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Ca^{2+}-INDUCED ISOTROPIC MOTION AND PHOSPHATIDYLCHOLINE FLIP-FLOP IN PHOSPHATIDYLCHOLINE-CARDIOLIPIN BILAYERS

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Summary

Ca^{2+} induces a structural change in phosphatidylcholine-cardiolipin bilayers, which is visualised by freeze-fracturing as lipidic particles associated with the bilayer and is detected by ^{31}P-NMR as isotropic motion of the phospholipids. In this structure a rapid transbilayer movement of phosphatidylcholine and a highly increased permeability towards Mn^{2+} are observed.

It is well established that in biological membranes lipid constituents can be arranged in a bilayer configuration [1—4]. In isolated form, however, particular lipid species have been shown to adopt non-bilayer phases [5—10] and the occurrence of non-bilayer configurations of lipids has been reported both for artificial membranes [11] and for biological membranes [12,13]. The functional implications of such non-bilayer configurations are readily envisaged for processes like fusion and flip-flop and for the barrier-properties of membranes [6,14]. In artificial membranes composed of phosphatidylcholine and cardiolipin, the addition of Ca^{2+} was shown to introduce a non-bilayer phase, characterised by an isotropic ^{31}P-NMR signal and visualised by freeze-fracturing as particles and pits on the fracture faces of a lipid bilayer [11,15]. This process is caused by the tendency of cardiolipin to adopt the hexagonal (H_{II}) phase in the presence of Ca^{2+} [9,11]. A rapid exchange of lipids between bilayer and particles was suggested from the ^{31}P-NMR linewidths and an inverted micelle model was proposed which could account for the dynamical nature of the system [11]. Recently it was shown that the Ca^{2+}-induced fusion of small phosphatidylcholine-cardiolipin vesicles is associated with the appearance of lipidic particles at the fusion interfaces [16], which strongly suggests that this fusion

event proceeds via intermediary inverted micellar structures. In this paper, we study the effects of the Ca^{2+}-induced non-bilayer configuration in phosphatidylcholine-cardiolipin model membranes on the transbilayer movement of phosphatidylcholine and the barrier properties of the membrane towards Mn^{2+}. The transbilayer movement of phosphatidylcholine is investigated by measuring the pool of phosphatidylcholine that is exchangeable with phosphatidylcholine exchange protein from bovine liver. The barrier properties of the membranes are investigated using ^{31}P-NMR with Mn^{2+} as a paramagnetic reagent which broadens the resonance of the accessible phospholipids beyond detection [17]. In cases of impermeability to Mn^{2+}, this also allows for the calculation of the outer monolayer pool of phospholipids from the decrease in ^{31}P-NMR signal intensity.

1,2-Dioleoly-*sn*-glycero-3-phosphocholine (18 : 1_c/18 : 1_c-phosphatidylcholine), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (18 : 0/18 : 1_c-phosphatidylcholine), 18 : 1_c/18 : 1_c-[^{14}C]phosphatidylcholine, 18 : 0/18 : 1_c-[^{14}C]phosphatidylcholine and [^{3}H]cholesterol oleate were synthesised as described before [18,19].

Cardiolipin was obtained from Sigma and [^{14}C]carboxyldextran (M_r 70 000) from New England Nuclear. Phosphatidylcholine exchange protein, purified from been liver according to Kamp et al. [20], was a gift from Dr. K.W.A. Wirtz. It was stored in 50% glycerol at −20°C and dialysed against incubation buffer before use. Multilamellar vesicles were made by dispersing a dry film of the phospholipid mixture in 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.4, at 23°C to give a concentration of 2 mM. Dispersions for ^{31}P-NMR experiments were made by dispersing 100 μmol of lipid in 1.5 ml 15% ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.0. Large unilamellar vesicles were prepared by the ether-injection method [21] as described previously [15], and used after sedimentation at 35 000 × *g* for 30 min. Small vesicles were prepared by sonication of a dispersion of 50—75 μmol of lipid in a 1.3 ml buffer under nitrogen, on ice, for 4 min using a Branson tip sonicator, power setting 4, followed by 60 min centrifugation at 150 000 × *g*.

Phosphatidylcholine exchange assays using phosphatidylcholine exchange protein from bovine liver and sonicated vesicles as acceptor particles were performed as outlined in the legend to Fig. 1. ^{31}P-NMR spectra were recorded at 36.4 MHz under conditions of proton decoupling, as described before [6,11]. Signal intensities were determined via computer integration with respect to an external triphenylphosphine reference. Freeze-fracture electron microscopy was performed as outlined previously [23]. Glycerol was added to the samples to prevent freeze damage.

The exchangeability of phosphatidylcholine in multilamellar vesicles, prepared from a mixture of 18 : 1_c/18 : 1_c-phosphatidylcholine and bovine heart cardiolipin, was studied with phosphatidylcholine exchange protein, in the absence and presence of 3 mM Ca^{2+} (Fig. 1). In the absence of Ca^{2+} a rapidly exchanging pool of about 17% of the phosphatidylcholine was detected. The presence of 3 mM Ca^{2+} caused a considerable increase in the size of the exchangeable pool; after 4 h of incubation, about 45% of the labeled phosphatidylcholine had been transferred. From phosphatidylcholine-cardiolipin-Ca^{2+} vesicles, prepared by the ether-injection method, nearly 90% of the

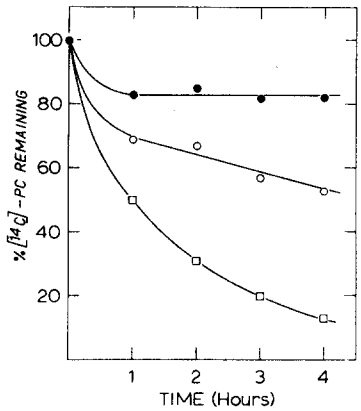

Fig. 1. Transfer of [^{14}C]phosphatidylcholine ([^{14}C]-PC) from 18 : 1_c/18 : 1_c-phosphatidylcholine-cardiolipin (1 : 1) multilamellar vesicles in the absence (●) and presence of 3 mM Ca^{2+} (○), and from 18 : 1_c/18 : 1_c-phosphatidylcholine-cardiolipin-Ca^{2+} vesicles, prepared by the ether injection method (□). For the experiments with multilamellar vesicles. 0.5 ml multilamellar vesicles (0.4 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine, 0.4 μmol cardiolipin, 10^5 dpm 18 : 0/18 : 1_c-[^{14}C]phosphatidylcholine, 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.4) were pre-incubated for 1 h at 20°C with 0.5 ml buffer with and without 6 mM Ca^{2+}. The actual incubation was started by the addition of 1.0 ml sonicated vesicles (30 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine, 10^6 dpm [^{3}H]cholesterololeate, 150 mM NaCl, 10 mM Tris/acetic acid pH 7.4), and 2.0 ml exchange protein (120 μg) and, for the incubation with Ca^{2+}, sufficient Ca^{2+} to maintain a concentration of 3 mM. The mixture was slowly rotated at 37°C and at time intervals of 1 h samples were withdrawn to determine the percentage of [^{14}C]phosphatidylcholine remaining in the multilamellar vesicles. After 2 h of incubation, 1.0 ml new exchange protein (60 μg) was added to the incubation mixtures.

For the experiment with the large, unilamellar vesicles, prepared by the ether injection method, 1.0 ml of large vesicles (0.5 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine, 0.5 μmol cardiolipin, $3 \cdot 10^4$ dpm 18 : 1_c/18 : 1_c-[^{14}C]phosphatidylcholine, 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.4) were incubated at 37°C with 1.0 ml sonicated vesicles (6 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine, $2.5 \cdot 10^5$ dpm [^{3}H]-cholesterololeate, 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.4) and 2.0 ml exchange protein (120 μg). After 1 h of incubation, a sample was withdrawn to determine the percentage of [^{14}C]phosphatidylcholine remaining in the large vesicles. The large vesicles remaining in the incubation mixture were sedimented at 35 000 × *g* for 30 min, the supernatant was discarded and the large vesicles were dispersed in buffer and subsequently reincubated with a new amount of sonicated vesicles and an aliquot of new exchange protein. This procedure was repeated three times. To determine the percentage of [^{14}C]phosphatidylcholine remaining in the multilamellar vesicles and the large unilamellar vesicles, the vesicles in the samples were sedimented at 35 000 × *g* for 30 min, washed three times with 1.0 ml buffer, and finally assayed for [^{14}C] and [^{3}H]radioactivity and phospholipid phosphorus [22]. The percentage [^{14}C]phosphatidylcholine remaining in the vesicles was calculated from [^{14}C]radioactivity and phospholipid phosphorus, after correction for small amounts (0.1—0.5%) of contaminating sonicated vesicles, as determined from [^{3}H]radioactivity.

labeled phosphatidylcholine had been transferred after four incubations of 1 h (Fig. 1).

Freeze-fracture electron microscopy of the multilamellar vesicles, incubated for 4 h at 37°C with or without 3 mM Ca^{2+} are shown in Fig. 2. Without Ca^{2+} smooth fracture faces are observed (Fig. 2A), whereas the presence of Ca^{2+} caused the appearance of particles and pits on the fracture faces of many vesicles (Fig. 2B). Incubation of multilamellar vesicles loaded with [^{14}C]-dextran, for 4 h at 37°C, caused the appearance of about 1.5% of [^{14}C]dextran in the supernatant after sedimentation of the vesicles at 35 000 × *g* for 30 min, both in the absence and presence of 3 mM Ca^{2+}. It is therefore unlikely that Ca^{2+} would make the membranes permeable to the exchange protein.

^{31}P-NMR spectra of multilamellar vesicles of an equimolar mixture of

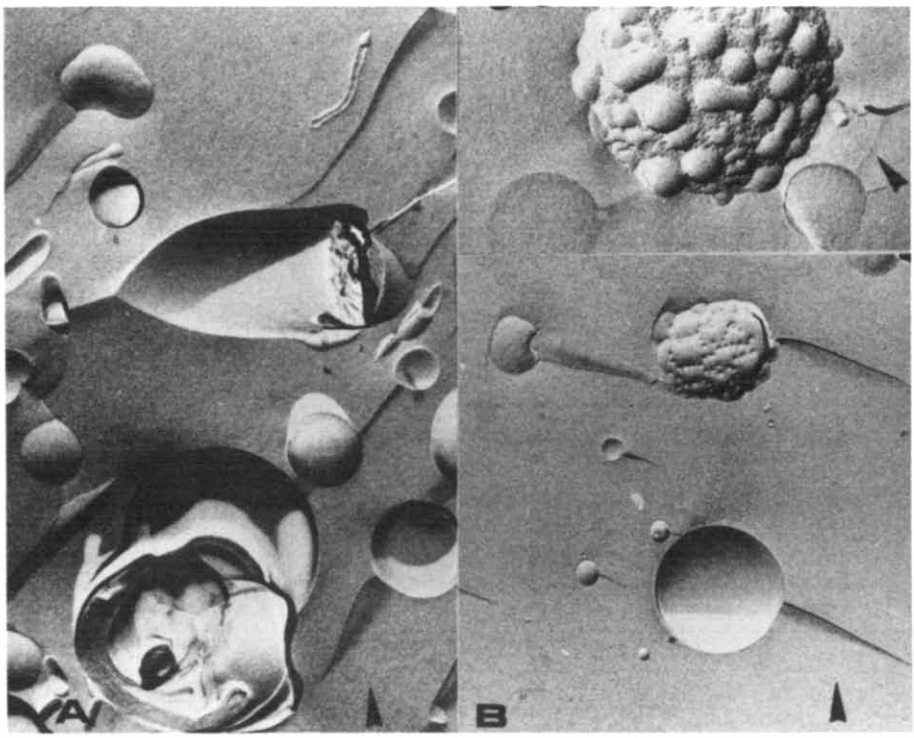

Fig. 2. Freeze-fracture electron micrographs of multilamellar 18 : 1_c/18 : 1_c-phosphatidylcholine-cardiolipin (1 : 1) vesicles, incubated for 4 h at 37°C in (A) 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.4, and (B) 3 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.4. After the incubation the vesicles were sedimented at 35 000 × *g* for 30 min. Magnification ×25 000.

18 : 1_c/18 : 1_c-phosphatidylcholine and cardiolipin, in the absence and presence of Ca^{2+}, and the effect of addition of Mn^{2+} to these systems, are shown in Fig. 3. In the absence of Ca^{2+} the characteristic bilayer spectrum was obtained (Fig. 3A). Upon addition of Mn^{2+} there was a decrease in the intensity of the signal, but no change in the shape of the spectrum (Fig. 3B). As shown in Fig. 4 the signal intensity could be reduced with Mn^{2+} to about 86% of its original value. Even after 2 h of incubation with 5 mM Mn^{2+}, 86% of the signal intensity was observed. This means that the bilayers are impermeable to Mn^{2+} and that the pool of phospholipids in the outer monolayer is about 14%. As a similar pool (17%) was found to be exchangeable with the exchange protein (Fig. 1), it is clear that the action of exchange protein is confined to the outer monolayer, and that there is no rapid transbilayer movement of phosphatidylcholine. The addition of Ca^{2+} to the multilamellar vesicles resulted in the appearance of a sharp signal at the resonance position of phospholipids undergoing isotropic motion. In the spectrum of Fig. 3C this isotropic signal contributes to 34% of the total signal intensity. Here the subsequent addition of Mn^{2+} caused the disappearance of the isotropic signal and part of the bilayer signal (Fig. 3D). With 5 mM Mn^{2+} a decrease was observed to 58% of the original intensity, and after 2 h of incubation this value had dropped to 42%. This suggests that the isotropic phase, introduced by Ca^{2+}, causes a highly

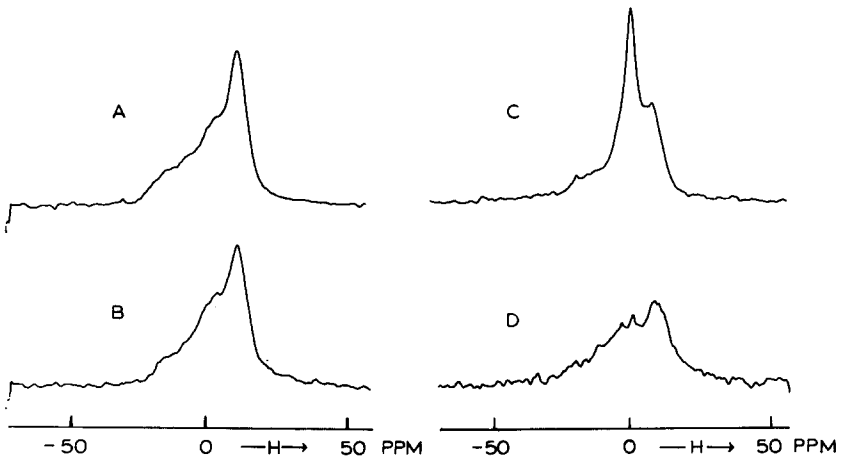

Fig. 3. Effects of Ca^{2+} and Mn^{2+} on the 36.4 MHz ^{31}P-NMR spectra of multilamellar 18 : 1_c/18 : 1_c-phosphatidylcholine-cardiolipin (1 : 1) vesicles. (A) Spectrum of 18 : 1_c/18 : 1_c-phosphatidylcholine-cardiolipin vesicles (100 μmol total phospholipid) in 1.5 ml ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.0. (B) Spectrum of the sample after the addition of Mn^{2+} (final Mn^{2+} concentration in the sample 5 mM). (C) Spectrum of a sample such as (A), after the addition of Ca^{2+} (final Ca^{2+} concentration in the sample 16 mM). (D) Spectrum of sample (C), after addition of Mn^{2+} (final Mn^{2+} concentration in the sample 5 mM).

increased permeability towards Mn^{2+}. In the same system a considerably increased amount of phosphatidylcholine was found to be exchangeable with the exchange protein (Fig. 1), which indicates that the isotropic phase also causes a rapid transbilayer movement of phosphatidylcholine. The ^{31}P-NMR spectrum of large unilamellar phosphatidylcholine-cardiolipin-Ca^{2+} vesicles has been described previously [11]. Upon addition of Mn^{2+}, most of the isotropic and bilayer components of this spectrum were broadened beyond detection,

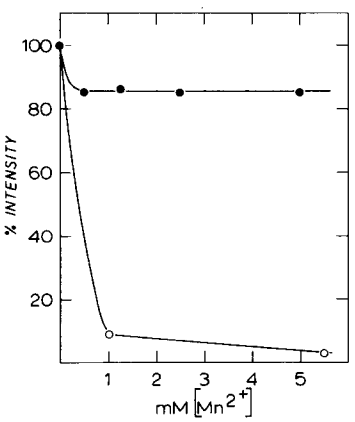

Fig. 4. Effect of Mn^{2+} on the 36.4 MHz ^{31}P-NMR signal intensity of 18 : 1_c/18 : 1_c-phosphatidylcholine-cardiolipin (1 : 1) multilamellar vesicles (●———●) and 18 : 1_c/18 : 1_c-phosphatidylcholine-cardiolipin-Ca^{2+} vesicles, prepared by the ether injection method (○———○). To vesicle suspensions in ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, pH 7.0, increasing amounts of $MnCl_2$ were added, and the intensity of the remaining ^{31}P-NMR signal was determined.

resulting in a decrease of the signal intensity to 0—10% of the original value (Fig. 4), which shows that the membrane is permeable to Mn^{2+}. That a rapid transbilayer movement of phosphatidylcholine also occurs, is shown by the exchangeability of about 90% of the phosphatidylcholine in these vesicles (Fig. 1).

The high permeability for Mn^{2+} and rapid transbilayer movement of phosphatidylcholine observed strongly support the inverted micelle model, proposed previously for the Ca^{2+}-induced lipidic particles in phosphatidylcholine-cardiolipin vesicles. A dynamic structure with a rapid exchange of lipids between bilayer and inverted micelle, can account for lipids and aqueous compartment within the inverted micelle moving rapidly across the bilayer, as depicted in Fig. 6 of Ref. 6. In the multilamellar vesicles, the presence of Ca^{2+} caused an increase in the pool of phosphatidylcholine, exchangeable with exchange protein to values more than twice the outer monolayer pool. This suggests that, in addition to the rapid flip-flop in the outer bilayer, phosphatidylcholine molecules from inner shells can move to the outer bilayer. Just as Ca^{2+} can cause fusion of phosphatidylcholine-cardiolipin vesicles via intermediary inverted micellar structures at the fusion interface [16], it can be envisaged that Ca^{2+} established contacts between two shells in a multilamellar vesicles. The presence of an inverted micelle at the contact points could allow for the movement of phosphatidylcholine molecules between two shells. Isotropic motion of membrane phospholipids has been reported for isolated rat, beef and rabbit liver microsomes [12—13] and intact rat liver tissue [17]. There exists a striking analogy between the properties of the phosphatidylcholine-cardiolipin (Ca^{2+}) model membrane, as described in this study, and results obtained for isolated microsomes with regard to the ^{31}P-NMR spectrum, Mn^{2+} permeability and transbilayer movement of phosphatidylcholine. In the absence of isotropic motion, which for microsomes means at low temperatures [13,17], the membranes are impermeable to Mn^{2+} [17] and only part of the phosphatidylcholine exchanges rapidly [24], which suggests that no rapid transbilayer movement of phosphatidylcholine occurs. As in the model membrane, isotropic motion of phospholipids in microsomes is accompanied by a high permeability to Mn^{2+} [17] and a rapid transbilayer movement of phosphatidylcholine [24,25].

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References

1 Wilkins, M.N.F., Blaurock, A.E. and Engelman, D.M. (1971) Nature New Biol. 230, 72—76
2 McLaughlin, A.C., Cullis, P.R., Hemminga, M.A., Hoult, D.J., Radda, F.K., Ritchie, G.A., Seeley, P.J. and Richards, R.E. (1975) FEBS Lett. 57, 213—218
3 Cullis, P.R. and Grathwohl, Ch. (1977) Biochim. Biophys. Acta 471, 213—226

4 Moore, N.F., Patzer, E.J., Wagner, R.R., Yeagle, P.C., Hutton, W.C. and Martin, R.B. (1977) Biochim. Biophys. Acta 464, 234—244
5 Rand, R.D., Tinker, D.O. and Fast, P.G. (1971) Chem. Phys. Lipid 6, 333—342
6 Cullis, P.R., and de Kruyff, B. (1978) Biochim. Biophys. Acta 507, 207—218
7 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 513, 31—42
8 Rand, R.P. and Sengupta, S. (1972) Biochim. Biophys. Acta 255, 484—492
9 Cullis, P.R., Verkleij, A.J. and Ververgaert, P.H.J.Th. (1978) Biochim. Biophys. Acta 513, 11—20
10 Weislander, A., Ulmius, J., Lindblom, A. and Fontell, K. (1978) Biochim. Biophys. Acta 512, 241—253
11 De Kruyff, B., Verkleij, A.J., van Echteld, C.J.A., Gerritsen, W.J., Mombers, C., Noordam, P.C. and de Gier, J. (1979) Biochim. Biophys. Acta 555, 200—209
12 De Kruyff, B., van den Besselaar, A.M.H.P., Cullis, P.R. van den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 514, 1—8
13 Stier, A., Finch, S.A.E. and Bösterling, B. (1978) FEBS Lett. 91, 109—112
14 Cullis, P.R. and Hope, M.J. (1978) Nature 271, 672—674
15 Verkleij, A.J., Mombers, C., Leunissen-Bijvelt, J. and Ververgaert, P.H.J.Th. (1979) Nature 279, 162—163
16 Verkleij, A.J., Mombers, C., Gerritsen, W.J., Leunissen-Bijvelt, J. and Cullis, P.R. (1979) Biochim. Biophys. Acta 555, 358—361
17 De Kruyff, B., Rietveld, A. and Cullis, P.R. (1980) Biochim. Biophys. Acta, in the press
18 Van Deenen, L.L.M. and de Haas, G. (1964) Adv. Lipid Res. 2, 168—229
19 De Kruyff, B. and Wirtz, K.W.A. (1977) Biochim. Biophys. Acta 468, 318—326
20 Kamp, H.H., Wirtz, K.W.A. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 318, 313—325
21 Deamer, D.H. and Bangham, A.D. (1979) Biochim. Biophys. Acta 443, 629—634
22 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494—496
23 Ververgaert, P.H.J.Th., Elbers, P.F., Luitingh, A.J. and van den Berg, H.J. (1972) Cytobiology 6, 86—96
24 Van den Besselaar, A.M.H.P., de Kruyff, B., van den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 510, 242—255
25 Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99—110