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Consequences of the interaction of calcium with dioleoylphosphatidate-containing model membranes: changes in membrane permeability

Erik B. Smaal [a,*], Jacqueline G. Mandersloot [a], Ben de Kruijff and Johannes de Gier [a]

[a] *Laboratory of Biochemistry and* [b] *Institute of Molecular Biology, State University of Utrecht, Padualaan 8, 3584 CH Utrecht (The Netherlands)*

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The permeability behaviour of dioleoylphosphatidate/dioleoylphosphatidylcholine (20:80, mol%) large unilamellar vesicles at low millimolar calcium concentrations is different for various solutes. Between 0.5 mM and 2.5 mM of calcium a selective influx of calcium and efflux of enclosed calcium chelating anions is observed. At higher calcium concentrations the membrane loses its barrier function for a large variety of solutes. These permeability increases are a specific consequence of calcium phosphatidate interactions, because control experiments in which calcium was replaced by magnesium or in which dioleoylphosphatidate was replaced by dioleoylphosphatidylglycerol showed under the same conditions no permeability changes. These results are discussed on the basis of various putative mechanistic models for phosphatidate-mediated calcium translocation across membranes. Furthermore a kinetical model is presented by which the observed selective calcium and calcium-chelator translocation can be explained.

Introduction

Cell stimulation by various hormones, neurotransmitters and growth factors is supposed to occur by triggering off the activity of a specific phospholipase C, which results in the breakdown of phosphorylated inositol phosphatides [1]. One of the products, inositol (1,4,5)-trisphosphate, has been shown to act as second messenger by stimulation of calcium release from intracellular pools (endoplasmic or sarcoplasmic reticulum) [2]. The simultaneously formed diacylglycerol is thought to stimulate protein kinase C, which catalyses protein phosphorylation in synergism with calcium-dependent protein kinases [3]. The cyclic character of events includes subsequent phosphorylation of the diacylglycerol to phosphatidate as a key intermediate in the reformation of phosphatidylinositides.

A direct role of phosphatidate in calcium mobilization is not clear but the last few years physiological studies with different cell types have indicated that phosphatidate may be important in the calcium mobilization over the plasma membrane [4–13]. In these experiments phosphatidate was either added externally to the cells or cell membrane vesicles [7–12] or generated by addition of an agonist of the membrane receptor or a phospholipase to the cells [11–14]. In most of these studies it is concluded that phosphatidate indeed plays a role in calcium mobilization over

* To whom correspondence should be addressed.

the plasmamembrane. To explain this there are at least two possibilities: either phosphatidate promotes the opening of calcium channels [5,6] or phosphatidate itself acts as a calcium ionophore [7–13]. To check the latter possibility several model studies were done [15–26]. The ability of phospholipids to transfer calcium from an aqueous phase into an organic phase was studied in two-phase partition systems [15–19]. The results showed that in general phosphatidate and cardiolipin are able to transfer calcium into the organic phase. However, the magnitude of this event is greatly depending on the nature of the organic solvent and whether or not an additional phospholipid is present [18]. Investigations on the calcium ionophoretic properties of phosphatidate with a more sophisticated membrane model, phosphatidate-containing phospholipid vesicles, gave conflicting results [19–26]. Serhan et al. [20,21] and Chauhan and Brockerhoff [23], using a spectrophotometric technique, and Deleers [19] and Nayar et al. [22], using a radiochemical technique all came to the conclusion that phosphatidate was able to translocate calcium across liposomal membranes. On the other hand Holmes and Yoss [24], using exactly the same assay and the same conditions as Serhan et al. [21] and Hunt et al. [25], using a ^{1}H-NMR technique, were unable to confirm these results. Recently we presented an improved calcium influx assay [26], in which large unilamellar vesicles enclosing the calcium chelating photoindicator arsenazo III are used [27]. With this method it is possible to discriminate between influx of calcium and efflux of the calcium chelator. Preliminary experiments indicated that calcium induces a permeability increase in phosphatidate-containing membranes not only for itself but also for arsenazo III [26]. Up till now there are no comprehensive data on the influence of calcium on the general permeability behaviour of phosphatidate-containing membranes.

In this communication we describe in more detail the permeability pattern of large unilamellar vesicles of dioleoylphosphatidate/dioleoylphosphatidylcholine (20 : 80, mol%), for which we focus on the calcium concentration dependency and the selectivity of the permeability. Next to calcium influx and arsenazo III efflux experiments, studies on the calcium-induced efflux of other solutes are presented. To see whether the results are unique for the calcium-phosphatidate system, control experiments were done using magnesium instead of calcium or using phosphatidylglycerol instead of phosphatidate. Furthermore, preliminary experiments are presented on the phosphatidate-mediated extraction of calcium from an aqueous phase into a benzene phase.

Materials and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesized from egg yolk phosphatidylcholine according to standard procedures [28]. 1,2-Dioleoyl-*sn*-glycero-3-phosphate (DOPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were prepared from DOPC using phospholipase D, isolated from Brussels sprouts [29]. A 100 mM calcium acetate buffer (pH 5.6) containing 2 mg/ml of the freeze-dried crude phospholipase D preparation (and, in the case of the DOPG synthesis, 50 vol% glycerol) was mixed with the same volume diethyl ether, containing 20 mg/ml DOPC. After 2 h of incubation at 37 °C the conversion was complete and the lipids were extracted according to Bligh and Dyer [30]. Traces of DOPA in the DOPG preparation were removed by chromatography over a silicagel column (57 × 2.5 cm; Polygosil 60, particle size 40–63 μm, Marchery-Nagel & Co., Düren, F.R.G.) with chloroform/methanol/water (85 : 20 : 2, v/v/v) as eluting fluid. Next, the DOPG- and DOPA-preparations were converted to their sodium salt form [31] and for further purification the DOPA was precipitated in ice-cold acetone [31]. The final products contained no detectable calcium (< 0.5 mol%) as determined by atomic absorbance spectrophotometry and the phospholipids were chemically pure (> 99%) as indicated by two-dimensional high-performance thin-layer chromatography.

6-Carboxyfluorescein (Eastman Kodak Co., Rochester, NY, U.S.A.) was purified by active carbon treatment, recrystallization in water/methanol (2 : 1, v/v) and Sephadex LH 20 column chromatography as described by Ralston et al. [32]. The product was chemically pure as indicated by high-performance thin-layer chromatography.

All other chemicals used were of analytical grade.

Lipid phosphorus was assayed according to Rouser et al. [33] or Böttcher et al. [34].

Phospholipid-mediated uptake of calcium into a benzene phase

A procedure essentially according to Cullis et al. [17] was used. To a series of incubation vessels (1.5 ml), containing 0.5 ml of 150 M KCl, 10 mM Tris-acetate (pH 7.4), 0.01–10 mM [^{45}Ca]$CaCl_2$ (about 1 μCi/μmol Ca^{2+}) solution, was added 0.3 ml of a 0.1 mM (or 0.2 mM) phospholipid solution in benzene. The two-phase systems were shaken gently so that the interface was continuously in movement, but not disrupted. After 3 h of incubation at 20°C samples (0.1 ml) were drawn from the benzene layer and the amount of extracted Ca^{2+} was determined by liquid scintillation counting (Packard, PRIAS model PLD). The amount of lipid in the benzene layer was not affected by the presence of the aqueous phase and was time independent during all experiments.

Vesicle preparation

Large unilamellar vesicles (LUV) were prepared in the appropriate buffered solution by the reverse-phase evaporation method at 20°C, followed by extrusion through a polycarbonate filter (Bio-Rad Uni-Pore; 0.4 μm pore size) [35]. When needed, the untrapped solution was replaced by a desired external solution using gelfiltration over a 1 × 10 cm Sephadex G-50 column. The vesicles had a trapped volume of 4–7 μl per μmol phospholipid.

Flux experiments

Unless otherwise stated, all experiments concerning calcium-induced fluxes over phospholipid membranes were carried out with large unilamellar vesicles suspended in 150 mM KCl, 10 mM Tris-acetate (pH 7.4) at 20°C. The vesicles, enclosing the desired probe, were incubated with extravesicular calcium (or magnesium) in the concentration range of 0.1–10 mM. An incubation was started by addition of the vesicle suspension (2.0–4.0 mM) to the calcium-containing solution in a 1 : 3 volume ratio. Addition of a concentrated calcium solution to the vesicle suspension was avoided as it causes unwanted temporarely locally high calcium concentrations. Samples were drawn after 1, 15, 60 and 180 min.

Calcium influx in and arsenazo III efflux from large unilamellar vesicles enclosing arsenazo III was assessed according to a previously described method [26]. The gist of this method is the addition of an excess of EDTA to a sample of the incubation suspension to discriminate between intra- and extravesicular calcium-arsenazo III complex, formed as a consequence of calcium influx and arsenazo III efflux, respectively.

EDTA and SO_4^{2-} efflux from large unilamellar vesicles was determined radiochemically. Vesicles were prepared of 20 mol% DOPA (or DOPG), 80 mol% DOPC with a trace amount of glycerol [^{3}H]trioleate (approx. 50 nCi/μmol phospholipid) and enclosing 5 mM [^{14}C]EDTA or 5 mM [^{35}S]Na_2SO_4 (specific radioactivity in both cases 1 μCi/μmol). An incubation was started by adding 250 μl of a 2–4 mM phospholipid vesicle suspension to 750 μl of a calcium-containing buffer. Samples (100 μl) were taken and immediately eluted over small Sephadex G-75 columns (5 × 60 mm) (20°C) with 1 ml of a EDTA/sulphate free 150 mM KCl, 10 mM Tris-acetate (pH 7.4) solution, to remove EDTA or sulphate, which had been leaked out of the vesicles. The ^{14}C/^{3}H or ^{35}S/^{3}H ratio in the eluate is a measure for the amount of trapped EDTA or sulphate per μmol phospholipid, respectively. The ratio was determined by liquid scintillation counting (Packard, PRIAS model PLD).

Potassium efflux from large unilamellar vesicles was assessed potentiometrically [36]. Vesicles were prepared in 150 mM KCl, 10 mM Tris-acetate (pH 7.4). The nontrapped solution was replaced by 150 mM choline chloride, 10 mM Tris-acetate (pH 7.4) by gelfiltration over a 1.5 × 15 cm Sephadex G-50 column. An incubation was started by adding 250 μl vesicle suspension (about 4 mM phospholipid) to 750 μl of calcium-containing 150 mM choline chloride, 10 mM Tris-acetate (pH 7.4). Samples (200 μl) were taken and added to 10 ml of 150 mM choline chloride, 10 mM Tris-acetate (pH 7.4) and nontrapped potassium was assayed with a potassium-selective electrode (Philips G15-K)-reference electrode (Philips, R44/2 SD/1) combination connected to a mV-meter (Radiometer, TTT2). Subsequently the amount of trapped potassium was determined by adding 100 μl 10% Triton X-100 solution, causing release of vesicle content.

Carboxyfluorescein release from large unilamellar vesicles was determined by fluorimetry [37]. Vesicles were prepared in 75 mM carboxyfluorescein, 10 mM Tris-acetate (pH 7.4). The external solution was replaced by 150 mM KCl, 10 mM Tris-acetate (pH 7.4) using gelfiltration over a 1.5 × 15 cm Sephadex G-50 column. An incubation was started by addition of 200 μl (about 3 mM phospholipid) to 800 μl of calcium-containing 150 mM KCl, 10 mM Tris-acetate (pH 7.4). Samples (30 μl) were drawn at time intervals and added to 3 ml of 150 mM KCl, 10 mM Tris-acetate (pH 7.4). The amount of non-trapped carboxyfluorescein was determined fluorimetrically (Perkin-Elmer LS 5). Carboxyfluorescein release from the vesicles causes dequenching of the fluorescence of the probe and results in an increase in emission signal (513 nm; excitation 430 nm). This signal is linear with the concentration of non-trapped carboxyfluorescein.

Results

Specific ionophoric activity is often thought to occur due to the formation of a reversible complex between the ion and the ionophore, which can dissolve in the hydrophobic core of the membrane barrier. The capability of phospholipids to act as such an ionophore was studied in the past with the help of simple models like two-phase partition systems [15,19]. In preliminary studies we also used this kind of system to see whether dioleoylphosphatidate is able to extract calcium into an organic layer. In view of the forthcoming permeability experiments with 20 : 80 mol% dioleoylphosphatidate/ dioleoylphosphatidylcholine vesicles, we were particularly interested in the influence of phosphatidylcholine on this translocation, the dependency of this event on the calcium concentration and the special requirement of phosphatidate for this process. To mimick the intrabilayer conditions as good as possible, we used benzene as organic layer. Benzene has a dielectric constant (2.28) which is close to the one of oleic acid (2.50). Chloroform which is often used as organic phase and which we also tried as an alternative, has the disadvantage of getting turbid under many conditions (compare also Ref. 15). Furthermore, we noticed sometimes a poor phase separation and the formation of a turbid layer at the interface.

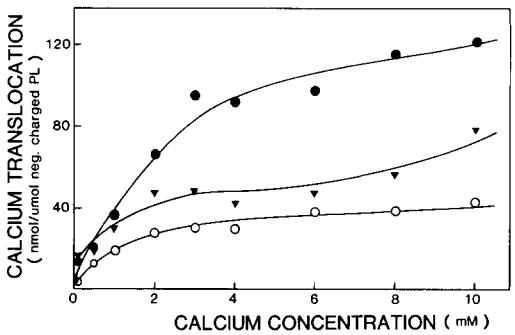

Fig. 1. Phospholipid-mediated uptake of calcium into a benzene phase. Two-phase partition systems consisting of 0.5 ml of an aqueous phase, containing 150 mM KCl, 10 mM Tris-acetate (pH 7.4), and [^{45}Ca]$CaCl_2$ and 0.3 ml of an benzene phase, containing 0.1 mM DOPA (●), 0.1 mM DOPG (▼) or 0.1 mM DOPA and 0.1 mM DOPC (○), were shaken gently for 3 h at 20 °C. After incubation the amount of calcium in the benzene phase was assessed by liquid scintillation counting. For further details see Materials and Methods.

Fig. 1 shows the calcium concentration dependency of the partition of calcium between an aqueous phase and a phospholipid-containing benzene phase. Pure DOPA showed a significant higher potency for calcium extraction than DOPG under these experimental conditions. This preferency is in accordance with earlier studies [15,18]. The shape of the curve for DOPA resembles a typical binding curve with a corresponding K_d of 1–2 mM. The binding of calcium takes place at the interface of the two phases and most of the lipid molecules will be in the bulk of the benzene layer. The rate of binding will be dependent on the exchange of lipids between the bulk and the interface. So the form of the curve is determined by the affinity of calcium for the phospholipids and the height of the curve by the rate of exchange of the phospholipids between bulk and interface. With our experimental conditions this exchange is quite slow and even in 3 h of incubation no equilibrium was reached. This explains why a binding stoichiometry of about 0.12 : 1 was found instead of 0.5 : 1 (Ca^{2+} : PA at 10 mM Ca^{2+}) which was found after vigorous mixing of the two phase system. In accordance with the findings of Tyson et al. [15] we found a strongly inhibiting influence of DOPC on the calcium translocation by DOPA.

This could be due to a competition of the phospholipids for a position at the interface. It is also possible that DOPC is inhibiting the rate of exchange of lipids between bulk and interface. Reusch [18], who studied two-phase systems in which the phospholipids were originally dispersed in the calcium-containing aqueous phase, found in contrary a stimulating effect of phosphatidylcholine on the phosphatidate-mediated translocation of calcium into a toluene phase. In these systems the calcium translocation rate is probably not dependent on the exchange between interface and bulk of the organic layer, but on the extractability of the phospholipids with associated calcium into the organic layer. Because of the kinetic incertainties and the risk of micro-emulsion formation when the system is shaken more vigorously, for further studies we focussed on translocation of calcium over the bilayer of large unilamellar vesicles.

Incubation of phosphatidate-containing vesicles, which enclose the calcium-chelating photoindicator arsenazo III, in a calcium-containing medium causes the formation of the calcium-arsenazo III complex [20,21,26]. In an evaluation study on this test method [26] we already showed that the calcium arsenazo III complex may not only be formed inside the vesicles as a consequence of calcium influx, but also outside the vesicles, as a consequence of arsenazo III efflux. In this study we confirm these findings and present results of experiments in which we studied the calcium concentration dependency and selectivity of the calcium-induced permeability. In Fig. 2 the calcium influx and arsenazo III efflux, expressed as the remaining amount of trapped arsenazo III, are shown for DOPA/DOPC (20 : 80, mol%) LUV. At the lowest tested calcium concentration (0.1 mM) only a slight time-dependent calcium influx was found. However, significant calcium permeability was induced at calcium concentrations of 1 mM and higher (Fig. 2). After a rather high initial rate of influx in the first few minutes (especially at 4 and 8 mM calcium) the calcium influx stops gradually and the amount of calcium trapped inside the vesicles reaches a plateau value. This equilibrium level does not increase with further increase in calcium concentration, the level at 8 mM is even slightly lower than at 4 mM calcium. Addition of the potassium

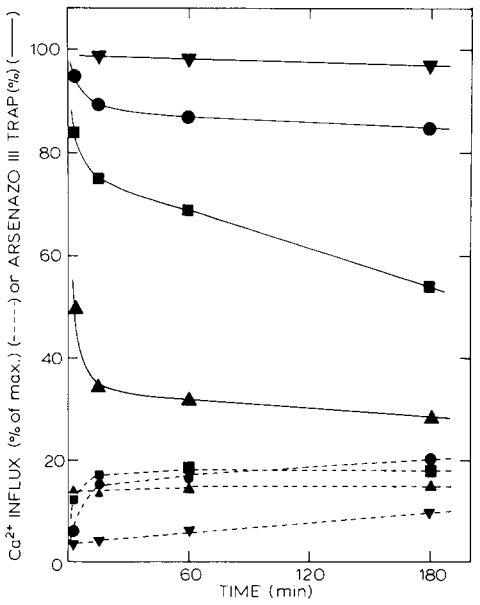

Fig. 2. Calcium and arsenazo III permeability of DOPA/DOPC (20 : 80, mol%) LUV incubated in a calcium-containing medium. The vesicles, enclosing 2.3 mM arsenazo III, 150 mM KCl, 10 mM Tris-acetate (pH 7.4) were incubated at 20 °C in a arsenazo III-free isotonic solution, containing different amounts of $CaCl_2$. The phospholipid concentration was about 0.6 mM. After various times of incubation aliquots were taken. The absorbance at 650 nm with the absorbance at 700 nm as reference was measured immediately (A_1), after addition of an excess of EDTA (A_2) and after subsequent addition of an excess of calciumionophore A23187 (A_3). For each incubation the potential maximal absorbance (A_m) was determined by addition of an excess of A23187 to a sample of the incubation suspension in the absence of EDTA. From these data were calculated: calcium influx (broken lines), $(A_2 - A_3)/(A_m - A_3) \times 100$ (% Ca·AIII formed inside vesicles) and AIII trap (solid lines), $100 - (A_1 - A_2)/(A_m - A_3) \times 100$ (total amount of AIII minus amount of Ca·AIII outside vesicles in %); for details see Ref. 26. ▼, 0.1 mM $CaCl_2$; ●, 1 mM $CaCl_2$; ■, 4 mM $CaCl_2$; ▲, 8 mM $CaCl_2$.

ionophore valinomycin (up to 1 μg/ml), the uncoupler FCCP (up to 2 μg/ml) or the combination of the two to the incubation suspension, did not influence the calcium influx characteristics (results not shown). This indicates that the influx of calcium is not controlled or driven by, respectively, a potential gradient or a pH gradient over the membrane and that the limited calcium influx is not a consequence of existence of one of these gradients. The use of vesicles, which enclosed 0.4 mM or 10 mM instead of 2.3 mM arsenazo III,

did not essentially change the percentual extent of the calcium influx (results not shown). This means that the absolute quantity of calcium that enters the vesicles is dependent on the amount of arsenazo III which is inside the vesicles.

At a calcium concentration of 0.1 mM hardly any efflux was detectable, but higher concentrations (> 1 mM) did cause extensive arsenazo III leakage from the vesicles in a calcium concentration dependent way (see also Fig. 4). As for the calcium influx, the percentual magnitude of the arsenazo III efflux was found to be independent of the concentration of the enclosed arsenazo III (results not shown). These findings suggest that the transmembrane diffusion of arsenazo III is an arsenazo III-gradient driven process, which is catalyzed by calcium ions.

In striking contrast to the findings with DOPA/DOPC vesicles are the results obtained

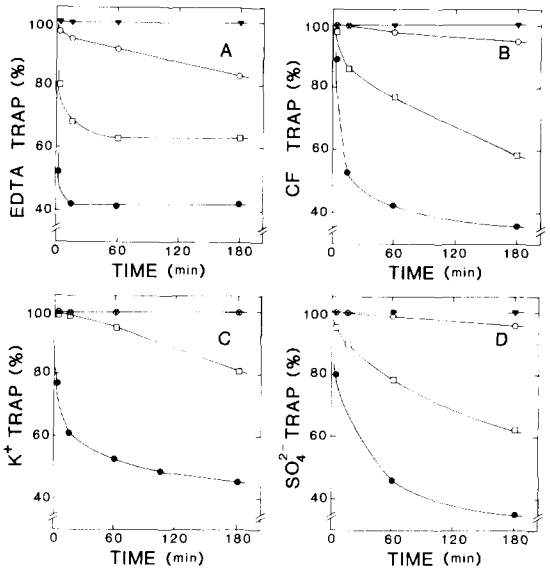

Fig. 3. Calcium-induced permeability changes of DOPA/DOPC (20:80, mol%) LUV for various solutes. The vesicles enclosed 5 mM [^{14}C]EDTA (A) or 5 mM [^{35}S]Na_2SO_4 (D) in 150 mM KCl, 75 mM CF (B) or 150 mM KCl (C). Incubation was carried out at 20 °C in 150 mM KCl (A, B, D) or 150 mM choline chloride buffer (C) containing different amounts of $CaCl_2$. All solutions were buffered with 10 mM Tris-acetate (pH 7.4). The phospholipid concentration was 0.5–1.0 mM. After different times of incubation samples were drawn and the amount of trapped solute was determined radiochemically (A, D), fluorimetrically (B) or potentiometrically (C). For further details see Materials and Methods. ▼, 0.1 mM $CaCl_2$; ○, 1 mM $CaCl_2$; □, 4 mM $CaCl_2$; ●, 10 mM $CaCl_2$.

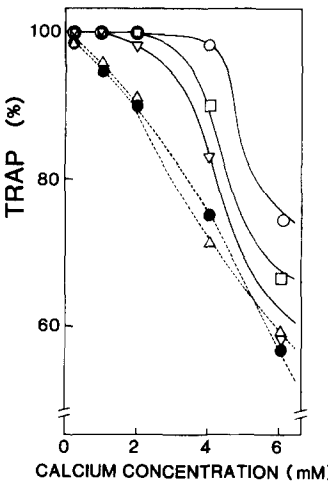

Fig. 4. Calcium-induced permeability changes of DOPA/DOPC (20:80, mol%) LUV for various solutes in relation to the calcium concentration. The vesicles were incubated for 15 min in media containing different amounts of $CaCl_2$. All details about conditions and methods are described in the legends of Figs. 2 and 3 and in Materials and Methods. Shown is the trapping capacity for K^+ (○), SO_4^{2+} (□), carboxyfluorescein (▽), EDTA (△) and arsenazo III (●).

with DOPG/DOPC (20 : 80, mol%) vesicles. These results showed that calcium is unable to change the permeability of bilayers containing DOPG as the negatively charged lipid. In concentrations up to 8 mM calcium no significant influx of calcium or efflux or arsenazo III could be noticed (results not shown).

To see whether the increased permeability for arsenazo III in the DOPA-containing vesicles is specific for this anion or not, we also studied the calcium-induced permeability characteristics of phosphatidate-containing membranes for several other solutes. For this purpose we chose EDTA, which just like arsenazo III froms chelates with calcium, the non-chelating organic anion 6-carboxyfluorescein (mol.wt. 376), the anorganic anion sulphate and the cation potassium. Fig. 3 shows that at 0.1 mM of calcium all studied solutes remained in the vesicles during the 3-h period of incubation. At 1 mM calcium a significant leakage of EDTA, which is comparable to the results of arsenazo III, whereas no efflux of potassium and only minor efflux of carboxyfluorescein and sulphate was found. Higher calcium concentrations (4 and 10 mM) did cause a permeabil-

ity increase of the vesicle membrane for all tested ions. When the results of the calcium induced permeability of DOPA/DOPC (20:80, mol%) LUV are compared with respect to the calcium concentration (Fig. 4) there appear to be three different membrane conditions. In the first, at low calcium concentration (< 1 mM) no specific calcium influx (Fig. 2) and no permeability increase for other ions (Fig. 4) is found. Intermediate calcium concentrations (1–2 mM) are causing specific permeability increase for calcium itself (Fig. 2) and calcium-chelating anions (Fig. 4, EDTA and arsenazo III). Higher calcium concentrations (> 2 mM) cause a general permeability increase.

When magnesium (0–10 mM) instead of calcium was used or DOPG/DOPC (20:80, mol%) LUV instead of DOPA/DOPC (20:80, mol%) LUV (0–10 mM Ca^{2+}) were used in parallel experiments no permeability changes of the vesicles under all comparable conditions were found (results not shown). So it can be concluded that the observed calcium induced permeability changes in DOPA/DOPC (20:80, mol%) LUV

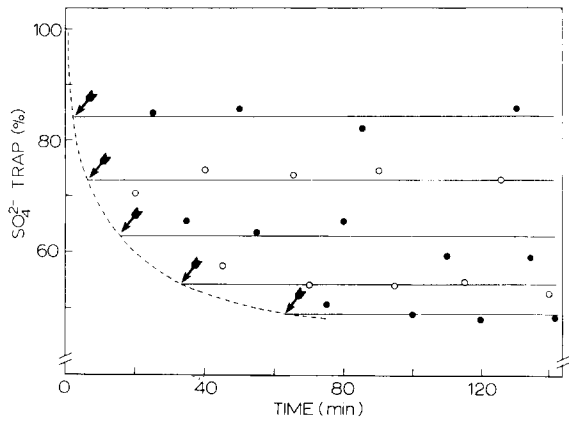

Fig. 5. Reversibility of the calcium-induced sulphate permeability increase of DOPA/DOPC (20:80, mol%) LUV. The vesicles, which enclosed 5 mM [^{35}S]Na_2SO_4, 150 mM KCl, 10 mM Tris-acetate (pH 7.4), were incubated at 20°C in a 5 mM $CaCl_2$, 150 mM KCl, 10 mM Tris-acetate (pH 7.4) solution. The phospholipid concentration was 0.7 mM. At times indicated with an arrow an excess if EDTA was added to the incubation suspension (25 mM) and the incubation was continued. From the different incubations samples were drawn with time intervals and the amount of trapped sulphate was determined by liquid scintillation counting. For further details see Materials and Methods.

are a specific consequence of calcium-phosphatidate interaction.

To see whether the observed changes in membrane permeability are reversible we did experiments in which the incubation of phosphatidate-containing vesicles with calcium was interrupted by removal of the external calcium with EDTA. The results are shown in Fig. 5. Addition of an excess of EDTA to the vesicle suspension after different incubation times caused a stabilization of the amount of trapped sulphate during further incubation. This demonstrates that the calcium-induced permeability increase of the vesicle membrane under these conditions is basically a consequence of a reversible calcium-vesicle interaction.

Discussion

The present experiments demonstrate that calcium is able to induce a permeability increase in lipid bilayers containing (dioleoyl)phosphatidate, not only for the cation itself as might be concluded from earlier studies [19–23] but also for other solutes. The permeability behaviour of DOPA/DOPC (20:80, mol%) large unilamellar vesicles in the presence of calcium is quite complex and is in nature different in various regions of calcium concentration. At concentrations below 0.5 mM no specific permeability increase is found. Intermediate calcium concentrations (1–2 mM) affect the membrane barrier in such a way that exclusively calcium and its chelators can pass the membrane. At higher concentrations calcium induces a general permeability increase. All ion fluxes are most pronounced in the first 15 min of incubation, which suggest transient changes in membrane organization. Interestingly, the leakage process could be stopped by chelating extravesicular calcium with an excess of EDTA. Apparently the cause of the permeability increase is reversible.

For the mechanism of the translocation of calcium over phosphatidate-containing model membranes Chauhan and Brockerhoff suggest a 'calcium ferry' in the form of a dehydrated $Ca(PA)_2$ coordination complex with intramolecular hydrogen bondings [23,38]. On the basis of experiments with two-phase partition systems Reusch [18] proposes a preferential formation of a calcium-phosphatidate-(neutral)phospholipid co-

ordination complex. Although we think that details of these proposals are subject to criticism, the basic idea, the formation of a lipid-soluble complex between ion and phospholipid, seems to be a valid model for the calcium translocation. However, to explain the specific efflux of chelating ions next to an influx of calcium, this hypothesis needs extension. Next to the translocation of a calcium-phosphatidate complex, transbilayer movement of a tri- or multi-complex formed by the phospholipid, calcium and the chelating anion can be postulated. A similar complex has already been suggested for calcium, phospholipid and inorganic phosphate [39,40].

Other factors than lipid solubility may also be of importance for the flip-flop movement of the complexes over the membrane. From monolayer experiments a condensation of the outer monolayer of the phospholipid vesicles, induced by calcium-phosphatidate interaction can be assumed [41,42]. This creates a difference in lateral surface pressure between inner and outer monolayer, which may be equilibrated through a rapid redistribution of the lipids between the two monolayers [43]. This may be of importance to understand the kinetics of the calcium translocation process, which shows a very rapid influx immediately after the addition of calcium. Furthermore, transient formation of non-bilayer structures after calcium membrane interaction could play an important role in the membrane translocation [44–46]. When calcium interacts with DOPA considerable change in the dynamic shape of the molecules can be expected, because of dehydration of the polar headgroup when the complex is formed. This is reflected in the phase behaviour of pure DOPA. When dispersed as the sodium salt DOPA forms a bilayer organization, but upon addition of calcium this organization can be converted into a hexagonal H_{II} phase [44,45]. In mixtures with phosphatidylcholine this tendency to form a H_{II} phase can result in the formation of lipidic particles as seen by freeze-fracture electron microscopy, which have been interpreted is inverted micelles [44,46]. Therefore the dynamic formation and resolution of inverted micelles in the bilayer of the DOPA-containing vesicles may enable calcium and chelator translocation via the calcium-rich internal aqueous compartments of the micelles [17,46]. In contrast to the DOPA system, H_{II} phase formation can not be observed when calcium is added to a pure DOPG dispersion [47]. This may explain why calcium is unable to induce significant permeability changes in our control vesicles of DOPG/DOPC and supports the view that non-bilayer structures are involved in the special permeability behaviour of phosphatidate-containing membranes.

Whichever underlying mechanism is responsible, it seems likely that an increased transbilayer movement of calcium-phosphatidate and calcium-phosphatidate-chelator complexes is the origin of the observed specific permeability increases in the calcium concentration range of 1–2 mM. In Fig. 6 a schematic presentation is given for the flux of calcium and calcium chelator over a membrane, mediated by those phosphatidate complexes. We have demonstrated that the calcium influx, shown in the left part of the figure, has initially a high rate followed by a stabilization of the amount of intravesicular calcium (15–20%; Fig. 2).

This equilibrium is not a consequence of the existence of a membrane potential or pH gradient, as we have demonstrated. Next it is neither a limited binding capacity of the enclosed arsenazo III, which inhibits the calcium influx, because the intravesicular arsenazo III is easily occupied by calcium ions after addition of an excess of calcium

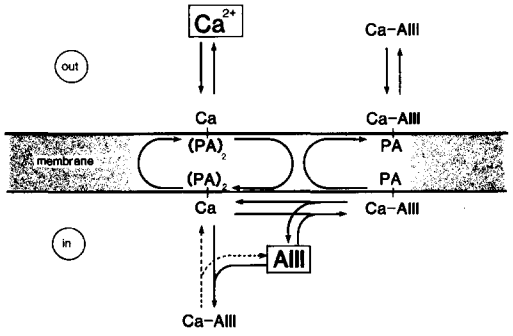

Fig. 6. Simplified model of the influx of calcium and the efflux of arsenazo III over a phosphatidate-containing membrane. This model is principally based on a phospholipid flip-flop-mediated translocation mechanism. Shown are the possible equilibria between calcium, arsenazo III (AIII) and phosphatidate (PA).

ionophore A23187 [26]. Furthermore, influx experiments with lower and higher amounts of arsenazo III showed that the percentual amount of intravesicular calcium-arsenazo III complex is determining the absolute extent of the calcium influx. So, the number of translocating calcium ions or more correctly, the number of cotranslocating phosphatidate molecules is not the limiting factor of the calcium influx. It therefore seems that the coherence of the equilibria of the coexisting intravesicular complexes between calcium, phosphatidate and chelator might be the cause of the limited calcium influx (Fig. 6). A theoretical explanation may be, that under equilibrium conditions the calcium influx is not inhibited, but only balanced by an equal counterflow of calcium through translocation of a calcium phosphatidate-chelator tri- or multi-complex from inner to outer monolayer. This model explains also the observed chelator efflux, which is still continuing during the equilibrium situation for the calcium translocation (Fig. 2, see also Ref. 26 for the results at 2 mM Ca^{2+}).

At calcium concentrations higher than about 2.5 mM a general permeability increase was observed (Fig. 4). Under these conditions most of the phosphatidate molecules are occupied by calcium ions (unpublished observations) and vesicle-vesicle interactions start to play a role, which can be noticed from increasing turbidity of the suspension (unpublished observations). This raises the possibility that vesicle aggregation and perhaps vesicle fusion become important in affecting the barrier function of the membrane. Fusion of lipid vesicles has been shown to be a direct cause of leakage of the vesicle content [48].

Earlier studies already showed calcium-induced fusion of egg PC-derived PA/egg PC (50:50, mol%) vesicles [49,50], but those results can not be translated directly to our system. Furthermore, phospholipid domain formation through calcium-induced lateral phase separation [51,52] could be a cause of increasing membrane permeability. Phospholipid packing defects at the boundary of the different domains might induce transient formation of pores in the membrane through which ions can escape from the vesicles [52,53].

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