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RAPID TRANSBILAYER MOVEMENT OF PHOSPHATIDYLCHOLINE IN UNSATURATED PHOSPHATIDYLETHANOLAMINE CONTAINING MODEL MEMBRANES

P.C. NOORDAM, C.J.A. VAN ECHTELD, B. DE KRUIJFF and J. DE GIER

Department of Biochemistry and Department of Molecular Biology, State University of Utrecht, Transitorium 3, Padualaan 8, 3584 CH Utrecht (The Netherlands)

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Aqueous dispersions of egg phosphatidylethanolamine/18 : 1_c, 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) undergo a temperature-dependent transition from extended bilayers to structures characterized by isotropic ^{31}P-NMR signals and visualized by freeze-fracturing as lipidic particles associated with the bilayer. This transition is accompanied by a 3-fold increase in the phosphatidylcholine pool which can be exchanged by phospholipid exchange protein demonstrating a direct relation between the occurrence of non-bilayer lipid structures and an increased transbilayer movement of phosphatidylcholine.

Transbilayer movements of phospholipids in model membrane systems, under equilibrium conditions, are usually extremely slow with half-times in the order of days [1]. Asymmetrical perturbation of the bilayer [2,3], gel to liquid crystalline phase transitions [4] and the incorporation of the erythrocyte membrane integral membrane proteins glycophorin [5] and band 3 [6] greatly enhance the transbilayer movement of the phospholipids. Furthermore, it has been hypothesized [7] that 'non-bilayer' lipid structures facilitate the transbilayer movement of lipids. It is well established now that Ca^{2+} addition to phosphatidylcholine/cardiolipin (1 : 1) bilayers results in the formation of a lipid organization characterized by an isotropic ^{31}P-NMR signal and lipidic particles on the fracture faces [9]. Recently the conclusion has been made that the presence of these particles greatly facilitates the phosphatidylcholine flip-flop [8]. Bilayer to non-bilayer transitions can also be induced by temperature changes, in particular in unsaturated phosphatidylethanolamine-rich systems [9–11]. Therefore we thought it of interest to investigate the phosphatidylcholine flip-flop in such systems. Using phosphatidylethanolamine/phosphatidylcholine/cholesterol-mixed bilayers it will be shown that there is a direct correlation between the occurrence of these temperature-induced non-bilayer structures and an increased transbilayer movement of phosphatidylcholine.

Cholesterol was purchased from Merck. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c, 18 : 1_c-phosphatidylcholine), 18 : 1_c, 18 : 1_c-[*N*-*methyl*-^{14}C]phosphatidylcholine, egg phosphatidylethanolamine (derived from egg phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylethanolamine (14 : 0, 14 : 0-phosphatidylethanolamine), 1,2-dioleoyl-*sn*-glycero-3-phosphate (18 : 1_c, 18 : 1_c-phosphatidic acid) and [^{3}H]cholesterol oleate were synthesized as described before [12–15]. Pure phosphatidylcholine exchange protein from bovine liver was a gift from Dr. K.W.A. Wirtz. It was stored in 50% (w/v) glycerol at −20°C and dialyzed against incubation buffer before use.

Multilayered liposomes were prepared by dispersing at 4°C (unless otherwise stated) a dry film of lipid in 150 mM NaCl (100 mM in the case of ^{31}P-NMR

experiments), 20 mM Tris-HCl, pH 7.4. Small unilamellar vesicles were prepared by sonication of a dispersion of 190 μmol lipid in 1.3 ml buffer under N_2, on ice, for 4 min using a Branson tip sonicator, power setting 5, followed by 60 min centrifugation at 150 000 × *g* to remove unbroken structures.

^{31}P-NMR spectra were recorded at 36.4 MHz under conditions of proton decoupling as described before [7]. Signal intensities were determined by integration. Precautions were taken to ensure direct comparibility of the spectra. For further details see the figure legends. Freeze-fracture electron microscopy was performed as outlined previously [16]. For our experiments we selected a mixture of egg phosphatidylethanolamine/18 : 1_c, 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) since this model system was already characterized in previous studies [11]. An aqueous dispersion of this mixture exhibits the following temperature-dependent phase behaviour: at 4°C the dispersion consists of bilayers as evidenced by ^{31}P-NMR and freeze-fracturing. Heating till 60°C results in a transition to the hexagonal H_{II} phase. Lowering the temperature results in the formation of structures characterized by an isotropic ^{31}P-NMR signal and visualized by freeze-fracturing as lipidic particles associated with the bilayer. The exchangeability of phosphatidylcholine in this lipid system was studied with phosphatidylcholine exchange protein (details in the legend to Fig. 3). As it is known that these lamellar and isotropic structures are impermeable for K^+ and glucose [11] it is likely that they are also impermeable for the exchange protein. Since in the exchange assay donor and acceptor vesicles are separated by low speed centrifugation it was necessary to repeat the ^{31}P-NMR- and electron microscopy measurements on structures obtained by low speed centrifugation to make a direct comparison possible. To obtain further structural information the effect of Mn^{2+} on the ^{31}P-NMR spectra of these systems was measured. The paramagnetic ion will broaden the resonance of those molecules which it can approach closely. The broadened resonances are not detected

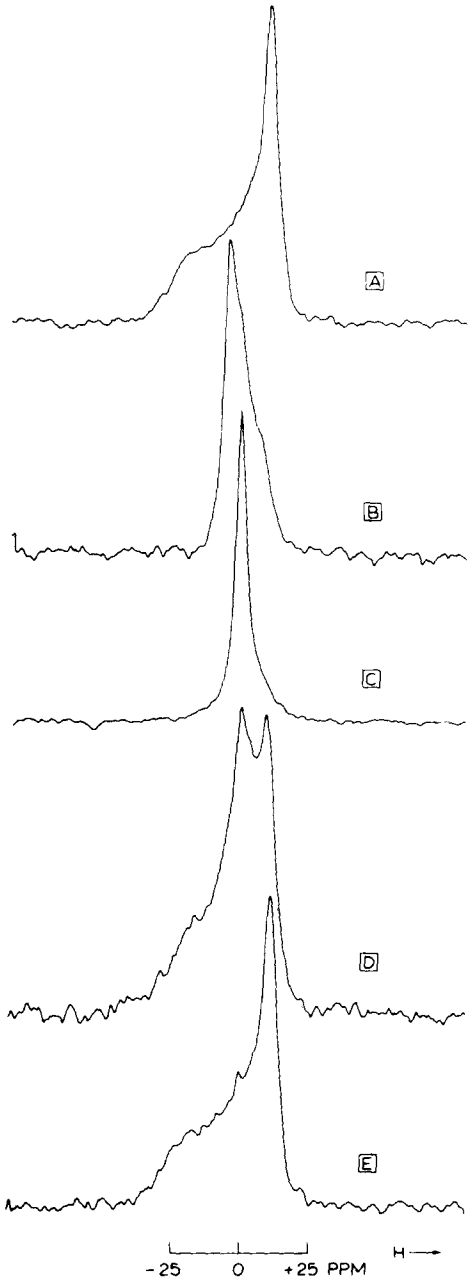

Fig. 1. 36.4 MHz ^{31}P-NMR spectra of at 4°C prepared aqueous dispersions of egg phosphatidylethanolamine/18 : 1_c, 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) at 20°C (A), at 60°C (B), at 20°C after being heated till 60°C (C), at 20°C after being heated till 60°C followed by freezing and thawing (D) and of an aqueous dispersion of 14 : 0, 14 : 0-phosphatidylethanolamine/18 : 1_c, 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) at 20°C after being dispersed at 60°C. Before recording the ^{31}P-NMR spectra at the indicated temperatures all samples were pelleted at 35 000 × *g* for 45 min. These low speed fractions contained 93% (A and B), 88% (C) and 95% (D) of the original lipid. After centrifugation the pellets were dispersed in 15% ^2H_2O containing buffer.

and hence the decrease in signal will give information on the fraction of the phospholipids present in the outer monolayer of the outer lipid bilayer of an impermeable liposomal system. The low speed pellet of an at 4°C prepared dispersion of egg phosphatidylethanolamine/18 : 1_c, 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) displays at 20°C and ^{31}P-NMR line-shape with a high-field peak and a low-field shoulder (Fig. 1A) characteristic for the bilayer orientation [17]. In agreement, freeze-fracturing reveals large lamellar structures (Fig. 2A). Upon addition of Mn^{2+} the shape of the spectrum remains unchanged but the signal intensity is reduced to 71 ± 3% of its original value, which means that the fraction of phospholipids in the outer monolayer is about 29%. Since from these multilamellar vesicles about 28% of the phosphatidylcholine is exchangeable with the exchange protein at 20°C in an incubation period of 4 h (Fig. 3) it can be concluded that the protein interacts only with the outer monolayer and that there is no rapid transbilayer movement of phosphatidylcholine. When the temperature is increased till 60°C the spectrum obtaines an opposite asymmetry and a reduced width (Fig. 1B) which is typical for the hexagonal H_{II} phase [7]. Heating did not cause any degradation of the lipids since the TLC patterns (eluent: chloroform/methanol/water/ammonia, 65 : 35 : 4 : 1, v/v) before and after heating were identical.

Upon cooling of the sample till 20°C the spectrum turns into one which indicates isotropic motion of the phospholipid molecules (Fig. 1C). It is highly unlikely that this isotropic signal originates from small vesicles since these are not present in the low-speed centrifugation fraction which contains 88% of the original lipid. Moreover, when the lipid in the low speed supernatant was pelleted at 150 000 × g for 20 min a typical bilayer ^{31}P-NMR spectrum was obtained (data not shown). That the isotropic signal is associated with non-bilayer lipid structures is indicated by freeze-fracturing which reveals bilayers with lipidic particles on the fracture faces (Fig. 2B). Addi-

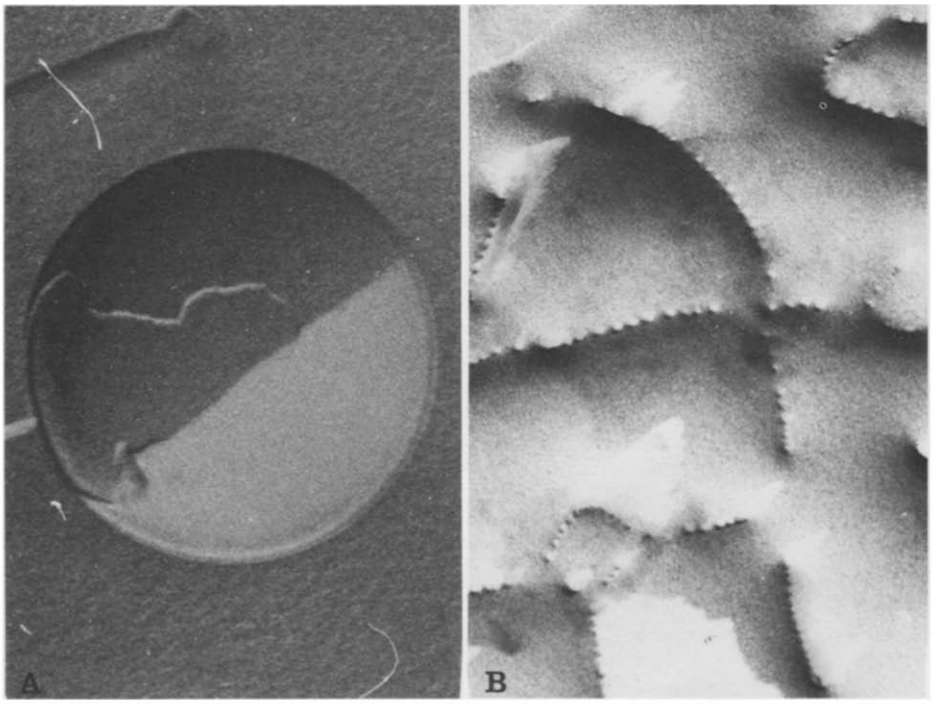

Fig. 2. Freeze-fracture electron micrographs of at 4°C prepared aqueous dispersions of egg phosphatidylethanolamine/18 : 1_c, 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) at 20°C (A) and at 20°C after being heated till 60°C (B). Magnification of the micrographs about 100 000×.

tion of Mn^{2+} to this dispersion results in the complete disappearance of the ^{31}P-NMR signal indicating that Mn^{2+} is highly permeable. Since from these structures about 74% of the phosphatidylcholine has been transferred after 4 h of incubation (Fig. 3) it can be concluded that in this system the transbilayer movement

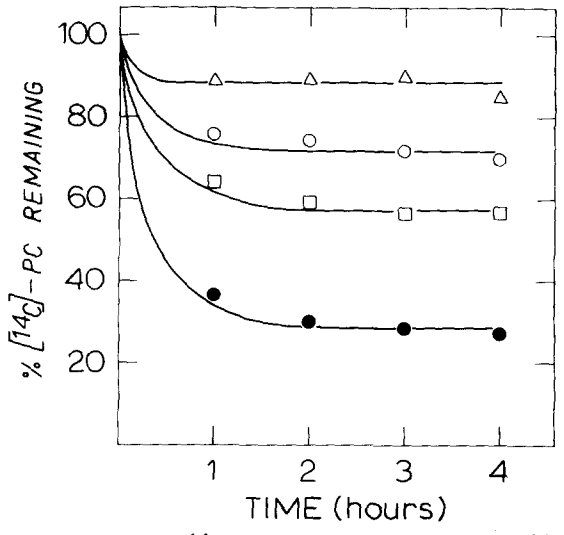

Fig. 3. Transfer of ^{14}C-labelled phosphatidylcholine ([^{14}C]-PC) at 20°C from at 4°C prepared dispersions of egg phosphatidylethanolamine/18 : 1_c, 18 : 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) (○———○), at 20°C after being heated till 60°C (●———●), at 20°C after being heated till 60°C followed by freezing and thawing (□———□) and at 20°C from an at 60°C prepared dispersion of 14 : 0, 14 : 0-phosphatidylethanolamine/18 : 1_c, 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) (△———△). The incubation mixture (4 ml 100 mM NaCl, 20 mM Tris-HCl, pH 7.4) contained in all four cases 2.4 μmol multilamellar vesicles ($7 \cdot 10^4$ dpm 18 : 1_c, 18 : 1_c-[^{14}C]phosphatidylcholine), 35 μmol sonicated vesicles (66% 18 : 1_c, 18 : 1_c-phosphatidylcholine, 30% cholesterol, 4% 18 : 1_c, 18 : 1_c-phosphatidic acid, $8 \cdot 10^5$ dpm [^{3}H]cholesterol oleate) and 100 μg exchange protein. The mixtures were slowly rotated at 20°C, and at time intervals of 1 h samples (1 ml) were withdrawn. The large vesicles remaining in these 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 [23]. The percentage ^{14}C-labelled phosphatidylcholine remaining in the vesicles was calculated from ^{14}C-radioactivity and phospholipid phosphorus, after correction for small amounts (±4%) of contaminating sonicated vesicles, as determined from ^{3}H-radioactivity. In the absence of exchange protein 1–2% of the ^{14}C-labelled PC was removed from the multilamellar vesicles.

of phosphatidylcholine and the permeability towards Mn^{2+} are greatly increased.

The original bilayer situation of the sample can be partially restored by freezing of the isotropic structures followed by warming-up till 20°C as indicated by the lineshape of Fig. 1D, and the disappearance of most of the lipidic particles. Parallel to this reduction in amount of non-bilayer lipid structures we observed that addition of Mn^{2+} to this sample broadens 75 ± 3% of the ^{31}P-NMR signal beyond detection while about 44% of the phosphatidylcholine is exchangeable with phosphatidylcholine exchange protein (Fig. 3).

Since saturated phosphatidylethanolamines do not undergo bilayer to hexagonal (H_{II}) transitions [13] it seemed us interesting to study the properties of a system in which egg phosphatidylethanolamine was replaced by 14 : 0, 14 : 0-phosphatidylethanolamine. Upon cooling till 20°C of an at 60°C (above the gel to liquid-crystalline phase transition of 14 : 0, 14 : 0-phosphatidylethanolamine) prepared dispersion of 14 : 0, 14 : 0-phosphatidylethanolamine/18 : 1_c, 18 : 1_c-phosphatidylcholine/cholesterol/18 : 1_c, 18 : 1_c-phosphatidic acid (50 : 16 : 30 : 4) a bilayer spectrum is obtained (Fig. 1E) and a freeze-fracture picture showing lamellar structures. Mn^{2+} addition to the sample does not change the ^{31}P-NMR line shape but broadens 16 (±3)% of the signal beyond detection. In the same system about 12% of the phosphatidylcholine molecules are exchangeable with the exchange protein (Fig. 3). This means that about 14% of the phospholipids are located in the outer monolayer and that there is no rapid transbilayer movement of phosphatidylcholine. So the presence of an unsaturated phosphatidylethanolamine which in its pure form adopts the hexagonal H_{II} orientation at increasing temperature is necessary for obtaining this structural and dynamical behaviour.

From these data it can be concluded that there is a direct relation between the occurrence of non-bilayer lipid structures induced by temperature variation in unsaturated phosphatidylethanolamine-containing model membranes and the increased transbilayer movement of phosphatidylcholine. The exact molecular architecture of the lipid membrane structure after the temperature treatment of the unsaturated phosphatidylethanolamine-containing system is not known but the most likely model is that of a

sponge- [11] or honey-comb- [18] like structure of a network of intersecting bilayers in which inverted micelles (representing the lipidic particles) are located at the nexus of intersecting bilayers or which are randomly dispersed in one bilayer. The presence of the lipidic particles (inverted micelles) apparently induces a dynamic state which is not present in the original pure bilayers and which allows transbilayer movement of phosphatidylcholine and penetration of Mn^{2+}.

The increased Mn^{2+} permeation contrasts with the impermeability of these systems towards K^+ and glucose [11]. This apparent discrepancy is at present not understood. One possible explanation might be that phosphatidic acid which for technical reasons is present in our systems might specifically mediate Mn^{2+} transport in particular in non-bilayer structures containing systems. Support for this suggestion comes from a recent study that phosphatidic acid is a very effective ionophore for Mn^{2+} in liposomal systems [21].

It is of interest to compare these results with observations on the metabolically very active rat liver endoplasmic reticulum membranes. At 4°C the ^{31}P-NMR spectrum indicates bilayer structure [19], phosphatidylcholine flip-flop is low [20] as is the Mn^{2+} permeability [19]. In strong contrast at 37°C an isotropic ^{31}P-NMR signal, fast phosphatidylcholine flip-flop and Mn^{2+} permeation is observed. Non-bilayer lipid structures, induced by the unsaturated phosphatidylethanolamine (which in isolated form prefers the H_{II} phase above 5°C) might be responsible for the high lipid dynamics in this membrane at 37°C, although other explanations cannot be excluded.

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