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Calcium-induced changes in permeability of dioleoylphosphatidylcholine model membranes containing bovine heart cardiolipin

Erik B. Smaal [a], Cor Schreuder [a], Jos B. van Baal [a], Pim N.M. Tijburg [a], Jacqueline G. Mandersloot [a], Ben de Kruijff [b] and Johannes de Gier [a]

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

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(1) At calcium concentrations up to about 4 mM a selective permeability increase of cardiolipin/dioleoylphosphatidylcholine (50:50, mol%) membranes for calcium and its chelator arsenazo III is observed. Under these conditions calcium does not occupy all the binding sites of cardiolipin at the membrane interface and no vesicle–vesicle interactions are found. (2) Lowering of the cardiolipin content of the vesicles to 20 mol% extends the calcium concentration range in which a selective permeability for calcium and arsenazo III is appearing up to about 12 mM. (3) We suggest that the observed selective permeability increase is caused by transient formation of inverted micellar structures in the membrane with cardiolipin as translocating membrane component for calcium and arsenazo III. (4) At calcium concentrations of 4 mM and higher for 50 mol% cardiolipin-containing vesicles a general permeability increase is found together with calcium-cardiolipin binding in a 1:1 stoichiometry, vesicles aggregation and, above 8 mM of calcium, vesicle fusion. (5) The loss of barrier function of the membrane under these conditions is correlated with vesicle aggregation and may be explained by a transition from a bilayer into a hexagonal H_{II} organization of the phospholipids.

Cardiolipin is a major component of the mitochondrial inner membrane of heart and skeletal muscle cells [1]. It is considered as a chemically unique phospholipid, because it contains four fatty acyl chains (predominantly linoleate [2]) and a backbone of three glycerol groups chained together by two phosphates. Recently, the lipid received considerable attention because of its polymorphic phase behaviour [3–6]. In aqueous dispersion at neutral pH the sodium salt of cardiolipin organizes in bilayer structures, but in the presence of divalent cations [3–5], at high sodium concentration [6] or at extreme low pH [6] a transition into a hexagonal H_{II} phase is observed. The structural behaviour of mixtures of cardiolipin with dioleoylphosphatidylcholine has also been studied in detail [5]. Aqueous dispersions of an equimolar mixture of these phospholipids at low millimolar calcium concentrations (< 5 mM) show lipidic particles in the freeze-fracture face as seen by electron microscopy [5]. These structures are interpreted as inverted micelles embedded in the hydrophobic part of the

Abbreviations: DOPC, dioleoylphosphatidylcholine; MLV, multilamellar vesicles; LUV, large unilamellar vesicles.

Correspondence: E.B. Smaal (present address), Gist-Brocades N.V., Research Development Division, P.O. Box 1, 2600 MA Delft, The Netherlands.

bilayer. At higher calcium concentrations (> 5 mM) again a hexagonal H_{II} organization is observed in which most of the phosphatidylcholine can be accommodated [5]. For dioleoylphosphatidate, a phospholipid which undergoes a similar polymorphic phase change in response to the presence of calcium, it has been suggested that this particular polymorphic behaviour may be coupled to calcium-induced permeability increase, aggregation and fusion of phosphatidate-containing vesicles [7,8]. For cardiolipin/dioleoylphosphatidylcholine (50 : 50, mol%) the effect of calcium on the structural behaviour has been correlated already with events like vesicle aggregation and fusion [5,9–12] and aselective increase in membrane permeability [9–13]. However, in relation to this less is known about the permeability of these membranes for calcium itself.

In this paper we report on the effect of increasing calcium concentration on the barrier function of cardiolipin-containing liposomal membranes.

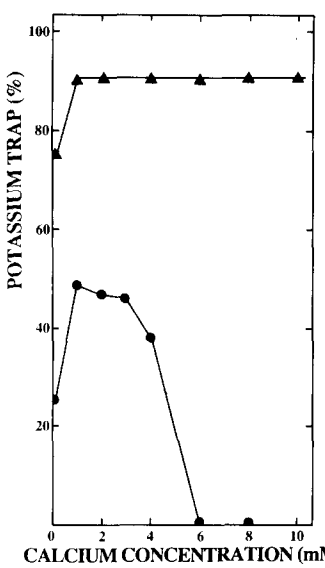

Fig. 1. Calcium-induced potassium permeability changes of cardiolipin/DOPC MLV. Vesicles, enclosing 150 mM KCl and 10 mM Tris-acetate (pH 7.4), were incubated for 90 min at 20°C in a 150 mM choline chloride, 10 mM Tris-acetate (pH 7.4) (isotonic) solution containing different amounts of $CaCl_2$. The lipid phosphorus concentration was about 4 mM [23]. After incubation, the amount of non-trapped and the total amount of potassium was determined potentiometrically before and after addition of Triton X-100, respectively. Shown is the calculated potassium trap as % of the initial potassium trap. For details see Ref. 13. ●, cardiolipin/DOPC (50 : 50, mol%) MLV; ▲, cardiolipin/DOPC (20 : 80, mol%) MLV.

We studied the trapping ability of liposomes for potassium ions at various concentrations of calcium in the outside medium. Furthermore, to see whether the presence of cardiolipin enables specific translocation of calcium, we measured the influx of the ion into cardiolipin-containing vesicles using a recently developped method [14], which is based on a method of Weissmann et al. [15]. In this method large unilamellar vesicles enclosing the calcium chelating photoindicator arsenazo III are used and a discrimination is made between influx of calcium and efflux of arsenazo III [14]. The results are discussed in relation to the results of calcium binding and vesicle aggregation and fusion experiments.

The sodium salt of cardiolipin was either isolated from beefheart and purified as described earlier [2] or purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesized from egg yolk phosphatidylcholine according to Van Deenen and De Haas [16].

Fig. 1 shows the potassium trapping capacity of cardiolipin/DOPC MLV after 90 min of incubation with various calcium concentrations. The liposomes containing the negatively charged cardiolipin were found to be leaky for potassium in the absence of calcium. The extent of this permeability was almost linearly with the amount of cardiolipin incorporated in the vesicles (results not shown). The potassium permeability may be caused by the high level of unsaturation of the acyl chains of cardiolipin [2] and the negatively charged membrane surface causing an increased surface concentration of potassium. In contrast with earlier findings [13], we found that calcium at low millimolar concentrations caused some inhibition of this non-specific potassium leakage (Fig. 1). Liposomes composed of an equimolar mixture of cardiolipin and DOPC showed a highly increased potassium permeability at calcium concentrations of 4 mM and higher. This is in accordance with an earlier study with the same system, in which above 3 mM of calcium also increased dextran leakage was demonstrated [13]. 20 Mol% cardiolipin-containing liposomes, which showed a high trapping capacity for potassium up to 10 mM of calcium (Fig. 1), were becoming increasingly leaky for potassium with increasing calcium con-

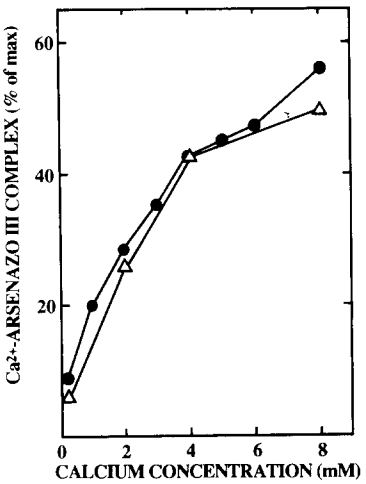

Fig. 2. Ca^{2+}-arsenazo III complex formation during incubation of cardiolipin/DOPC LUV with entrapped arsenazo III in a calcium-containing medium. LUV [24] enclosing 2.3 mM arsenazo III, 150 mM KCl, 10 mM Tris-acetate (pH 7.4) were incubated at 20°C in an arsenazo III-free isotonic solution, containing different amounts of $CaCl_2$. The lipid phosphorus concentration was about 0.5 mM [23]. The amount of Ca^{2+}-arsenazo III complex formed after 1 h of incubation was determined by measuring the absorbance at 650 nm with the absorbance at 700 nm as reference [14]. This absorbance is expressed as the percentage of the potential maximal absorbance as determined by the addition of an excess of calcium ionophore A23187. For details see Ref. 14. ●, cardiolipin/DOPC (50:50, mol%) LUV; △, cardiolipin/DOPC (20:80, mol%) LUV.

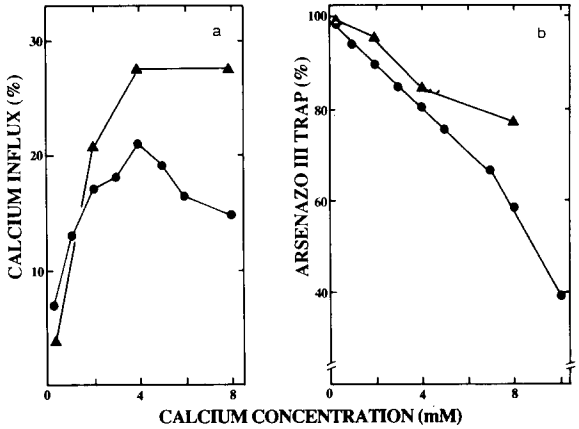

Fig. 3. Calcium and arsenazo III permeability of cardiolipin/DOPC LUV incubated in a calcium-containing medium. The experiment was performed as described in the legend of Fig. 2. After 1 h of incubation the absorbance at 650 nm with the absorbance at 700 nm as reference was measured immediately (A_1), after addition of an excess of EDTA (A_2) and after subsequent addition of an excess of 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: (a) calcium influx: $((A_2 - A_3)/(A_m - A_3)) \times 100$ (% Ca^{2+}-arsenazo III formed inside vesicles) and, (b) arsenazo III trap: $(1-(A_1 - A_2)/(A_m - A_3)) \times 100$ (total amount of arsenazo III minus amount of Ca^{2+}-arsenazo III outside vesicles; in %). For further details see Ref. 14. ●, cardiolipin/DOPC (50:50, mol%); ▲, cardiolipin/DOPC (20:80, mol%).

centrations above 12 mM of calcium (53% of maximum trap at 20 mM Ca^{2+}). A similar calcium concentration dependent potassium permeability behaviour was found for dioleoylphosphatidate/DOPC (20:80, mol%) vesicles [7], and it was proposed that this increasing leakage is a consequence of intermembrane contact of the vesicles [8].

It has already been suggested that cardiolipin may be able to translocate calcium specifically over a membrane [5,17–21]. However, it could not be demonstrated in experiments with MLV [22]. Recently we presented an adapted version of a calcium influx assay [15] in which large unilamellar vesicles enclosing the calcium-chelating photo indicator arsenazo III are used [14]. In Fig. 2 the results are depicted of experiments on the calcium translocation over membranes of cardiolipin/DOPC LUV. Apparently, formation of the calcium-arsenazo III complex is possible under conditions where only a limited non-specific potassium leakage is found. This deviant permeability behaviour is most striking for 20 mol% cardiolipin- containing vesicles. The formation of the calcium-arsenazo III complex can be a consequence of either calcium influx or arsenazo III efflux. In an earlier paper we presented a method in which these two possible fluxes are determined separately [14]. In this assay an excess of EDTA is added to samples of the vesicles suspension after incubation with calcium, causing chelation of extravesicular calcium and dissociation of extravesicular Ca^{2+}-arsenazo III complex, which can be present because of arsenazo III efflux. Subsequent addition of an excess of the calcium ionophore A23187 causes efflux of calcium and consequently dissociation of intravesicular Ca^{2+}-arsenazo III complex, giving a measure for the amount of translocated calcium during the incubation [14]. Fig. 3 shows the results of this assay applied on

the cardiolipin/DOPC system. It can be concluded that calcium is able to induce a permeability increase for itself but also for arsenazo III, even in low millimolar concentrations. The calcium influx for cardiolipin/DOPC LUV measured over a period of 1 h is calcium concentration dependent. Up to about 4 mM of calcium the amount of trapped calcium increases with the extravesicular calcium concentration. At increasing calcium concentration (4–10 mM Ca^{2+}) the calcium trap stabilizes for vesicles containing 20 mol% cardiolipin and decreases for vesicles containing 50 mol% cardiolipin. The limited calcium influx may be explained by the possibility that the influx of calcium is counteracted by an efflux of calcium ions bound to effluding arsenazo III (Fig. 3B). A coupling between calcium influx and arsenazo III efflux has already been suggested for phosphatidate- containing vesicles [7,8] for which we proposed the formation of a hydrophobic complex between calcium, calcium-chelator and phosphatidate as translocating shuttle for arsenazo III. Below 4 mM of calcium the induced translocation of calcium and arsenazo III over cardiolipin/DOPC (50 : 50, mol%) membranes appears to be selective because no increase in permeability was found for potassium (Fig. 1). A similar conclusion can be drawn for 20 mol% cardiolipin-containing vesicles for a much larger concentration range (up to about 12 mM Ca^{2+}). The influx of calcium also gives a plausible explanation for the inhibition of the potassium permeability at low calcium concentrations (Fig. 1). Translocated calcium ions may accumulate in the diffuse double layer at the negatively charged inner surface of the outer bilayers of the liposomes and thus decrease the surface concentration of potassium ions.

It is possible to draw a parallel between the present permeability results and those of our previous study with dioleoylphosphatidate containing vesicles [7]. Low calcium concentrations cause a limited calcium influx together with a calcium chelator efflux, which both are selective because no dextran permeability is found [13] and the non-specific potassium permeability is even decreased (Fig. 1). Above 4 mM and 20 mM of calcium for, respectively, 50 mol% cardiolipin- and 20 mol% cardiolipin-containing vesicles the permeability barrier of the vesicular membrane is destroyed. For dioleoylphosphatidate/DOPC (20 : 80, mol%) vesicles this typical permeability behaviour is also found [7]. For these vesicles below about 2.5 mM of calcium an increased selective calcium and calcium-chelator permeability increase is found, which shows a similar extent and the same characteristics as the selective permeability observed for cardiolipin/DOPC vesicles. At calcium concentrations higher than 2.5 mM for dioleoylphosphatidate/DOPC (20 : 80, mol%) vesicles also an increase in aselective permeability is appearing [7]. For dioleoylphosphatidate-containing vesicles the permeability behaviour can be correlated with calcium-vesicle association, vesicle aggregation and phospholipid phase behaviour [8]. To see whether a similar relation applies for the cardiolipin/DOPC mixture, experiments were

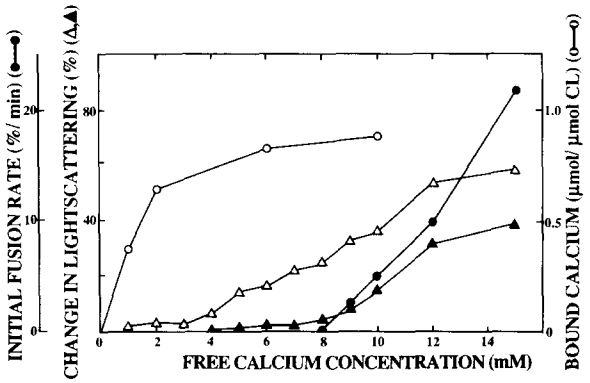

Fig. 4. Cardiolipin/DOPC (50:50, mol%) vesicles: calcium association and vesicle–vesicle interactions. Binding of calcium to MLV (○) was measured radiochemically [5,8]. The vesicles were incubated for 1 h at 20°C in 150 mM KCl, 10 mM Tris-acetate, (pH 7.4) containing different amounts of [^{45}Ca]$CaCl_2$ and 25 µg/ml A23187. After subsequent centrifugation (10 min, 100000 × *g*) the amount of bound calcium and the free calcium concentration was determined by liquid scintillation counting of samples of the pellet and supernatant, respectively. For details see Ref. 8. Aggregation of LUV [24] was determined as described in Ref. 8. The increase in lightscattering at 450 nm of a vesicle suspension was measured during incubation with various $CaCl_2$ concentrations at 20°C. After 1 h of incubation an excess of EDTA was added, causing a decrease in lightscattering. Shown are the EDTA-induced decrease (△) and the remaining lightscattering with respect to that of the calcium-free suspension (▲), both expressed as the percentage of the maximal lightscattering (at 20 mM $CaCl_2$). Fusion of LUV (●) was assayed according to Wilschut et al. [9] and is expressed as the initial rate of increase in fluorescence signal (% of maximum fluorescence/min).

done on the calcium-vesicle and calcium-promoted vesicle–vesicle interactions (Fig. 4).

Calcium binds to cardiolipin/DOPC (50 : 50, mol%) vesicles with an apparent dissociation constant of 1–2 mM and a stoichiometry which is close to one calcium ion per cardiolipin molecule (Fig. 4). These results are in full agreement with earlier findings [5]. At low calcium concentrations, where calcium does not occupy all the cardiolipin binding sites, no significant interaction between the vesicles was found (Fig. 4). Calcium in a concentration of 4 mM and higher is causing an increase in lightscattering at 450 mm. Under these conditions the negatively charged membrane surface is almost completely neutralized by calcium ions. Below 8 mM of calcium the increase in lightscattering can almost completely be reversed by the addition of an excess of EDTA (Fig. 4). This points out that vesicle aggregation is the cause of the lightscattering increase. At calcium concentrations higher than 8 mM a residual fraction of the lightscattering increase is found (Fig. 4) probably as a consequence of the presence of fused structures. Measurements of membrane fusion with the method of Wilschut et al. [9] indeed yield a calcium threshold concentration for fusion of 9 mM (Fig. 4), which is in agreement with earlier findings [9,10]. When the results of Fig. 4 are compared with the permeability results it seems that vesicle aggregation can be directly correlated with the observed increased general permeability of the vesicular membrane. This general permeability is already appearing under conditions where no membrane fusion is found. Apparently, the intermembrane contact affects the organization of the phospholipids in such a way that the permeability barrier is broken. De Kruijff et al. showed that cardiolipin/DOPC (50 : 50, mol%) mixtures tend to organize in a hexagonal H_{II} phase at higher calcium concentrations [5]. At low millimolar calcium concentrations this bilayer destabilization leads to the formation of lipidic particles in the membrane face as was shown with freeze-fracture electron microscopy, in parallel with the appearance of an isotropic signal in the ^{31}P-NMR spectrum [5]. These lipidic particles are interpreted as intramembrane inverted micelles [5]. The appearance of these structures is attended with a highly increased transbilayer movement of phosphatidylcholine [11], which may be explained by the fact that the transient formation of inverted micelles permits high phospholipid dynamics. Because the calcium–cardiolipin interaction is the trigger of this event, the calcium-lipid complex would certainly take part in the process, thus facilitating the translocation of calcium. The ability of cardiolipin to form together with calcium such inverted micellar structures is also mentioned as mechanistical explanation for the cardiolipin mediated uptake of calcium from an aqueous phase into an organic phase [21]. Phosphatidylcholine, known as promoter of the bilayer organization in aqueous phospholipid dispersions, inhibits the cardiolipin-mediated calcium transfer into an organic phase [17,21]. Furthermore, ruthenium red, an inhibitor of the calcium- induced formation of non-bilayer structures in cardiolipin-containing membranes [19], also inhibits cardiolipin-mediated calcium translocation [17,19]. (In the context of the present investigation it is of interest that ruthenium red is well known because of its inhibitory action on mitochondrial calcium transport.) The experiments with these two phase systems, which can be regarded as a simplified model for a phospholipid membrane, generally confirm the idea that hydrophobic inverted micelle-like structures are involved in the transmembrane transport of calcium. In parallel with earlier studies with phosphatidate-containing vesicles [7,8] it can be suggested that these structures also can enclose the calcium chelator arsenazo III as a part of a cardiolipin-calcium-chelator tri-complex.

About the physiological implications of the present findings can only be speculated. Cardiolipin is the only major negatively charged phospholipid of the inner mitochondrial membranes of heart cells (20 mol% of the total lipid content) [25]. Calcium translocation across the membranes is of crucial importance for the regulation of the cytoplasmic calcium level in the metabolically active cells. It may be possible that cardiolipin is playing a role in the electrical silent calcium efflux from mitochondria, a process of which the mechanism is still unclear. Although the mitochondrial calcium level is much lower than the calcium concentrations we used in our model experiments, it is conceivable that other inner mitochondrial membrane components, like membrane proteins, are

involved in the cardiolipin-mediated calcium translocation by enabling local formation of calcium-cardiolipin non-bilayer structures and transbilayer movement of the calcium-cardiolipin complex.

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