alkyl chain length. In the case of DMPS and the DMPA derivatives with polyalcohols (Table I), here too the halftimes can be correlated with the hydrophilic properties of the polar part. Based on the roughly estimated hydration data of Cevc [18] we calculated the hydration numbers * to be 7–8 for DMPA; 8–9 for DMPEthyleneglycol; 8–11 for DMPG (see also Ref. 19), DMPErythritol, and DMPS (see also Refs. 20, 21). These numbers

* The term 'hydration number' is used without specifying the strength of the interaction.

qualitatively correlate with the transfer speed (DMPA < DMPEthyleneglycol < DMPG, DMPErythritol, DMPS).

Yet, it remains difficult to give a picture detailing the accommodation of each group esterified to the phosphate. With the simple alkyl-headgroup phospholipids for instance the decrease in T_t with increasing chain length may be caused (i) by a gradual increase in the cross-sectional area of the headgroup [22] which facilitates interchain separation [23] and/or (ii) possibly by a (partial) interdigitation of the (longer) alkyl chains in the bilayer matrix, thereby causing some packing defects [24]. In any case, a methyl(ene) group present in the polar region modulates the intrinsic transfer capacity of a phospholipid molecule in a different way as compared with a methylene group in the fatty acyl side chains (see Table II). Qualitative information about the hydration state of the alkyl chains can be gathered from the Arrhenius plots. The 'apparent' activation energy probably is determined by the so-called hydrophobic effect, i.e. with the energy needed to disrupt the strong attractive forces between water molecules when a solute (e.g. nonpolar) is dissolved in water [25]. For DMPA and DMPButanol, both having 26 $-CH_2-/-CH_3$ groups in their fatty acyl chains, we found a similar activation energy. This suggests that during the escape of a DMPButanol molecule from the donors no (or only a few) additional water molecules are forced to come into contact with the butyl residue. We must stress, however, that this conclusion does not necessarily imply that the alkyl chain would not infiltrate the membrane. Blume [26] for instance claims that there may be more water molecules present in the hydrophobic part of the bilayer than commonly thought.

Beside chemical manipulations, changes in the polar headgroup can also be triggered by altering the pH. For this purpose PA is the most likely candidate to investigate since it can appear in three different states: neutral, singly and doubly charged. For pure PA membranes the first proton is released between pH 1 and 3 and the second one between pH 7.5 and 10 [27]. Also in the transfer curve of DMPA (Fig. 3) three different zones are distinguished: $t_{1/2} > 160$ min below pH 4.5; $t_{1/2} \simeq 160$ min between pH 4.5 and 7.0 and $t_{1/2} < 10$ min above pH 7.2. Similarly around pH 7.0, Massey et al. [28] observed a drastic change in the partitioning rate of a pyrene-labeled PA between apolipoprotein-phospholipid recombinants. Thus, apparently, the charge on the headgroup is a major factor in determining the transfer properties of DMPA. Unfortunately, no information is available about the ionization state of DMPA in our mixed vesicles at the prevailing ionic strength of the incubation media. Consequently, it remains difficult to ascertain whether or not the pK values of DMPA coincide with the inflection point(s) in the transfer curve.

As discussed above, again the transfer rates can be correlated with the degree of solvatation (the approximate hydration numbers equal 5 for $H_2PO_4^-$, 7–8 for a single and 9–11 for a double negatively charged phosphate residue; [18]). Undoubtedly, other factors will also affect the DMPA jump from the donors. For instance, at high pH we may expect that the higher the degree of intermolecular electrostatic repulsions is (particularly in our low ionic strength media), the faster the transfer will occur. In a similar sense, Felgner et al. [29] found that trisialoganglioside moves at a faster rate than monosialoganglioside from their micelles to PC vesicles. In addition to this direct effect of electrostatics, in pure PA membranes at high pH, destabilization is further promoted by the disruption of the lattice of hydrogen bonds [30]. In the low pH-range the interpretation of our DMPA transfer results is further complicated by the possibility that the matrix DMPC lipid molecules in the donors will become positively charged (pK of the phosphate of PC equals 2–3; [16]). In these circumstances, we may reasonably expect that the transfer of the remaining monovalent DMPA molecules will be considerably inhibited or even completely abrogated. This situation is indeed similar to that of DMPG in cationic vesicles as described previously [7].

Conclusions

The studies presented here complete our picture of the spontaneous intervesicular phospholipid transfer event in one essential point: the partitioning behavior of phospholipids between membranes and the aqueous solvent can be modulated

by modifying the phospholipid polar headgroup characteristics. This statement may also explain the observations of Nichols and Pagano [31], concerning the enhancement of the spontaneous transfer of a broad range of phospholipids by bovine liver nonspecific lipid transfer protein. The transient interaction of the protein with the bilayer-embedded lipid molecules indeed may inprove the water solubility of the latter and consequently their escape from the donor particles. As donors we used artificial vesicles, existing in a 'liquid-like' state and bearing negative charges as biological membranes generally do. Possibly, our observations may be of relevance with respect to the biogenesis of new membranes or the maintenance of existing ones. For instance, near neutral pH, small shifts in pH may exert a regulating influence on the spontaneous intermembraneous transfer of PA, e.g. from outer to inner mitochondrial membrane as recently described by Baranska and Wojtczak [32].

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