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Fusion, Leakage and Surface Hydrophobicity of Vesicles Containing Phosphoinositides: Influence of Steric and Electrostatic Effects

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Abstract. Calcium and lanthanum ion-induced fusion of lipid vesicles containing phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP), phosphatidylinositol-4,5-bisphosphate (PIP_2) or phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and its associated membrane properties, e.g., surface dielectric constant and vesicle leakage, were studied by fluorescence methods. The presence of poly-phosphorylated phosphoinositides (PPI) in lipid vesicles enhanced fusion, depending on the PPI phosphorylation level and the PPI concentration, as determined by the lipid mixing assay. This correlation held even at physiologically relevant small concentrations of PPI in vesicle membranes. However, the presence of nonphosphorylated PI inhibited fusion due to the steric effect of the inositol ring. The cation threshold concentration for the lipid mixing of vesicles made of mixtures of phosphatidylserine (PS) with PI increased with increasing PI contents. For all vesicle systems studied, a decrease in vesicle surface dielectric constant and an increase in vesicle leakage accompanied fusion. The presence of the nonphosphorylated inositol ring in PI did not interfere with the changes in the surface dielectric constant caused by fusogenic cations. Therefore, we deduce that the reduction of the surface dielectric constant is a necessary condition for membrane fusion to occur but it does not correlate with membrane fusion when interacting membranes are blocked for close approach as by the nonphosphorylated inositol ring.

Key words: Phospholipids — Phosphoinositides — Membranes — Fusion — Leakage — Surface hydrophobicity

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Introduction

Phosphoinositides are a group of phospholipids that is directly involved in signal transduction and other cellular processes. In spite of their low abundance in cellular membranes (5 to 10% of cellular lipids), the phosphoinositides are of enormous physiological importance. The phosphoinositide turnover acts as pathway to transmit extracellular signals through cell membranes by formation of the second messengers inositol-l,4,5-trisphosphate (IP_3) and diacylglycerol (DG) (Berridge & Irvine, 1989; Berridge, 1993). A second signaling pathway includes the 3-phosphorylated phosphoinositides, e.g., phosphatidylinositol-3,4,5-trisphosphate (PIP_3). They appear to regulate multiple cell functions, such as the vesiculation from and the fusion of vesicles with the cellular membrane (Kapeller & Cantley, 1994; Carpenter & Cantley, 1996; Scales & Scheller, 1999).

The phosphoinositides differ from other phospholipids by the inositol ring system at the hydrophilic headgroup. Here, they can be phosphorylated by phosphokinases. While PI has one negative charge at the phosphodiester group, the charge of the PPI increases with increasing number of phosphomonoester groups at the inositol ring in the order $PIP < PIP_2 < PIP_3$. The pK_a values of these groups vary between 6 and 8 depending on the nature of the phosphoinositide, experimental parameters such as local pH and salt concentration, and the binding of ions or proteins (van Paridon et al., 1986; Marsh, 1990). Consequently, the net charge of a certain phosphomonoester group varies between -1 and -2. Therefore, the charge on PIP_2, for example, can be -3, -4, or -5 (Toner et al., 1988; McLaughlin et al., 2002).

PI, PIP and PIP_2 are found almost exclusively on the inner leaflet of the plasma membrane (Gascard et al., 1991). Due to their high negative charge, they can act as binding sites for cytosolic cations and cationic

domains of proteins (McLaughlin, 1989; Isenberg, 1991; Janmey, 1995). Alterations in local PPI-concentrations or the clustering of these lipids would lead to changes in the membrane physical properties, such as local charge density, local curvature, packing properties and lateral tension.

The physical characteristics of PPI-containing bilayer systems are not yet fully understood. ^{2}H-NMR, neutron diffraction and x-ray diffraction were used to determine the molecular conformation of PPI in model membranes and the orientation of the inositol ring (Hansbro et al., 1992; Bradshaw et al., 1996, 1997, 1999). The electrophoretic mobility of PPI-containing vesicles, their surface electrostatics as well as their cation binding properties have been studied both experimentally and theoretically (McDaniel et al., 1984; Toner et al., 1988; Langner et al., 1990; Denisov et al., 1998).

Membrane fusion is influenced by the physicochemical properties of the participating membranes. Because of their specific properties, the phosphoinositides are possibly involved in fusion processes of cellular membranes (Jones & Clague, 1995; Summers, Guebert & Shanahan, 1996; Ruiz-Arguello et al., 1998). Membrane fusion occurs in various intra- and intercellular processes, such as exocytosis, endocytosis, membrane genesis, and fertilization. It was established by phospholipid vesicle studies that all the diverse membrane fusion events have definite physical principles in common (Rand & Parsegian, 1986; Papanadjopoulos, Nir & Düzgünes, 1990; Ohki, 1991; Ohki & Arnold, 2000). Cation-induced aggregation and fusion experiments were carried out to elucidate the role of surface forces and membrane destabilization. Differences in the charge state of the participating lipids are expected to influence the cation-induced fusion especially, via electrostatic forces. Other factors related to membrane fusion (membrane stability and hydrophobicity of the vesicle surface) are specifically influenced by the lipid species.

Sundler and Papahadjopoulos (1981) have found that pure PI-LUV do not fuse even at very high Ca^{2+} concentrations (up to 50 mM), and concluded that PI inhibits the cation-induced fusion of vesicles due to the steric effect of the uncharged inositol ring, preventing the close approach of vesicle surfaces. Summers et al. (1996) showed that the addition of phosphate groups at the outer inositol ring resulted in an increase of the fusion ability of vesicles containing rather high concentrations of PPI (25 mol%).

In the present study, we investigated fusion of PC vesicles containing PI, PIP, PIP_2, or PIP_3 by fluorescence methods monitoring different steps of membrane fusion (membrane dehydration, vesicle leakage and phospholipid mixing). For the first time, these experiments were conducted in vesicle systems containing PPI at a physiologically relevant concentration of only 5 mol% in a PC membrane. Further, similar experiments were carried out on vesicles prepared from mixtures of PI with phosphatidylserine (PS). All measurements were performed by varying the bulk Ca^{2+} or La^{3+} concentrations to compare the effects of di- and trivalent cations.

Materials and Methods

Chemicals

Egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS) and the ammonium salt of bovine liver phosphatidylinositol (PI) were purchased from Avanti Polar Lipids (Alabaster, AL). The sodium salt of phosphatidylinositol-4-phosphate (bovine brain, PIP) was obtained from Sigma (Deisenhofen, Germany). The ammonium salt of phosphatidylinositol-4,5-bisphosphate from bovine brain (PIP_2) was obtained from Calbiochem (Bad Soden, Germany). The ammonium salt of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) containing saturated fatty acid chains (1,2-dipalmitoyl-PIP_3) was obtained from Biomol (Hamburg, Germany). Lipids obtained as powders were dissolved in chloroform. However, for the PPI, the addition of methanol and a slight acidification by the addition of a small amount of HCl were required. All lipids were characterized for molecular weight and purity by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Schiller et al., 1999; Müller et al., 2001) and used without further purification. The fluorophore-labeled phospholipids dansyl phosphatidylethanolamine (DPE), nitrobenzoxadiazole phosphatidylethanolamine (NBD-PE) and lissamine rhodamine B sulfonyl phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids. Aminonaphthalene trisulfonic acid (disodium salt, ANTS) and xylene-bis-pyridinium bromide (DPX) were purchased from Molecular Probes Inc. (Eugene, OR). All other chemicals were of reagent grade and obtained from Sigma (Deisenhofen, Germany).

Vesicle Preparation

Multilamellar vesicles (MLV) were prepared using the method of Bangham, Hill & Miller (1974). Aliquots of lipids were combined in organic solvent. The lipid solvents were evaporated in a round-bottom flask at a pressure of 20 mbar and subsequently dispersed in buffer solution (10 mM HEPES, 100 mM NaCl, pH 7.4) prepared in purified water (0.2 mM EDTA present for measurements in the absence of cations). Then, the lipid suspension was intensely vortexed using a Vortex Genie 2 (Scientific Industries, Bohemia, NY) for 10 min and subsequently shaken in a water bath at 37°C for another 10 min. Small unilamellar vesicles (SUV) were prepared by sonication of the MLV-suspension with a tip sonifier (Branson Tip Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT) for 10 min.

Particle size of vesicles was measured by dynamic light scattering using a Malvern Zetasizer 4 (Malvern Instruments, Malvern, UK). The instrument was equipped with a 5-mW Helium-Neon laser, and the scattered light was detected at an angle of 90°. Signals were analyzed by multimodal exponential fit of the autocorrelation function. All SUV preparations showed a relatively broad size distribution around an average vesicle diameter of 60–80 nm. No indications for micelle formation of pure PPI were found.

Fluorescence Measurements

All fluorescence measurements were carried out on a Perkin-Elmer fluorescence spectrometer (model LS-50B, Beaconsfield, UK) at a temperature of 37°C using quartz cuvettes equipped with magnetic

stirrer. If not stated otherwise, aliquots of vesicle suspensions were diluted in 3 ml buffer solution to reach a final lipid concentration of 20 μM. Before starting the experiment, the system was allowed to equilibrate for about 5 min. All measurements were carried out at least in duplicate. The agreement between different runs was well within a 10% error margin.

Phospholipid Mixing

Phospholipid mixing was investigated by the NBD-PE/Rh-PE assay (modified from (Struck, Hoekstra & Pagano, 1981)). The method is based on the fluorescence energy transfer (FRET) from NBD, acting as donor, to Rhodamine, acting as acceptor. Both fluorophores were present in chemically modified lipids (PE). They were incorporated into vesicle membranes by mixing the organic solutions of NBD-PE (1 mol%), Rh-PE (1 mol%) and the matrix lipids, and preparing fluorophore-labeled vesicles from this mixture as described above. A second vesicle population was prepared from the same lipids but in the absence of fluorophore-labeled lipids. For the measurements, 31 μl of a 1 mM stock of labeled vesicles and 31 μl of a 1 mM stock of unlabeled vesicles were combined in 3 ml buffer solution representing a 1:1 ratio of labeled and unlabeled vesicles. The fluorescence measurements were carried out by exciting the NBD at 460 nm and recording the fluorescence emission of both NBD and Rh in emission spectra ranging from 500 to 600 nm. When phospholipid mixing between fusing labeled and unlabeled vesicles occurs, the average distance between NBD- and Rh-labeled molecules increases on the membrane. In this case, the acceptor emission intensity at 588 nm decreases, whereas the donor emission intensity at 520 nm increases, since the FRET efficiency strongly depends on the distance between donors and acceptors (Förster mechanism). The extent of phospholipid mixing M was calculated from

$$M = (q - q_0)/(q_{\text{Triton}} - q_0) \cdot 100\% \quad (1)$$

where $q = I_{\text{NBD}} / I_{\text{Rh}}$ is the ratio of the intensity of NBD at 520 nm and the intensity of Rhodamine at 588 nm. The values for q_0 were measured in the absence of the fusion trigger. The values for q_{Triton} were measured to define the 100% value of phospholipid mixing by solubilization of the vesicles in 0.2% (v/v) Triton X-100. By using the ratio q instead of the NBD-intensity, no intensity correction for light scattering or for quenching of the NBD-fluorescence by Triton X-100 was necessary.

Surface Dielectric Constant

For measurements of the vesicle surface polarity, 1 mM vesicle stock suspensions were prepared containing 1 mol% dansyl-phosphatidylethanolamine (DPE) (Ohki & Arnold, 1990). The wavelength position of maximum dansyl emission intensity is related to the polarity of the probe environment (Kimura & Ikegami, 1985) and decreases in wavelength with increase in the surface hydrophobicity of vesicle membranes (Ohki & Arnold, 1990). For the measurements, 62 μl of the vesicle stock solution was diluted in 3 ml buffer solution to match the concentration conditions of the NBD/Rh assay. Fluorescence of DPE was detected by exciting at 340 nm and recording the fluorescence signal from 400 to 600 nm. To avoid influences of light scattering on the estimation of the maximum emission wavelength $\lambda_{\max}^{em}$, the spectra were fitted to a combination of Gaussian scattering peaks and one asymmetric double-sigmoidal emission peak, using a commercial peak fitting software (PeakFit, Jandel Scientific, Erkrath, Germany). The surface dielectric constant ε was calculated using an empiric formula from measurements in standard organic solvents

$$\lambda_{\max}^{em} = 462.8 + 31.4 \cdot \log \varepsilon. \quad (2)$$

Vesicle Leakage

Leakage of vesicle contents was studied using a procedure based on the dynamic quenching of ANTS fluorescence by DPX (Ellens, Bentz & Szoka, 1985). Vesicle stock solutions of 12 mM total lipid were prepared in a solution containing both fluorescent dyes (20 mM NaCl, 12.5 mM ANTS, 45 mM DPX). As checked with a semi-microosmometer (KNAUER GmbH, Berlin, Germany) this solution had the same osmolarity as the buffer. Nonencapsulated fluorophores were removed from vesicles by passing the vesicle suspension through a Sephadex-G 75 column. The lipid concentration after column was determined by performing a phosphorus assay (Chen, Toribara & Warner, 1956). For the measurements, 67 μM total lipid in 3 ml buffer were used. The excitation was at 384 nm and the total fluorescence emission intensity was measured at wavelengths >530 nm due to the weak ANTS fluorescence. The time course of ANTS fluorescence was recorded and the leakage extent, L, was calculated according to

$$L = (I - I_{\text{scatter}} - I_0)/(I_{\text{Triton}} - I_{\text{scatter}} - I_0) \cdot 100\% \quad (3)$$

where I is the maximum fluorescence intensity obtained after addition of di- or trivalent cations, I_0 is the initial fluorescence of intact vesicles containing both ANTS and DPX in buffer solution. I_{Triton} is the fluorescence intensity of lysed vesicles (using Triton X-100 as described above) and was used as the value for 100% leakage. Intensities were corrected for light scattering intensity I_{scatter} measured using unlabeled vesicles only (Düzgünes & Bentz, 1988).

Abbreviations

ANTS:	8-aminonaphtalene-1,3,6-trisulfonic acid
DPE:	*N*-(6-dimethylaminonaphtalene-2-sulfonyl)-l,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine
DPX:	*p*-xylene-*bis*-pyridiniumbromid
HEPES:	2-[4-(2-hydroxyethyl]-1-piperazinyl]ethane sulfonic acid
LUV:	large unilamellar vesicles
NBD-PE:	*N*-(7-nitrobenz-2-oxa-l,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine
PC:	phosphatidylcholine
PE:	phosphatidylethanolamine
PI:	phosphatidylinositol
PIP:	phosphatidylinositol-4-monophosphate
PIP_2:	phosphatidylinositol-4,5-bisphosphate
PIP_3:	phosphatidylinositol-3,4,5-trisphosphate
PPI:	(poly-)phosphoinositides
PS:	phosphatidylserine
Rh-PE:	*N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine
SUV:	small unilamellar vesicles

Results

PPI/PC Mixtures

Phospholipid Mixing

Figure 1 shows the Ca^{2+} and La^{3+}-induced phospholipid mixing of SUV consisting of 5 mol% PI, PIP, PIP_2 or PIP_3 and 95 mol% egg PC. The measurements indicate a strong dependence of phospholipid mixing on the phosphorylation state of the phosphoinositide. The concentration of divalent (Ca^{2+}) or trivalent

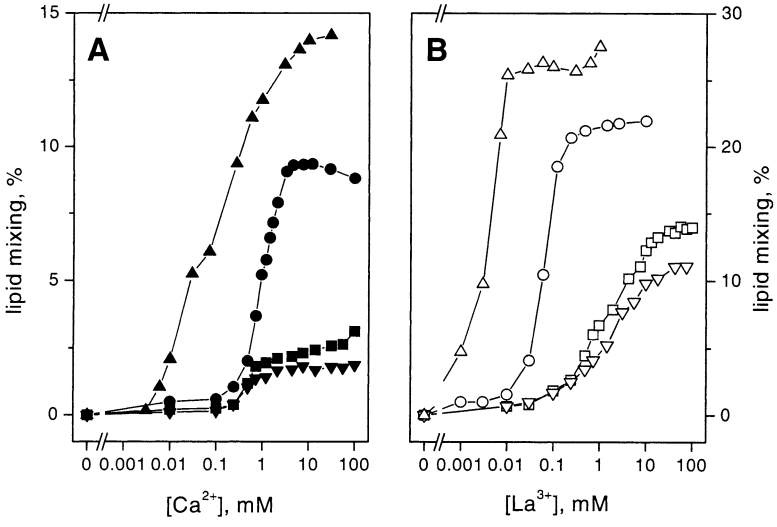

Fig. 1. Cation-induced phospholipid mixing of SUV prepared from 5 mol% of different phosphoinositides in mixture with PC. Measurements were carried out in a buffer containing 100 mM NaCl and 10 mM HEPES at pH 7.4. Ca^{2+}- induced (panel *A*, filled symbols) and La^{3+}-induced phospholipid mixings (panel *B*, empty symbols) were measured using the NBD-PE/Rh-PE assay. For the measurements in the absence of cations, 0.2 mM EDTA was present. Data are shown for vesicles prepared from 5 mol% PIP_3 (▲ and △), PIP_2 (● and ○), PIP (■ and □), or PI (▼ and ▽) in mixture with PC.

(La^{3+}) cations necessary to induce vesicle fusion decreased and the maximum phospholipid mixing extent increased with increasing phosphorylation level of the PPI. Under the influence of Ca^{2+} (Fig. 1*A*), both PI- and PIP-containing PC-SUV showed only a negligible lipid mixing up to Ca^{2+} concentrations of 100 mM, whereas the mixing effect was well observed for PIP_2- or PIP_3-containing SUV. The Ca^{2+}-threshold concentration for lipid mixing of PIP_2-containing PC-SUV was about 2 mM. For PIP_3-containing vesicles, a measurable phospholipid mixing effect was already observed at approx. 0.01 mM Ca^{2+} and the extent of lipid mixing was higher than for PIP_2-containing SUV. For the La^{3+}-induced phospholipid mixing, the same phosphorylation-state dependence as in the Ca^{2+} case was observed (Fig. 1*B*). The critical La^{3+} concentrations for lipid mixing were approx. 1.2 mM for PI-, approx. 1 mM for PIP-, approx. 0.05 mM for PIP_2-, and approx. 0.003 mM for PIP_3-containing PC-SUV.

In Fig. 2, phospholipid mixing experiments are shown for PC-SUV of varying PIP_2-content. Critical cation concentrations were determined to be approx. 2 mM Ca^{2+} or approx. 0.05 mM La^{3+} for PC-SUV containing 5 mol% PIP_2 as before, approx. 1.5 mM Ca^{2+} or approx. 0.03 mM La^{3+} for PC-SUV containing 10 mol% PIP_2, and approx. 0.7 mM Ca^{2+} or approx. 0.007 mM La^{3+} for PC-SUV containing 20 mol% PIP_2. The decrease in the critical cation concentrations with increasing PIP_2 content was accompanied by an increase in the phospholipid mixing extent. For SUV of different PIP/PC mixtures, qualitatively the same dependence of critical concentrations and mixing extents on the phosphoinositide content of the membrane was found (*data not shown*).

Surface Dielectric Constant

In Fig. 3, the Ca^{2+}- and La^{3+}-concentration dependence of the surface dielectric constant is plotted for SUV prepared from PC (94 mol%), DPE (1 mol%) and PI, PIP, or PIP_2 (5 mol%), respectively. In the absence of Ca^{2+} or La^{3+}, the measured dielectric constant values were about $\varepsilon = 32$. The variation in the observed values for vesicles containing differently phosphorylated phosphoinositides was in the range of the experimental errors. Comparable values were also found for PS- and PA-SUV (Ohki & Arnold, 1990; Ohki & Zschörnig, 1993). Addition of Ca^{2+} at fusion-inducing concentrations (>2 mM) led to a decrease of the surface dielectric constant of PIP_2-containing PC-SUV to a value of $\varepsilon = 23$. This decrease was much greater than that found for PIP- or PI-containing PC-SUV. The latter showed only small effects at Ca^{2+} concentrations higher than 10 mM. On the other hand, La^{3+} decreased the vesicle surface dielectric constant considerably when it exceeded a certain threshold concentration. These threshold concentrations were lower by at least two orders of magnitude in comparison to Ca^{2+} and decreased with increasing degree of PPI phosphorylation. For PC-SUV containing 5 mol% PIP_2, concentrations of 0.01 mM La^{3+} were sufficient to obtain a surface dielectric constant of 25. The lowest surface dielectric constants measured at high concentrations (>1 mM) were 18 for PC-SUV containing 5 mol% PI, 14 for PC-SUV containing 5 mol% PIP, and 12.5 for PC-SUV containing 5 mol% PIP_2. This sequence was in agreement with the results of the phospholipid mixing assay.

For PC-SUV containing 5 mol% PIP_3 with saturated acyl chains ($DPPIP_3$), the DPE emission maximum in the absence of cations (508.6 nm) revealed a surface dielectric constant of 28.7 (*data not shown*). Ca^{2+} or La^{3+} at fusion-relevant concentrations did not shift the dansyl emission peak to lower wavelengths, but resulted in a remarkable splitting of the emission signal into two peaks. One of them had a constant position at 508.6 nm ($\varepsilon = 28.7$), but the other shifted with increasing Ca^{2+} or La^{3+} concentration to

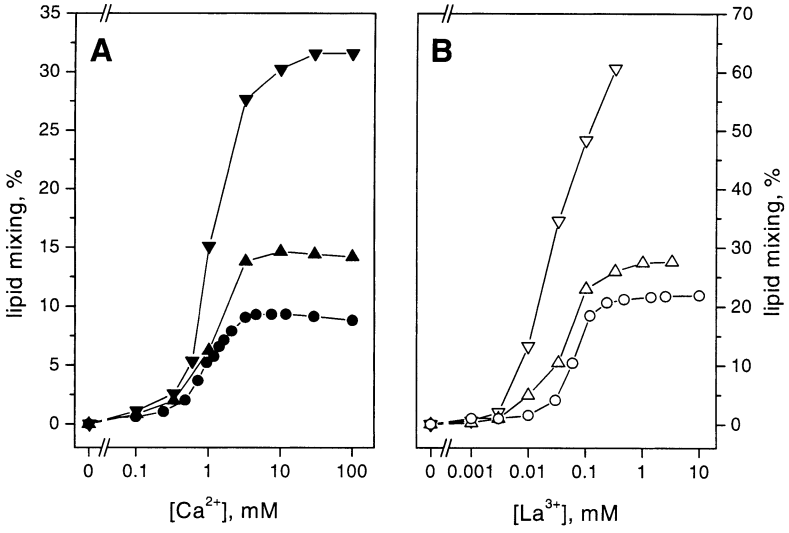

Fig. 2. Phospholipid mixing of PIP_2/PC-SUV with varying PIP_2 concentration. Cation-induced phospholipid mixing of SUV prepared from PC and different concentrations of PIP_2 (5 mol%: ● and ○, 10 mol%: ▲ and △, or 20 mol%: ▼ and ▽) was measured using the NBD-PE/Rh-PE assay. All other conditions were as in Fig. 1. Filled symbols (panel *A*) refer to the case of Ca^{2+}; empty symbols (panel *B*) refer to that of La^{3+}.

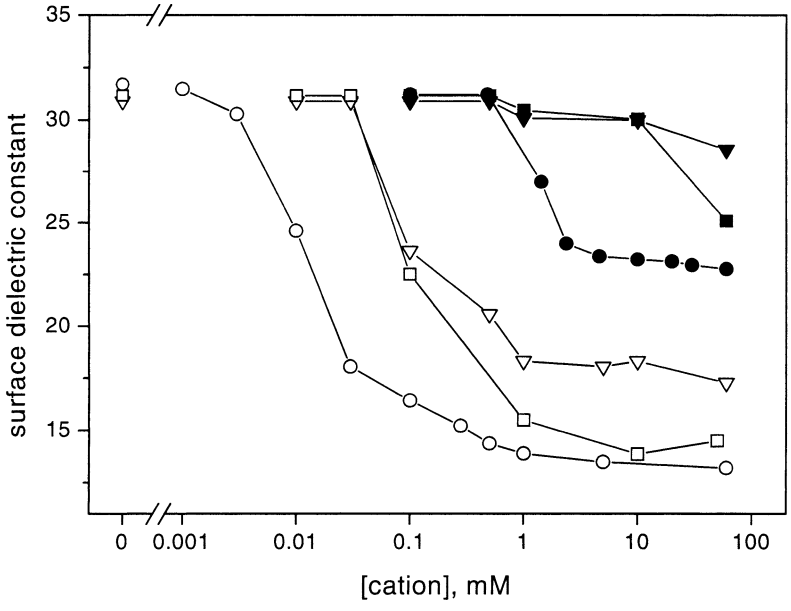

Fig. 3. Cation-induced changes of the surface dielectric constant of SUV prepared from 5 mol% phosphoinositide and PC. The surface dielectric constant was calculated from the position of the fluorescence emission maximum of the membrane-incorporated fluorophore dansyl-PE (DPE, 1 mol%). SUV were prepared from 5 mol% of PIP_2 (● and ○), PIP (■ and □), or PI (▼ and ▽) in mixture with PC. Filled symbols refer to the case of Ca^{2+}; empty symbols refer to that of La^{3+}. The measurements were carried out at 100 mM NaCl, 10 mM HEPES, pH 7.4.

lower wavelengths (lowest observed: 485 nm corresponding to $\varepsilon \approx 5$). Therefore, the spectra could not be fitted by a combination of one emission peak and scattering peaks. The integral intensity ratio of both emission peaks changed with increasing cation concentration. While the constant peak reduced its intensity, the second peak gained intensity when Ca^{2+} or La^{3+} concentrations increased. We suppose that this behavior is related to a lateral separation of two DPE species in the $DPPIP_3$ containing membrane. Although preliminary FT-IR studies revealed that $DPPIP_3$ is in the liquid-crystalline phase at 37°C, lateral separation processes may be intensified by the different acyl chain composition of dipalmitoyl PIP_3 and egg PC.

In Fig. 4 (panel *A*), the effects of Ca^{2+} or La^{3+} on SUV containing 5 mol% PIP_2 in mixture with PC are compared with those on SUV containing 20 mol% PIP_2 in PC. In the absence of di- or trivalent cations, the measured surface dielectric constants were 31.7 for SUV of 5 mol% PIP_2 in PC and 25.1 for SUV of 20 mol% PIP_2 in PC. These differences are exceeding the error margin of our measurements and can be related to the hydration properties of the phosphorylated inositol ring. The addition of cations decreased the surface dielectric constant when they exceeded a certain threshold concentration. In agreement with the results of the phospholipid mixing experiment (Fig. 2), an increase of the content of negatively charged PIP_2 resulted in a decrease of this threshold. The values for SUV containing 20 mol% PIP_2 were approx. 0.6 mM Ca^{2+} or approx. 0.004 mM La^{3+}. When the Ca^{2+} or the La^{3+} concentrations were increased clearly over this threshold, finally values below 10 were achieved.

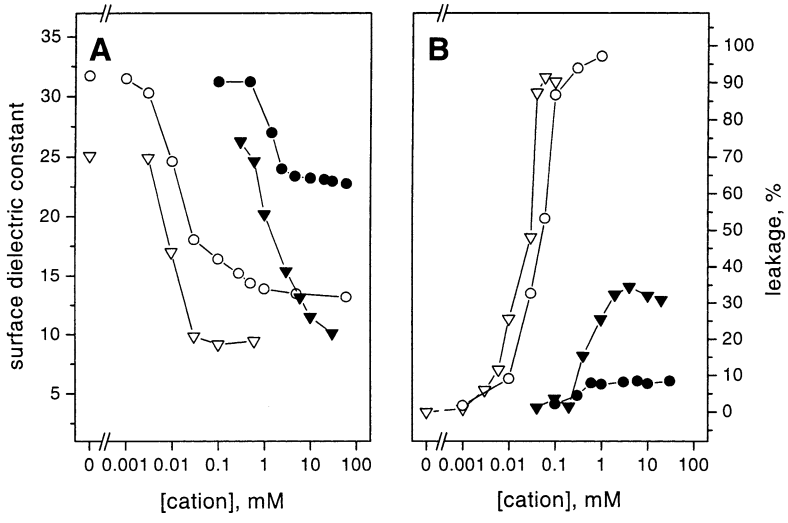

Fig. 4. Cation-induced changes of the surface dielectric constant (panel *A*) and cation-induced vesicle leakage (panel *B*) of PIP_2/PC-SUV. SUV were prepared from different concentrations of PIP_2 (5 mol% (● and ○) or 20 mol% (▼ and ▽)) in mixture with PC. The buffer contained 100 mM NaCl and 10 mM HEPES at pH 7.4. The surface dielectric constant was measured as in Fig. 3. Leakage of internal contents was measured using the ANTS/DPX assay. Filled symbols refer to the case of Ca^{2+}; empty symbols refer to that of La^{3+}.

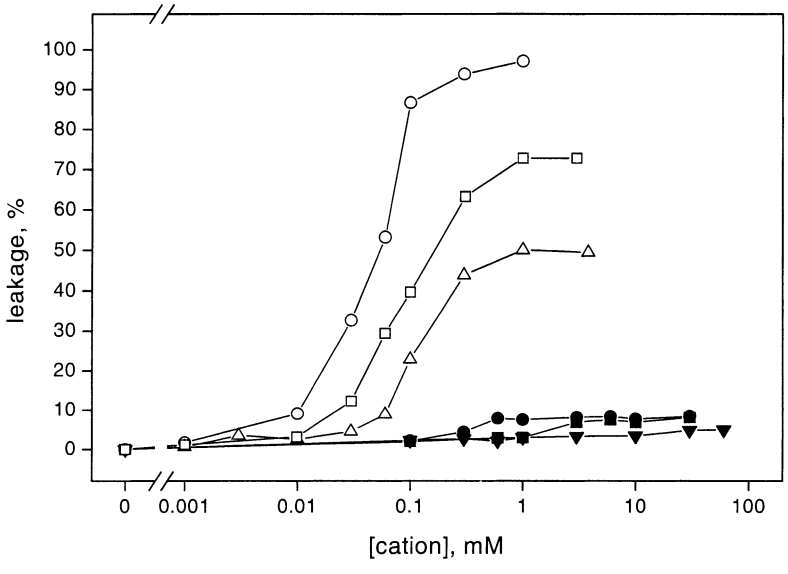

Fig. 5. Cation-induced vesicle leakage of SUV made of 5 mol% of different phosphoinositides and 95 mol% PC. The Ca^{2+}-induced (filled symbols) and La^{3+}-induced (empty symbols) leakage of internal contents was measured using the ANTS/DPX assay. Vesicles were prepared from 5 mol% of PIP_2 (● and ○), PIP (■ and □), or PI (▼ and ▽) in mixture with PC. Experimental conditions were as in Fig. 4*B*.

Leakage of Vesicle Contents

Panel *B* of Fig. 4 gives the results of the leakage assay for both the influence of Ca^{2+} and La^{3+} on SUV prepared from 20 mol% PIP_2 in mixture with 80 mol% PC. Again, the curves for SUV containing 5 mol% PIP_2 in mixture with PC are given for comparison. The critical concentrations for contents leakage from vesicles containing 20 mol% PIP_2 (approx. 0.5 mM Ca^{2+} or approx. 0.008 mM La^{3+}) were lower than for vesicles containing 5 mol% PIP_2 (approx. 0.9 mM Ca^{2+} or approx. 0.02 mM La^{3+}).

The Ca^{2+}- or La^{3+}-induced leakage of internal contents from PC-SUV containing 5 mol% PI, PIP or PIP_2, respectively, is shown in Fig. 5. Experiments with PIP_3 were not conducted because of the large amount of PIP_3 necessary for these experiments. Surprisingly, in all cases the measured maximum extent of Ca^{2+}-induced leakage was relatively small (below 10%). Besides, the leakage rate was very low. The ANTS fluorescence increased only very slowly after the addition even of a high concentration of Ca^{2+} (up to 60 mM). Typically, the leakage maximum was reached after 30 to 45 min, with no remarkable Ca^{2+} concentration dependence. The leakage induced by La^{3+} was much more pronounced compared to Ca^{2+}. The La^{3+} effects were in the same sequence as the extent of phospholipid mixing ($PI < PIP < PIP_2$).

PI/PS Mixtures

Phospholipid Mixing

Figure 6 shows the Ca^{2+}- and La^{3+}-induced phospholipid mixing of SUV prepared from different mixtures of PI and PS. Pure PS-SUV fused at threshold concentrations of approx. 1 mM Ca^{2+} or

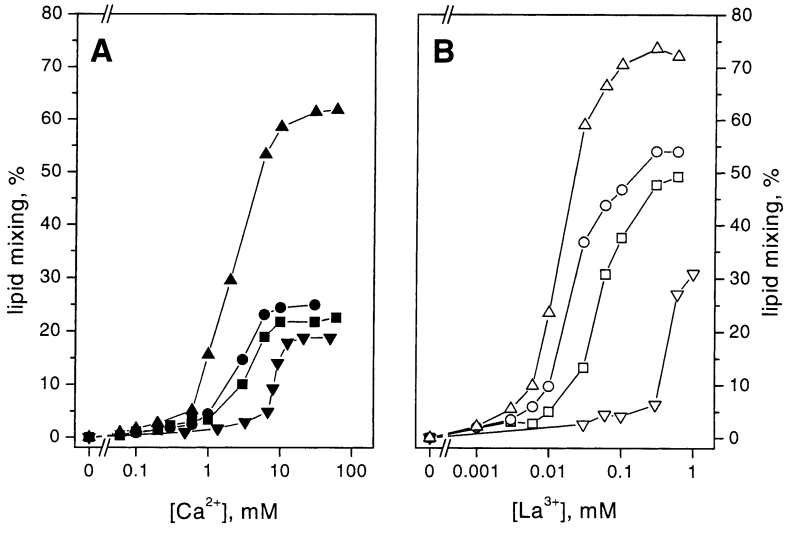

Fig. 6. Cation-induced phospholipid mixing of SUV prepared from various mixtures of PI and PS. Ca^{2+}-induced (panel *A*, filled symbols) and La^{3+}-induced phospholipid mixing (panel *B*, empty symbols) were measured as in Figs. 1 and 2. Data are shown for SUV made of 100 mol% PS (▲ and △) and those prepared from 20 mol% PI in PS (● and ○), 50 mol% PI in PS (■ and □), or 100 mol% PI (▼ and ▽). All other conditions were as in Fig. 1.

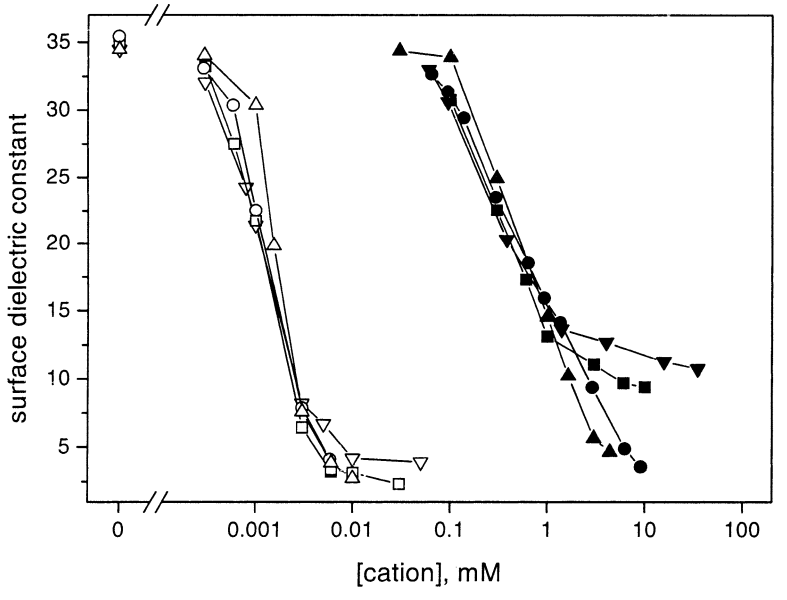

Fig. 7. Cation-induced changes in the surface dielectric constant for SUV prepared from various mixtures of PI and PS. The surface dielectric constant was determined as in Fig. 3 for SUV made of 100 mol% PS (▲ and △) and those prepared from 20 mol% PI in PS (● and ○), 50 mol% PI in PS (■ and □) or 100 mol% PI (▼ and ▽). Filled symbols refer to the Ca^{2+} case; empty symbols refer to the La^{3+} case.

approx. 0.01 mM La^{3+} leading to relatively high lipid mixing extents of approx. 60% in the Ca^{2+} case and approx. 72% in the La^{3+} case. These data are in agreement with Papahadjopoulos et al. (1974) and Ohki & Arnold (1990). For SUV prepared from 80 mol% PS and 20 mol% PI, we determined critical concentrations of approx. 2.5 mM Ca^{2+} or approx. 0.015 mM La^{3+}. For SUV prepared from 50 mol% PS and 50 mol% PI, they were approx. 3.5 mM Ca^{2+} or approx. 0.03 mM La^{3+}. SUV prepared from PI only showed threshold concentrations of approx. 10 mM Ca^{2+} or approx. 0.4 mM La^{3+}. Thus, as the PI concentration in PS-SUV increased, the Ca^{2+}- or La^{3+}-threshold concentration increased and the extents of phospholipid mixing reduced.

Surface Dielectric Constant

Figure 7 shows the effects of Ca^{2+} and La^{3+} on the surface dielectric constant of SUV made of PI/PS mixtures with varying PI concentration. In the absence of di- or trivalent cations, the surface dielectric constants ε for all vesicle cases were around 34 and differed only slightly. The addition of Ca^{2+} or La^{3+} led to a drastic decrease in the surface dielectric constant at a certain cation concentration. For PS-SUV, this was at approx. 0.002 mM La^{3+} or approx. 0.2 mM Ca^{2+}, respectively. There was no significant shift of the threshold when the PI content in the vesicles increased. However, under the influence of Ca^{2+}, there was a dependence of the minimum di-

electric constant at high Ca^{2+} concentrations on the PI content of the vesicles. It was 12 for pure PI-SUV, 9 for SUV containing 50 mol% PI and 5 for SUV containing 20 mol% PI and 80 mol% PS. The minimum surface dielectric constant for PS-SUV was obtained at approx. 0.01 mM La^{3+} or approx. 10 mM Ca^{2+}, respectively, and was below 5. This was also the case for the effect of La^{3+} on SUV prepared from different PI/PS mixtures.

Discussion

Phosphorylated PPI Promote Fusion at Physiological Concentrations

Our experiments clearly demonstrate a fusion-promoting effect of PPI phosphorylation. The higher the negative charge of the incorporated phosphoinositide in a PC membrane, the more vesicle fusion was observed (*see* Fig. 1). The effect might be related to the finding that phosphoinositides are necessary for fusion of some types of cellular membranes.

Our results are in agreement with Summers et al. (1996), who compared the Ca^{2+}- and Mg^{2+}-induced fusion of LUV prepared from relatively high concentrations (25 mol%) PI, PIP or PIP_2 in mixture with phosphatidylethanolamine (PE). With a decrease in the concentrations of PPI in a mixture, the extent of fusion decreases (*see* Fig. 2). However, even for vesicles containing only 5 mol% PIP_2, we detected relevant fusion effects by using the lipid-mixing assay. This might be of biological relevance. Other negatively charged lipids (PS, PG, PA) incorporated into PC vesicles at such low concentrations do not lead to fusion by di- or trivalent cations (*data not shown*). Vesicles made of only zwitterionic phospholipids (PC or PE) do not fuse in the presence of either divalent (Ca^{2+}, Mg^{2+} ...) or trivalent cations (e.g., La^{3+}) in the concentration range employed here (Nir et al., 1983). However, PE is known to decrease the critical cation concentrations for fusion when present in negatively charged membranes (Sundler, Düzgünes & Papahadjopoulos, 1981). In contrast, observed cation effects on vesicles made from PPI-PC mixtures (*see* Figs. 1–5) can be directly attributed to the effect of PPI.

The fusion tendency for vesicles with higher curvature (e.g., SUV as used in our study) is greater than that of LUV or for less curved membranes, since membrane curvature contributes to the energy profile between two interacting membranes (Ohki, 1984; Ohki & Arnold, 2000; Malinin, Frederik & Lentz, 2002; Markin & Albanesi, 2002). In cells, phosphoinositides are reported to be enriched in membrane areas with relatively high curvature (Pike & Casey, 1996; Scales et al., 1999).

The physiologically interesting lipid PIP_3 was not studied by Summers et al. (1996). We found that the Ca^{2+} concentration necessary to induce fusion of PC-SUV containing 5 mol% PIP_3 decreased to some μM. This is remarkable with respect to cellular functions, because intracellular Ca^{2+} concentrations in activated cells were reported to achieve higher levels (Barritt, 1993; Capuozzo et al., 1997). Effects of La^{3+} were still more pronounced than those of Ca^{2+}. Due to its higher charge, La^{3+} causes measurable effects even at concentrations at which Ca^{2+} does not show any effect.

Phophatidylinositol Inhibits Fusion

The presence of PI in lipid mixtures always leads to a reduction of fusion. An increasing PI content in SUV prepared from mixtures of PS and PI decreased the fusion ability of the PS vesicles (*see* Fig. 6). Cation threshold concentrations for fusion of PI-SUV were much higher than that for other negatively charged lipids and pure PI-LUV do not fuse under the influence of cations (Sundler et al., 1981), although PI has a negative charge of −1e like PS or PG, which do undergo cation-induced fusion.

The fusion-inhibiting effect of PI arises from steric constraints of the nonphosphorylated inositol ring. It extends into the aqueous phase (Hammond et al., 1984; Hansbro et al., 1992; Bradshaw et al., 1999) and provides the major barrier for the close approach of PI-containing bilayers (McDaniel & McIntosh, 1989). Additionally, the bulky inositol headgroup interferes with the formation of cation-*trans*-complexes between phosphate groups from apposing membranes. In the phosphorylated phosphoinositides at least one additional negatively charged phosphate group is present at the outer segment of the inositol ring. This enhances the cation binding to the lipids (Toner et al., 1988) and the tendency to form cation bridges between two apposing vesicle surfaces.

Surface Hydrophobicity

To achieve fusion, a certain increase in vesicle surface hydrophobicity by the depletion of water from the vesicle-contact region is necessary (Arnold, 1995).

The vesicle hydrophobicity was monitored by measuring the surface dielectric constant (Ohki & Arnold, 1990). Under the influence of Ca^{2+} and La^{3+}, the surface dielectric constant for all investigated phosphorylated and nonphosphorylated phosphoinositides decreased. For SUV containing 5 mol% PPI in mixture with PC, the critical cation concentrations for this effect followed the same sequence as those for phospholipid mixing: PI > PIP > PIP_2. Although the NBD/Rh assay revealed that PI inhibited lipid mixing, it did not inhibit the cation-induced membrane dehydration, since cation binding also occurs at PI. While the threshold con-

centrations for lipid mixing increased with increasing PI concentrations in PI/PS-SUV, the ion concentrations at which the surface dielectric constants changed greatly were almost the same for pure PS-SUV, pure PI-SUV and all investigated PI/PS mixtures (*see* Fig. 7). This demonstrates that an increase in membrane hydrophobicity is a necessary but not a sufficient condition for membrane fusion to occur. When the close approach of the two membranes is blocked due to the additional energy barrier arising from the steric properties of the inositol ring, the two membranes do not fuse.

For vesicles consisting of purely negatively charged phospholipids, a dielectric constant of 10 was discussed to be typical (Ohki & Arnold, 1990; Arnold, 1995). Values below 10 were not measured for pure PI-SUV in the presence of Ca^{2+}. The presence of PS in mixtures with PI facilitates the formation of cation bridges and the depletion of water from the contact region of two apposing membranes. Therefore, the surface dielectric constants decreased below 10 for all systems containing PS when exceeding the respective cation concentration thresholds. In the presence of La^{3+}, surface dielectric constants below 10 were measured even for systems with high PI content and low fusion tendency.

The minimum surface dielectric constants for vesicles from all investigated mixtures of 5 mol% PPI and 95 mol% PC were higher than 10 (*see* Fig. 3). In mixed membranes containing low amounts of negatively charged PPI as well as zwitterionic phospholipids, areas with and areas without cation binding coexist. Here, only the dielectric constant at the surface of membrane areas that bind cations is reduced. The polarity probe DPE is supposed to be homogeneously distributed over the whole membrane and reports an averaged value for the surface dielectric constant. Then, the actual value in the presence of cations is affected by the portion of charged lipids in the mixture as seen in Fig. 4.

The higher the phosphoinositide charge is, the more cations accumulate at the vesicle surface due to its increasing electrostatic potential (Langner et al., 1990). This effect can be influenced by the local pH as well as by the salt concentration of the solution. It may additionally increase at low ionic strength, depending on the experimental conditions (Galneder et al., 2001) or can lead to an enhanced binding of cations also to uncharged lipids (Huster, Arnold & Gawrisch, 2000) and influence the surface dielectric constant as well.

Leakage of Vesicle Contents

A local destabilization of the bilayer structure is a prerequisite of a complete vesicle fusion. The destabilizing action of cations on vesicles made (partly) of negatively charged phospholipids leads to a release of aqueous contents as seen by the leakage of encapsulated dyes. This can be a consequence of membrane instability resulting from membrane-stressing forces either with or without fusion (Nir, Bentz & Wilschut, 1980; Ellens et al., 1985; Ohki 1991, 2000).

As discussed for the effects of PI on the surface dielectric constant, PI did not inhibit the leakage induced by Ca^{2+} or La^{3+}. We determined approx. 1.5 mM Ca^{2+} or approx. 0.008 mM La^{3+} as critical cation concentrations for vesicle-contents leakage for PI-SUV, and approx. 1 mM Ca^{2+} or 0.005 mM La^{3+} for PS-SUV. That means that PI does not differ from PS in terms of vesicle leakage under the influence of cations.

The low Ca^{2+}-induced leakage for vesicles containing 5 mol% PPI in mixture with PC (*see* Fig. 5) was unexpected. This holds for the low measured leakage extent as well as for the small leakage rate especially with the vesicles containing PIP_2, where significant lipid mixing was observed. Although we cannot obtain data on the membrane structure or the lateral distribution of PIP_2 under the influence of Ca^{2+} with the techniques used in this study, we assume that the low leakage is a consequence of the small amount of cation-binding lipids present in these systems. Accordingly, the leakage extent as well as the leakage rate increased with increasing concentration of PPI (*see* Fig. 4*B*). We suppose that the differences in the effects of Ca^{2+} or La^{3+} are caused by a combination of the composition of the lipid mixture and the binding geometry of the respective cation.

Conclusions

The present experimental data suggest that the influence of phosphoinositides on membrane fusion events must be described as a combination of fusion-promoting and fusion-inhibiting surface properties of PPI-containing membranes. On the one hand, the presence of highly phosphorylated PPI in phospholipid vesicles clearly enhanced fusion. This holds even at very low PPI concentrations. On the other hand, the presence of nonphosphorylated PI reduced fusion, since the inositol ring of PI hinders the cation-induced merging of membranes sterically.

However, this qualitative difference between nonphosphorylated and phosphorylated phosphoinositides is not found for the surface dielectric constant and for vesicle leakage. This leads us to the conclusion that PI does not differ from other phospholipids with one single negative net charge (PS, PG) with respect to the effects of cations on non-adhered membranes, i.e., the cation-induced decrease in surface dielectric constant and the cation-induced vesicle leakage.

The latter two effects will be increased by non-phosphorylated as well as by phosphorylated phos-

phoinositides in membranes from complex mixtures containing both types of phosphoinositides. However, the inhibition of cation-induced aggregation and fusion by PI and the promotion of fusion by phosphorylated phosphoinositides will compete in such mixtures.

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