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Consequences of the interaction of calcium with dioleoylphosphatidate-containing model membranes: calcium–membrane and membrane–membrane interactions

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(1) Calcium binds to dioleoylphosphatidate/dioleoylphosphatidylcholine (DOPA/DOPC) (20:80, mol%) multilamellar vesicles in the presence of a calcium ionophore with stoichiometry of about 0.6 nmol calcium per nmol phosphatidate and an apparent dissociation constant of about 1.7 mM. (2) Experiments on the behaviour of monomolecular films at an air/water interface show that calcium-phosphatidate binding results in a decrease in the area of the polar region of the phosphatidate molecule, probably caused by headgroup dehydration and partial charge neutralization. (3) At calcium concentration higher than about 3 mM calcium neutralizes the negatively charged membrane surface of DOPA/DOPC (20:80, mol%) large unilamellar vesicles, and vesicle aggregation is observed. At 10 mM of calcium this results in a low level of vesicle fusion. (4) These observed processes are not attended with calcium-induced phosphatidylcholine transbilayer movement in the membranes of DOPA/DOPC (20:80, mol%) large unilamellar vesicles. (5) When these findings are compared with the results of a previous study on the permeability behaviour of large unilamellar vesicles of the same phospholipid composition under comparable conditions (Smaal, E.B., Mandersloot, J.G., De Kruijff, B. and De Gier, J. (1986) Biochim. Biophys. Acta 860, 99–108) the following conclusions can be drawn. At low millimolar calcium concentrations (< 2.5 mM) calcium does not occupy all the binding sites of the membrane, no membrane-membrane interactions are observed and a selective translocation of calcium and calcium-chelating anions is appearing. The mechanism of this translocation may be explained by the formation of uncharged dehydrated complexes of calcium, phosphatidate and calcium chelator, which can pass the membrane via transient occurring non-bilayer structures. Between 3 and 10 mM of calcium an aselective permeability increase of the vesicular membrane is found, which is not a consequence of vesicle fusion but apparently of vesicle aggregation, possibly causing packing defects in the membrane.

Introduction

Phosphatidic acid is a key intermediate in the main phospholipid biosynthesis route [1] and, in addition, one of the products of the agonist-induced cyclic metabolic phosphoinositide route in the plasma membrane of a large number of excitable cells [2]. Although it is only a minor membrane component, phosphatidate is considered to play a direct role in transmembrane transport of calcium [3]. Several research groups already have studied these potential calcium-mobilizing properties of

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phosphatidate in model experiments. Holmes and Yoss [5] stated that earlier findings of Serhan et al. [4] about calcium ionophoretic properties of phosphatidate probably were due to oxidation of the acyl chains of the phospholipids. However, more recent studies in which phosphatidate oxidation was excluded confirmed the results of Serhan again [6,7]. Our group presented an improved calcium influx assay [8] and a study on the calcium-dependent permeability behaviour for different kinds of solutes of phosphatidylcholine large unilamellar vesicles containing 20 mol% phosphatidate [9,10]. We concluded that this membrane permeability is more complex than suggested by other groups [4,6,7]. The permeability is dependent on the calcium concentration and is a direct consequence of calcium-phosphatidate interaction [9,10]. Vesicles containing dimyristoylphosphatidate are releasing their content at low millimolar calcium concentrations, while calcium influx is not demonstrable [10]. For dioleoylphosphatidate-containing vesicles [9], however, at low calcium concentrations (< 0.5 mM) no permeability changes were observed. Between 0.5 mM and 2.5 mM of calcium a selective influx of calcium and efflux of enclosed calcium-chelating anions was found, whilst at higher calcium concentrations the membrane did loose its barrier function for a variety of solutes (sulphate, potassium, 6-carboxyfluorescein). All the observed permeability changes were specific for both calcium and phosphatidate [9].

The background of this calcium concentration dependent permeability behaviour is not yet fully understood. Next to the possibility that non-bilayer structures play a role in these events [4,11], a number of physical chemical aspects of the calcium-dioleoylphosphatidate interaction, observed in earlier studies [12,13], might be relevant for the observed phenomena.

In the first place, binding of calcium to the outer surface of phosphatidate-containing vesicles may result in a condensation of the phosphatidate molecules [12] causing a difference in surface pressure between outer and inner monolayer. This difference could be equilibrated by redistribution of phospholipids through transbilayer movement, in which mechanism phosphatidate might act as a shuttle for calcium ions. Secondly, calcium ions could act as a bridge between the membranes of neighbouring vesicles, causing vesicle-aggregation or even fusion [13]. It is already well known that vesicle fusion can give rise to leakage of the vesicle content [14].

To test these possibilities we have investigated the calcium-membrane and membrane-membrane interactions of dioleoylphosphatidate/dioleoylphosphatidylcholine (20 : 80 mol%) systems at different calcium concentrations. We present the results of studies on calcium binding, behaviour in monomolecular films at the air/water interface, vesicle-aggregation and -fusion and phosphatidylcholine transbilayer movement. On basis of these results we suggest a strong correlation between phenomena observed in this study and the results of permeability studies in a previous paper [9].

Materials and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) were synthesized as described earlier [9]. Phosphatidylcholine transfer protein (PC-transfer protein) from bovine liver was a gift from Dr. K.W.A. Wirtz. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE) were from Avanti Polar Lipids (Birmingham, AL); 1-[^{14}C]DOPC and [1,2(n)-^{3}H]cholesteryl hexadecyl ether were from New England Nuclear Research Products (Boston, MA); glycerol tri[9,10(n)-^{3}H]oleate and [^{45}Ca]$CaCl_2$ from Amersham International (Amersham, U.K.). All other chemicals were of a analytical grade.

Vesicle preparation

Large unilamellar vesicles (LUV) were prepared in a 150 mM KCl, 10 mM Tris-acetate (pH 7.4) solution by the reverse-phase evaporation method of Szoka et al. [17] and successive extrusion through a polycarbonate filter (Bio-Rad, Unipor; 0.4 μm pore size).

Multilamellar vesicles (MLV) were prepared by dispersing a dry film of 40 μmol of phospholipid in 3 ml of a 150 mM KCl, 10 mM Tris/acetate (pH 7.4) solution.

Small unilamellar vesicles (SUV) were prepared from MLV by sonication, using a Branson B-12 sonifier (medium tip), under a continuous flow of nitrogen at 0°C for 5 times 0.5 min, followed by centrifugation (30 min; $100000 \times g$) to remove titanium particles and MLV.

Lipid phosphorus was analysed according to Böttcher et al. [15] or Rouser et al. [16].

Cation-vesicle association

Association of calcium with multilamellar vesicles was determined essentially as described by De Kruijff et al. [18]. After preparation, MLV labeled with glycerol tri[^{3}H]oleate (10 nCi/μmol phospholipid) were pelleted by 20 min of centrifugation ($10000 \times g$) and resuspended to a volume of 1 ml in 150 mM KCl, 10 mM Tris-acetate (pH 7.4). Next 75 μl of this suspension (about 40 mM of phospholipid) was incubated with 25 μl of a 150 mM KCl, 10 mM Tris-acetate (pH 7.4) solution, containing 0–10 mM [^{45}Ca]$CaCl_2$ (10 nCi/μmol $CaCl_2$). The suspensions were incubated for 1 h at 20°C in the presence or absence of an excess of the calcium ionophore A23187 (2.5 μg/μmol phospholipid). Subsequently 10 μl samples were taken and the calcium and phospholipid content was determined by liquid scintillation counting (Packard, PRIAS, model PLD). From the remaining suspension the vesicles were removed by centrifugation (15 min, $150000 \times g$; Beckman Airfuge) and the concentration of free calcium was analyzed by liquid scintillation counting of a sample of the supernatant. In all experiments no ^{3}H radioactivity (MLV) was detectable in the supernatant, so the vesicles were pelleted quantitatively. The amount of calcium associated to the vesicles was obtained by substraction of the free-calcium concentrations from the total calcium concentration in the vesicle suspension and subsequent division by the phospholipid concentration.

Magnesium association to MLV was assessed in a similar manner. Instead of [^{45}Ca]$CaCl_2$ solutions (unlabeled) $MgCl_2$ solutions were used. The magnesium content of the samples was determined by atomic absorption spectrometry (Varian Techtron 1200) [19].

Monolayer studies

The surface tension was measured by the Wilhelmy plate method using a recording electrobalance (Beckman LM 500). All aqueous solutions were made using triplely distilled water. The experiments were carried out at 20°C.

Force-area measurements were performed according to Demel et al. [20]. 50.0 nmol of phospholipid dissolved in chloroform was carefully spread by means of an Agla micrometer syringe on a 150 mM KCl, 10 mM Tris-HCl (pH 7.4) subphase (with or without 10 mM $CaCl_2$) in a teflon trough (dimensions 32.2×17.2). The film was compressed by constant mechanical propulsion of a teflon barrier (rate 0.258 $mm^2 \cdot mol^{-1} \cdot min^{-1}$) and the surface pressure was monitored continuously.

Change in surface pressure at constant area. A phospholipid solution in chloroform was carefully spread by means of a glass capillary tube on the surface of a 150 mM KCl, 10 mM Tris-HCl (pH 7.4) solution in a teflon trough, until a surface pressure of about 35 mN/m was reached. The trough (dimensions $5 \times 5 \times 0.5$ cm; volume about 15 ml) had an injection hole to add material to the subphase without disturbing the monolayer and a depression of 0.5 cm depth in the middel for a teflon coated bar to stir the subphase [21]. After stabilization of the surface pressure an aliquot of a 100 mM $CaCl_2$, 10 mM Tris-acetate (pH 7.4) solution was added to the subphase and the change in surface pressure was measured.

Phosphatidylcholine transbilayer movement

The transbilayer movement of phosphatidylcholine in large unilamellar vesicles was assessed essentially according to Noordam et al. [22]. In this assay the exchangeability of [^{14}C]DOPC in the outer monolayer of the vesicles is measured with the aid of PC-transfer protein. An incubation suspension (1.3 ml) contained DOPA/[^{14}C]DOPC (20 : 80, mol%) LUV (2 μmol of phospholipid; 4 nCi 1-[^{14}C]DOPC), DOPC SUV (20 μmol of phospholipid with 1.1 μCi [^{3}H]cholesteryl-hexadecyl ether as non-exchangeable marker) and 35 μg of PC-transfer protein in 150 mM KCl, 10 mM Tris-acetate (pH 7.4) and 0, 1.5 or 5 mM of $CaCl_2$. In some experiments $CaCl_2$ was added to the suspension after 2 h of incubation with the transfer protein. The mixture was incubated at 20°C under continuous gentle movement and at

time intervals samples (175 μl) were drawn. The LUV in these samples were sedimented by centrifugation (15 min, 150000 × g, Beckman Airfuge), washed with 150 μl of buffer and assayed for $^{14}C/^{3}H$-radioactivity and lipid phosphorus. The percentage [^{14}C]DOPC remaining in the LUV was calculated from the ^{14}C/lipid phosphorus ratio, after correction for contaminating SUV (max 9%) as determined from ^{3}H radioactivity.

Vesicle aggregation

Vesicle aggregation, caused by interaction with calcium was determined by measuring the increase in lightscattering of the vesicle suspension. 0.5 ml of a suspension of LUV (1.0 mM of phospholipid) was mixed with an equal volume of 150 mM KCl, 10 mM Tris-acetate (pH 7.4) containing $CaCl_2$. After 30 min of incubation at 20°C the absorbance at 450 nm of the suspension was measured with a calcium-free vesicle suspensions as a reference (Hitachi; Perkin-Elmer model 356 double-beam spectrophotometer in a special setting: distance cuvettes-photomultiplier tube 32 cm). Increase in absorbance, interpreted as increase in lightscattering, was expressed as the percentage of the maximal absorbance increase of the vesicle suspension (i.e. at 20 mM of calcium). Finally 50 μl of 0.5 M EDTA, 10 mM Tris-acetate (pH 7.4) was added, to check the reversibility of the cause of the lightscattering increase.

Membrane fusion

Membrane fusion was determined by a fluorescence resonance energy transfer method as described by Hoekstra [23]. Two populations of large unilamellar vesicles (0.8 mM phospholipid), containing respectively 0.5 mol% N-NBD-PE or 0.5 mol% N-Rh-PE, were mixed in a one to one ratio. 1.5 ml of this suspension was added to 1.5 ml of a $CaCl_2$- or $TbCl_3$-containing 150 mM KCl, 10 mM Tris-acetate (pH 7.4) solution. Resonance energy transfer between the two fluorescent labels as a consequence of intervesicular lipid mixing, was determined by measuring the N-Rh-label fluorescence at 590 nm after excitation of the N-NBD label at 450 nm. To exclude vesicle-aggregation as cause of an increase in the 590 nm emission signal (intervesicular resonance energy transfer or lightscattering contributions), finally, 100 μl of a 0.5 M EDTA (excess), 20 mM Tris-acetate (pH 7.4) solution was added to the incubation suspension.

Furthermore the results were controled by a variant of the resonance energy transfer assay [24]. A suspension of LUV labeled with 2% NBD-PE and 2% Rh-PE was mixed in a 1 : 19 ratio with an unlabeled LUV suspension. 0.2 ml of the vesicle mixture (3.5 mM phospholipid) was diluted with 1.8 ml of a $CaCl_2$- or $TbCl_3$-containing 150 mM KCl, 10 mM Tris-acetate (pH 7.4) solution.

Subsequently the emission of the N-NBD label (530 nm) was measured after excitation at 450 nm. Fusion causes dilution of the fluorescent labeled lipids with the phospholipids from the unlabeled membrane, resulting in a decrease of the resonance energy transfer and therefore an increase of the emission of the N-NBD label.

Freeze-fracture electron microscopy

A 25 mM suspension of LUV was added to a $CaCl_2$-containing 150 mM KCl, 10 mM Tris-acetate (pH 7.4) solution in a 3 : 2 ratio. The samples were incubated for 1 h at room temperature and subsequently quenched with the jet-freezing technique [25] without the use of cryoprotectants.

Freeze-fracture electron microscopy was performed according to established procedures.

Results and Discussion

Cation–phospholipid association

In Fig. 1 the results of calcium binding experiments with DOPA/DOPC (20 : 80, mol%) MLV are depicted. We have chosen for a multilamellar instead of an unilamellar system for technical reasons. In contrast with LUV, MLV are easily quantitatively separated from the external medium by centrifugation. This allows a simple determination and derivation of the free calcium concentrations and the amount of associated calcium, respectively (see Materials and Methods). In some experiments A23187 was added to ensure accessibility for calcium to all possible binding sites in the multilamellar system. In the presence of the ionophore for phosphatidate-containing vesicles a typical binding curve was found (Fig. 1). When these findings are plotted according to Scatchard,

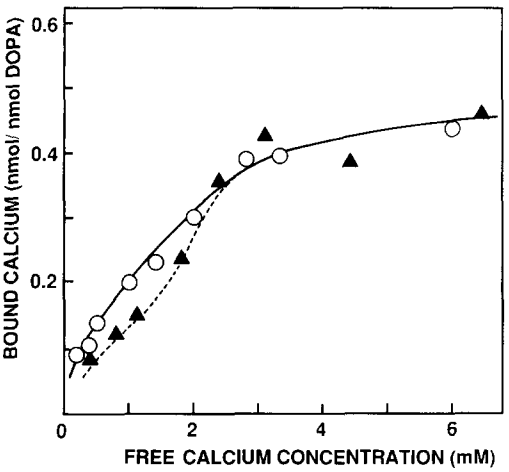

Fig. 1. Calcium binding to DOPA/DOPC (20:80, mol%) MLV in media with various calcium concentrations. The vesicles were incubated for 1 h at 20°C in a 150 mM KCl 10 mM Tris-acetate (pH 7.4) solution, containing different amounts of [^{45}Ca]$CaCl_2$. The phospholipid concentration was about 30 mM. The determination of the amount of bound calcium and the free calcium concentration and further experimental details are described in Materials and Methods. ○, incubation in the presence of A23187 (2.5 μg/μmol phospholipid); ▲, incubation in the absence of A23187.

a straight line can be drawn (Fig. 2). This suggests a description of the binding characteristics by a first-order reaction equation. With this assumption the reciprocal slope of the line can be interpreted as the apparent dissociation constant of the binding reaction, which is than about 1.7 mM.

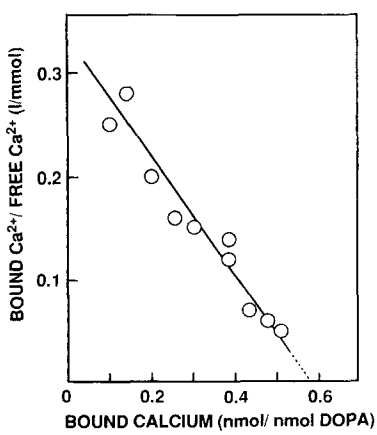

Fig. 2. Scatchard plot of the calcium binding to DOPA/DOPC (20:80, mol%) LUV in the presence of A23187. The results, as depicted in Fig. 1, are presented according to Scatchard. Experimental conditions are described in the legend of Fig. 1.

The binding stoichiometry, which is equal to intercept on the *X*-axis, is 0.6 nmol calcium per nmol phosphatidate. This is close to a stoichiometry of one calcium ion per two phosphatidate molecules, what is in accordance with the predominantly singularly negatively charged nature of phosphatidate at pH 7.4 [12]. It should be noted that in this simple approximation the calcium association caused by electrostatic interaction as described by the Stern-Gouy-Chapman theory is neglected [26]. When those findings are compared with the results of the permeability studies for the same system [9], it is striking that the value of the apparent dissociation constant is just in the middle of the calcium concentration region (0.5–2 mM), in which the membranes are found to be selectively permeable for calcium and its chelators. The general permeability increase found at calcium concentrations higher than 2.5 mM is manifesting when the negative charge of the membrane is practically neutralized by calcium ions (Fig. 1).

Incubation in the absence of the ionophore shows a result which deviates only slightly from the curve found in the presence of the ionophore A23187 (Fig. 1). A similar result was found by De Kruijff et al. [18] for cardiolipin/DOPC (50:50, mol%) MLV. At low calciumconcentrations the binding level without A23187 tends to be somewhat lower. However, it is higher than should be expected when only the outer monolayer of the MLV is reachable for calcium. This means that calcium can pass the bilayer barrier without the aid of A23187, confirming earlier findings about the ionophoretic properties of phosphatidate. In the binding studies the calcium influx seems to be higher than the limited influx found in earlier permeability studies [9]. This discrepancy is possibly due to the differences in the experimental conditions, such as the use of a multilamellar instead of an unilamellar system, the considerably higher vesicle consideration, the absence of a calcium sink inside the vesicles and the non-attendance of a calcium-chelator counter transport.

The association of calcium to DOPG/DOPC (20:80, mol%) MLV was much less. For calcium concentrations up to 8 mM we found a maximal level of about 0.07 nmol calcium per nmol DOPG. This association of calcium is probably a consequence of electrostatic interaction [26] and not of

chemical binding as was observed for DOPA-containing vesicles. We also performed the same kind of experiments with magnesium instead of calcium and found for DOPA-containing MLV no magnesium binding for magnesium concentrations up to 8 mM (results not shown).

For a further characterization of the interaction between calcium and phosphatidate we studied the behaviour of phospholipid monolayers at the air-water interface as a model for a membrane surface. Fig. 3 shows surface pressure-area curves of different phospholipids in the presence and absence of calcium in the subphase, yielding information about the specific molecular area of the lipids. In contrast with DOPC (results not shown) and DOPG, for which no influence of calcium on the surface characteristics was found, pure DOPA monolayers showed film condensation in the presence of calcium. Interestingly, calcium induced a lowering of the collapse pressure to about 36 mN/m. This condensation and destabilization effect of calcium was also found by Patil et al., who used completely ionized DOPA (pH 12) [12] and Ohki et al., who used egg phosphatidylcholine-derived phosphatidate [27]. For DOPA/DOPC (20 : 80, mol%) mixed monolayers in the presence of calcium also a condensation of the monolayer was found. However, a film collapse was not detected within the measuring limits (< 45 mN/m). Apparently, the phosphatidylcholine stabilizes the film under these conditions. The magnitude of the change in compressibility suggests that only the phosphatidate molecules contribute to the condensation. Measurement at constant area showed that when calcium is added to the subphase below a phosphatidate-containing monolayer an immediate decrease (within 1 s) followed by a stabilization in surface pressure is observed (time curve not shown), depending on the calcium concentration (Fig. 4). In pure DOPA monolayers this decrease is already observed in the micromolar calcium concentration range and it is maximal above 3 mM of calcium. The condensations in DOPA/DOPC (20 : 80, mol%) monolayers are less pronounced and higher calcium concentrations are needed for a detectable effect. When these latter results are compared with the calcium-vesicle binding results of the same mixture (Fig. 1), the calcium concentration dependency of the magnitude of the condensation is in agreement with the binding characteristics. The origin of this condensation effect of calcium of molecular level would be the decrease in volume of the polar region of the phosphatidate molecule. This can be a consequence of a dehydrating effect

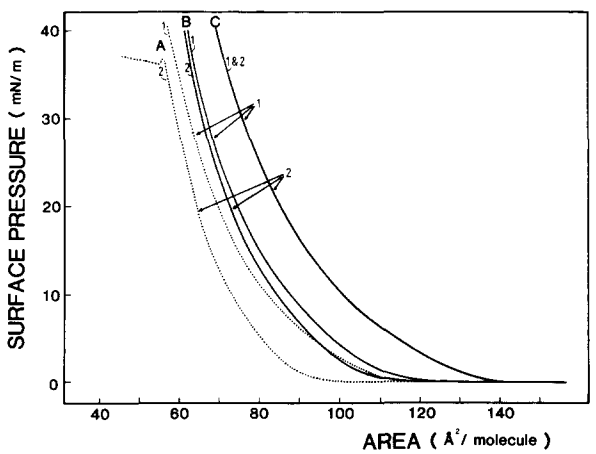

Fig. 3. Force–area characteristics of monolayers with different phospholipid content in the presence and absence of calcium in the subphase. Monolayers of DOPA (A), DOPA/DOPC (20 : 80, mol%) (B) or DOPG (C) were spread on a subphase of 150 mM KCl, 10 mM Tris-acetate (pH 7.4) with (2) or without (1) 10 mM $CaCl_2$. The curves were recorded by measuring the surface pressure continuously during constant compression of the monolayer at 20°C. Details are described in Materials and Methods.

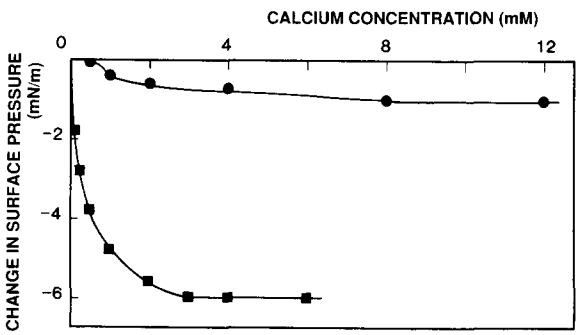

Fig. 4. Change in surface pressure in monolayers of different phospholipids at constant area, induced by calcium. Monolayers of DOPA (■) or DOPA/DOPC (20:80, mol%) (●) (surface pressure 32–35 mN/m) were spread on a subphase of 150 mM KCl and 10 mM Tris-acetate (pH 7.4). Changes in surface pressure were measured 3 min after addition of an aliquot of 100 mM $CaCl_2$, 10 mM Tris-acetate (pH 7.4) to the subphase. The experiments were performed at 20°C. Experimental details are described in Materials and Methods.

of the calcium binding and/or a neutralization of the negative charge of phosphatidate by calcium.

When the results of the monolayer studies are translated to a bilayer membrane of phospholipid vesicles, addition of calcium to vesicles containing phosphatidate initially should result in a condensation of the outer monolayer of the vesicle. This causes a lower surface pressure in the outer monolayer, which may be abolished by a transient redistribution of the phospholipid molecules between the two monolayers through phospholipid transbilayer movement. When phosphatidate is involved in this flip-flop calcium bound to phosphatidate can pass the hydrophobic interior of the membrane.

Phosphatidylcholine transbilayer movement

Unfortunately, at present, there is no unambiguous method to study the transbilayer movement of phosphatidate. An explicit experimental demonstration of the proposed phosphatidate flip-flop mechanism as the origin of calcium translocation can consequently not be given. However, transbilayer movement of phosphatidylcholine, which is possibly accompanying the phosphatidate flip-flop, can be studied with a PC exchange assay [22]. The results of such phosphatidylcholine exchange experiments in suspensions containing DOPA/DOPC (20:80, mol%) LUV in the presence (1.5 and 5 mM) and absence of calcium are shown in Fig. 5. Under both conditions only about 50% of the phosphatidylcholine of the LUV is exchangeable with phospholipids from the SUV. This means that only the phosphatidylcholine pool in the outer monolayer of the LUV is reachable for the PC transfer protein. Consequently, during the time course of the incubation (5 h) no demonstrable transfer of PC molecules from the inner to the outer monolayer has occurred. However, when calcium induces only an initial disturbance in the membrane organization which is reequilibrated by a flip-flop in a short time, this can not be detected in our experimental set up, because the exchange process of the PC-transfer protein is too slow. To get insight in this possibility calcium was added to the suspension in which the [^{14}C]DOPC in the outer membranes of the LUV is already exchanged by unlabeled DOPC (after 2 h of incubation). Then a flip-flop should result in renewed

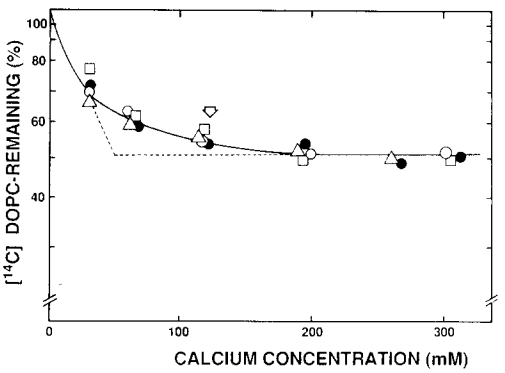

Fig. 5. Transfer of [^{14}C]DOPC from DOPA/DOPC (20:80, mol%) LUV to DOPC SUV mediated by PC-transfer protein. A suspension (1.3 ml) of DOPA/[^{14}C]DOPC (20:80, mol%) LUV (2 μmol of phospholipid; 4 nCi 1-[^{14}C]DOPC), DOPC SUV (20 μmol of phospholipid) and 35 μg of PC-transfer protein in 150 mM KCl, 10 mM Tris-acetate (pH 7.4) was incubated at 20°C in the presence of 0 (■), 1.5 (△) and 5 (○) mM of $CaCl_2$. In one experiment (●) 5 mM of $CaCl_2$ was added after 2 h of incubation (indicated with an arrow). In samples taken at different timepoints, the LUV were sedimented by centrifugation and the amount of remaining [^{14}C]DOPC was determined by liquid scintillation counting. For further details see Materials and Methods.

exchange of labeled DOPC. We also performed the assay in this way, but no extra exchange was found (Fig. 5). So, we can conclude that 1–5 mM of calcium does not induce a phosphatidylcholine flip-flop. This emphasizes the possible exclusive role of phosphatidate as transbilayer calcium carrier.

Intermembrane interactions

Earlier investigations already showed that addition of calcium to membrane systems containing negatively charged phospholipids may result in membrane–membrane interactions like vesicle aggregation and fusion [13,28]. As we mentioned in the introduction, this latter event could be the cause of membrane permeability changes. Here we present the result of intermembrane interaction studies for DOPA/DOPC (20:80, mol%) LUV in the presence of calcium.

Vesicle aggregation. Fig. 6 shows the calcium-induced lightscattering at 450 nm of a DOPA/DOPC (20:80, mol%) LUV suspension after 30 min of incubation with calcium. In this period the absorbance at 450 nm reaches a stable level. Above about 3 mM of calcium a strong increase in

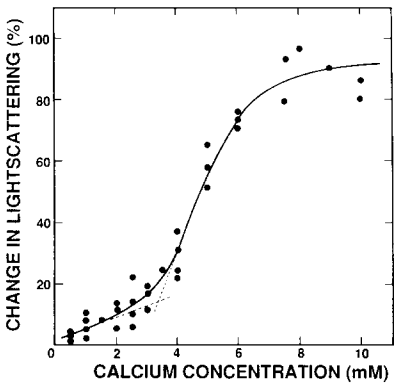

Fig. 6. Aggregation of DOPA/DOPC (20:80, mol%) LUV in media with various $CaCl_2$ concentrations. The vesicles were incubated for 30 min at 20°C in a 150 mM KCl, 10 mM Tris-acetate solution containing $CaCl_2$. The phospholipid concentration was about 0.5 mM. Subsequently the lightscattering was determined as the increase in absorbance at 450 nm of a sample with respect to that of the calcium free suspension. The lightscattering is expressed as the percentage of the maximal lightscattering increase (at $[Ca^{2+}] = 20$ mM). Further details are described in Materials and Methods.

lightscattering is observed. At calcium concentrations below 6 mM, chelating of the calcium ions by addition of an excess of EDTA causes a decrease in lightscattering to the same absorbance value as that of the incubation without calcium. These results demonstrate that calcium causes vesicle aggregation, which can be fully reversed by the addition of EDTA. At higher calcium concentrations a relatively small residual lightscattering is observed after EDTA addition (up to 10% of the total absorption decrease), probably caused by the presence of fused structures in these incubation suspensions. So, at the vesicle concentration we used (0.5 mM of phospholipid), noticeable aggregation is found only at calcium concentrations higher than about 3 mM (Fig. 6). The slight increase in lightscattering observed at lower concentrations is possibly due to other aspects of the calcium–phosphatidate interaction (see also freeze-fracture electron microscopy result in Fig. 9: at 1 mM calcium no aggregation is observed). The value of 3 mM can be regarded as the calcium threshold concentration for aggregation of DOPA/DOPC (20:80, mol%) LUV under the mentioned conditions. Below this concentration of calcium specific translocation is observed at the same phospholipid concentration [9], which

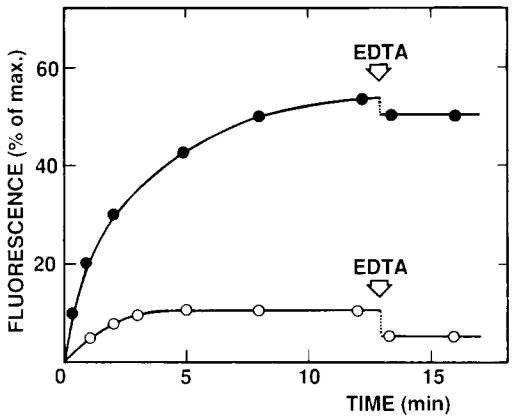

Fig. 7. Membrane fusion of DOPA/DOPC (20:80, mol%) LUV. An equal amount of two populations of vesicles, containing resp. 0.5 mol% N-NBD-PE and 0.5% N-Rh-PE, were incubated at 20°C in a 150 mM KCl, 10 mM Tris-acetate (pH 7.4) solution, containing 5 mM of $TbCl_3$ (●) or 10 mM of $CaCl_2$ (○). The phospholipid concentration was 0.4 mM. Membrane fusion was followed by measuring the increase in fluorescence at 590 nm (excitation at 450 nm) caused by resonance energy transfer. The fluorescence increase is expressed as the percentage of the fluorescence at theoretically maximal resonance energy transfer. At points indicated with EDTA, an excess of EDTA was added to the incubation suspension (25 mM). For further details see Materials and Methods.

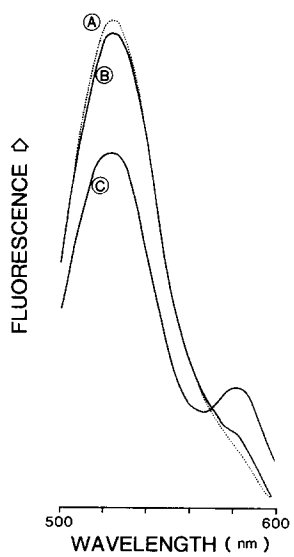

Fig. 8. Fluorescence spectra of the incubation mixture described in Fig. 7. Vesicles were incubated for 15 min in the absence (A) (dotted line) or the presence (B) of 10 mM of $CaCl_2$ or 5 mm of $TbCl_3$ (C). Subsequently an excess of EDTA was added to the vesicle-suspension and the emission spectrum was recorded after excitation at 450 nm. For further details see legend of Fig. 7 and Materials and Methods.

is apparently not related with vesicle aggregation. Chauhan et al. [29] came recently to the same conclusion. The general permeability increase observed in earlier studies [9] seems to be coupled to vesicle-vesicle interaction. Further experiments confirmed this idea. Lowering or increasing the vesicle concentration, causing respectively an increase or a decrease in the threshold calcium concentration for aggregation, did influence the threshold calcium concentration for the general permeability in the same extent (results not shown).

The same as for the permeability behaviour [9] the vesicle aggregation under these conditions is exclusively a consequence of phosphatidate–calcium interaction. Magnesium did not cause aggregation of DOPA-containing vesicles for concentrations up to 8 mM (results not shown). For DOPG/DOPC (20 : 80, mol%) LUV a low level calcium-induced reversible vesicle aggregation was observed with a calcium threshold concentration of 8 mM (at 10 mM calcium 20% of the lightscattering of DOPA/DOPC (20 : 80, mol%) LUV).

Vesicle fusion. The observed coupling between vesicle–vesicle interaction and permeability changes might be a direct consequence of a leaky fusion process. In the lightscattering studies described above; appearance of vesicle fusion was already mentioned. We next investigated fusion between vesicles in more detail with established methods.

Membrane fusion, or better: the intermixing of membrane components of different vesicles, was assayed by measuring the transfer of the resonance energy of N-NBD-PE to N-Rh-PE. Resonance energy transfer is only possible when the two fluorescent probes, which are originally in different vesicles, are in close contact. At calcium concentrations up to 6 mM no resonance energy transfer was detectable for DOPA-containing LUV, even after 90 min of incubation with calcium (results not shown). At 10 mM calcium a small, but significant increase in fluorescence at 590 nm was observed within 5 min (Fig. 7). This increase could be partially annuled by the addition of an excess of EDTA. From the emission spectra before (spectrum not shown) and after (Fig. 8) addition of EDTA it can be concluded that the decrease in fluorescence signal is a consequence of deaggregation of the vesicles. Deaggregation causes the disappearance of either the lightscattering contribution to the emission or the resonance energy transfer between neighbouring vesicles. The remaining emission at 590 nm is caused by intravesicular resonance energy transfer (Fig. 8). So it can be concluded that at 10 mM of calcium and a vesicle concentration of 0.4 mM membrane fusion is detectable. This conclusion was confirmed by the results of a different resonance energy transfer assay, in which fusion causes dilution of the two fluorescently labeled phosphatidylethanolamines from a minor population of vesicles into an excess of unlabeled vesicles (see Materials and Methods; results not shown).

On the other hand, terbium as a trivalent cation capable of a stronger interaction with negatively charged lipids [30], caused extended fusion of DOPA/DOPC (20 : 80, mol%) LUV even at low concentrations (Figs. 7 and 8)). This shows the validity of the assay. Terbium, which is often used as a calcium analogue [31], was already known for

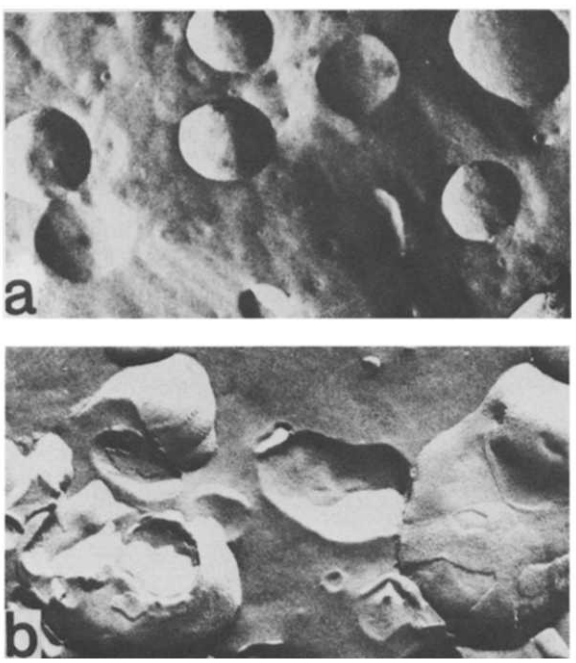

Fig. 9. Freeze-fracture electron micrographs of DOPA/DOPC (20 : 80, mol%) LUV incubated in the presence of 1 mM (a) and 10 mM (b) of $CaCl_2$. Final magnification 56000×. For experimental details see Materials and Methods.

its more potent interaction with phospholipids than calcium [30].

Freeze-fracture electron microscopy of the phosphatidate-containing LUV after incubation with 1 mM of calcium showed a homogeneous suspension of intact unilamellar vesicles (Fig. 9). At 10 mM calcium, however, extended fusion to large multilamellar aggregates was observed (Fig. 9). This high fusion level, compared with the results of the former method, is most probably a consequence of the high vesicle concentration of the used suspension (15 mM, which is 50-times more concentrated than in the resonance energy transfer assay), because the fusion process is second order with respect to the vesicle concentration.

The overall conclusion of these different kinds of fusion studies is that calcium can act as fusogen for DOPA/DOPC (20 : 80, mol%) LUV, albeit only under extreme conditions (high vesicle concentration and relatively high calcium concentration). However, specific intermembranal exchange of phosphatidate molecules, coupled to vesicle aggregation can not be excluded.

Summarizing discussion

When the results of this study are compared with the results of a previous study [9] on the permeability behaviour of DOPA/DOPC (20 : 80, mol%) LUV under the same conditions, the following conclusions can be drawn. At low calcium concentrations (< 2.5 mM) these DOPA-containing LUV show a selective influx of calcium and efflux of calcium chelating anions, apparently due to the binding of calcium to phosphatidate, which is demonstrated in the present binding studies. However, calcium does not occupy all the binding sites of the membrane, so, the membrane surface is still negatively charged, and no vesicle–vesicle interactions are observed. To explain this translocation the principles of the model as proposed by Chauhan et al. [7,29] and Reusch [32] are plausible. Phosphatidate could act as 'ferry' for the calcium ions, and, furthermore, for calcium-chelating anions by forming an uncharged dehydrated tri- or multicomplex with these ions [9]. Blau et al. [33] already suggested that the effectiveness of calcium ionophores as calcium carrier is dependent upon their ability to form neutral complexes with divalent cations which complexes can traverse membranes. The driving force for the transbilayer movement of the uncharged complexes of Ca^{2+}-DOPA is possibly the calcium-induced condensation in the outer monolayer. The phospholipid transbilayer movement required for such a process has to be specific for the phosphatidate molecules, as no phosphatidylcholine flip-flop was observed. The basis of the mechanism of the translocation process can be found in the tendency of phosphatidate to organize in hexagonal structures in the presence of calcium [34,35]. This property distinguishes phosphatidate from most other negatively charged phospholipids (such as DOPG used as control lipid in this and the previous [9] study) and can be explained by a dynamic shape of the lipid molecules upon calcium binding from cylindrical to more conical (headgroup dehydration) [11]. At low pH in pure phosphatidate dispersions addition of calcium results in a transition from a bilayer to a hexagonal H_{II} organisation of the lipid molecules [34,35]. Under the same conditions in mixed systems of DOPC and DOPA in a 1 : 1 ratio calcium induces lipidic particles as can be seen by freeze-fracture electron microscopy, which have been interpreted as stable inverted micellar structures [11,34]. These structures are considered as intermediates in the membrane fusion process. In DOPA/DOPC (20 : 80, mol%) LUV such calcium-induced lipidic particles are not yet observed. However, transient formation of such or related inverted lipid structures, which should be hard to detect, may enable transbilayer movement of the involved phospholipids (i.e. Ca^{2+}-DOPA and Ca^{2+}-DOPA-Ca^{2+} chelator complexes) and thus mediate calcium translocation [4,36]. Using NMR techniques we found very recently that at physiological pH calcium forms complexes with DOPA in which the polar headgroup is immobilized and dehydrated but in which the acyl chains have increased disorder and are in a liquid-crystalline state [37]. In the near future we will report on this investigations.

At calcium concentrations higher than about 2.5 mM the membrane surface is neutralized and a general loss of barrier properties is observed, accompanied by vesicle aggregation. According to our experiments no membrane fusion is involved at calcium concentrations below 10 mM. So, the

calcium-induced non-specific leakage in this region (2.5–10 mM Ca^{2+}) can not be due to fusion events. The exact molecular basis of this intermembranal interaction cannot be derived from the experiments in this paper. The results of Graham et al. [28], suggesting a lateral phase separation of DOPA and DOPC, which is not a consequence but a cause of vesicle aggregation, could be describing our system. Further experiments on this subject will be published in a subsequent paper [37]. For the mechanism of the increased general permeability packing defects at the boundaries of the areas in close intermembranal contact of the aggregated vesicles could be of importance.

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