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I. PHOSPHOLIPID RELEASE FROM SONICATED VESICLES INDUCED BY A 'CRITICAL' FATTY ACID CONCENTRATION

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Summary

Dipalmitoyl phosphatidylcholine vesicles incubated in the presence of increasing amounts of myristic acid showed a progressive translocation of phospholipid molecules across a dialysis membrane. The rate of phospholipid translocation increased abruptly at a 'critical' value of myristic acid concentration. The translocation rate of mixed dipalmitoyl phosphatidylcholine/myristic acid vesicles obtained by cosonicating the two components was also dependent on a 'critical' fatty acid concentration. A marked release of K^+ and different responses of fluorescent probes to the fatty acid addition were observed at this concentration.

Introduction

The membrane interactions occurring between phospholipid vesicles lead to vesicle aggregation [1], phospholipid exchange [1—4] and increase in vesicle size [5—10]. The rates of lipid exchange and of vesicle size enlargement are increased by the presence of fatty acids [6—9]. In recent years, membrane interactions have aroused great interest because they may represent a simple model for investigating important biological processes such as cell-cell recognition, membrane fusion and cell interactions [11—15]. On the other hand, fatty acids having a higher concentration in heart and brain than in other tissues, may play an important role in regulating many biological functions.

However, three problems are still under debate: (1) the rationale of vesicle aggregation, of phospholipid exchange and of vesicle size enlargement; (2) the mechanism of vesicle interaction; (3) the role of fatty acids. In the present and following paper the role of fatty acids in phospholipid exchange and in size

increase of phosphatidylcholine vesicle is investigated. Phospholipid exchange was followed by a new analytical method based on a dialysis technique. By means of this technique the dependence of the aforementioned process on a 'critical' fatty acid concentration is presented. At this 'critical' concentration the membrane structure seems to be modified.

Methods and Materials

Single bilayer vesicles were prepared by exposing a lipid suspension to ultrasonic radiation under a nitrogen atmosphere for 20 min in a medium of 0.1 M KCl/0.01 M Tris-HCl/$2 \cdot 10^{-4}$ M EDTA-Tris pH 7.2, with a Branson sonifier model B30. Temperature was constantly kept above the phase transition temperature. Undispersed phospholipids and titanium particles were removed by centrifugation at $40\,000 \times g$ for 20 min. Phospholipid vesicles were maintained at 4 °C for 48 h.

Dialysis experiments. Experiments were performed in a dialysis jacketed apparatus consisting of two polycarbonate cells, separated by a cellulose dialysis membrane and maintained at constant temperature by a circulating bath. The upper and lower cell volumes, 1 and 2 ml respectively, were magnetically stirred. Dialysis membranes were either Uni-Pore polycarbonate membrane with an average pore diameter of 300 Å, purchased from Bio-Rad Laboratories, or a cellulose membrane with an average pore diameter of 48 Å, purchased from Thomas Apparatus Co. Stretched cellulose membranes were obtained in a controlled apparatus as described by Craig and King [16]. An increase of the average pore diameter was obtained with application of hydrostatic pressure and longitudinal stretching to moisted dialysis tubing.

Kinetics of phospholipid translocation were measured as follows: the cells were filled with the sonication medium, and 2.3 μmol dipalmitoyl phosphatidylcholine vesicles were added to the upper cell. The amount of phospholipid translocated across the dialysis membrane was measured by determining the inorganic phosphate content in the lower cell solution according to Bartlett [17]. The phospholipid translocation rate expressed as nmol of inorganic phosphate/h was estimated from the slope of the straight line obtained by plotting the amount of inorganic phosphate in the lower cell solution vs. the time.

Column chromatography. Analytical sieve chromatography on a Sepharose 4B column was used to determine vesicle size distribution. A sample of 2 ml containing vesicles was applied to a 15×1 cm column previously saturated with phospholipids. Elution was carried out at a rate of 5 ml/h. Fractions of 1 ml were collected and analyzed by absorbance measurements at 280 nm.

K^+ measurements. Measurements of K^+ concentration were obtained by a Beckman K^+ electrode connected to a Beckman pH meter. When K^+ trapped had to be measured, the vesicles were dialyzed at 4 °C against 0.1 M cholinechloride/10^{-2} M Tris-HCl pH 7.2/$2 \cdot 10^{-4}$ M EDTA-Tris for 20 h. The amount of K^+ trapped into the dialyzed vesicles was determined by lysing the vesicles with Triton X-100, according to Block et al. [18]. The final detergent concentration was 0.4% in a solution containing 10^{-3} M phospholipids.

Optical measurements. Fluorescence experiments were performed with a

Hitachi Perkin-Elmer spectrofluorimeter model MPF-2A. Temperature was kept constant by a circulating bath. The medium in the cell was magnetically stirred. Absorbance measurements were performed with a double beam spectrophotometer Perkin-Elmer model 124.

Materials. Diacyl phosphatidylcholines and fatty acids were purchased from Sigma, Koch and Light Laboratories, respectively, and were used without further purification. 8-Anilino-1-naphthalene sulfonic acid (Sigma) was purified according to Dodd and Radda [19]. Pyrene was purchased from Fluka and was used without further purification. [1-^{14}C]myristic acid (Amersham) had a spec. act. for 38 Ci/mol.

Results and Discussion

A. Phospholipid translocation across a dialysis membrane

Myristic acid added to a dipalmitoyl phosphatidylcholine vesicle suspension in the upper cell of a dialysis apparatus induced a phospholipid translocation across a dialysis membrane. Fig. 1 shows the time-dependent change of the amount of lipids in the two dialysis compartments when 5 mol% myristic acid was added to the upper dialysis cell. The increase of the amount of phospholipids recovered in the lower cell was linear during the first 5—7 h. The distribution of fatty acids, measured after 5 h with [1-^{14}C]myristic acid was: 85% in the upper cell; 5% in the lower cell and the residual 10% was bound to the dialysis membrane and cell walls.

Since the dialysis membrane properties may influence the phospholipid translocation rate, dialysis membranes having different chemical structures and average pore diameters have been used. Fig. 2A shows the rate of phospholipid translocation across different dialysis membranes versus the amount of externally added myristic acid. The rate increased abruptly at a 'critical' myristic acid concentration (approx. 3 mol%) and reached a plateau at higher fatty acid concentrations. The values of the translocation rate when the curves reached the 'plateau' were dependent on the dialysis membrane properties.

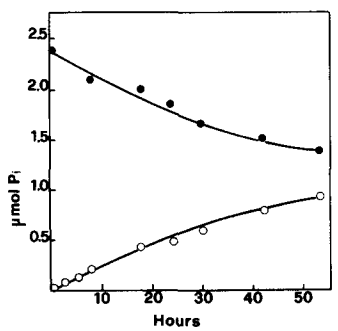

Fig. 1. The kinetics of phospholipid translocation across a dialysis membrane. The amount of phospholipid in the dialysis cells was measured as described in the Methods. 5 mol% myristic acid was added to the upper dialysis cell containing dipalmitoyl phosphatidylcholine vesicle suspension (2.3 µmol P_l) in 0.1 M KCl, 10^{-2} M Tris-HCl and $2 \cdot 10^{-4}$ M EDTA-Tris pH 7.2. ●———●, phospholipid in the upper dialysis cell, ○———○, phospholipid in the lower dialysis cell. Temperature was 50° C. Each experimental point represents a single dialysis experiment. A cellulose dialysis membrane was used.

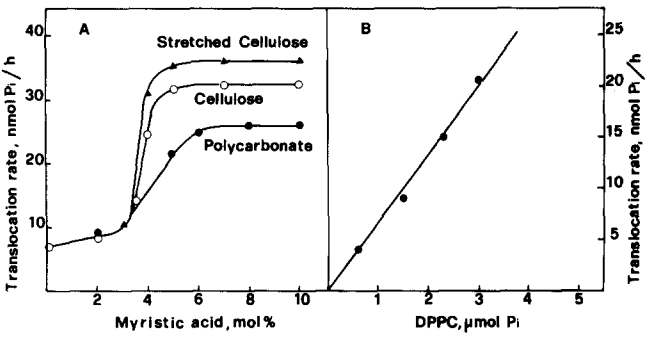

Fig. 2. A. Phospholipid translocation rate across various dialysis membranes as a function of the amount of externally added myristic acid. Variable amounts of myristic acid were added to the upper cell of the dialysis apparatus containing dipalmitoyl phosphatidylcholine vesicle suspension (2.3 μmol P_i). The medium was the same as in Fig. 1. Temperature 50° C. B. Translocation rate dependence on the lipid concentration. Myristic acid 5 mol% was added to the upper dialysis cell containing variable amounts of dipalmitoyl phosphatidylcholine vesicle. The medium was the same as in Fig. 1. Temperature 36° C.

Neither increasing temperature, nor increasing phospholipid and fatty acid concentration were obtained at values higher than those reported in Fig. 2A. Therefore, the rate-limiting step seems to be dependent on the dialysis membrane structure: the pore density of polycarbonate membranes is much lower than that of cellulose membranes, whereas stretched cellulose membranes still maintaining the original pore density, possess a higher average pore diameter. In this paper, dialysis experiments were done on cellulose membranes.

The rate of phospholipid translocation was directly proportional to the vesicle concentration as shown in Fig. 2B. First-order kinetics may be explained by two mechanisms: (a) phospholipid molecules, released from the vesicles, translocated across the dialysis membrane or (2) vesicles collide with the dialysis membrane and release phospholipids. Both mechanisms would explain the abrupt increase in translocation rate when fatty acids are above their 'critical' concentration: either fatty acids facilitate phospholipid release by interacting with the vesicles or, once bound to the dialysis membrane, they induce phospholipid release after collision of the vesicles with the dialysis membrane. As shown in Fig. 3, myristic acid above a 'critical' concentration induces an abrupt increase in translocation rate dependent on the length of the phospholipid chain. In both cases the amount of myristic acid bound to the dialysis membrane remained unchanged. The breaks observed in the translocation curve should therefore be attributed to a property of the vesicle-fatty acid system rather than to the dialysis membrane.

Dipalmitoyl phosphatidylcholine vesicles were equilibrated for 50 h in the presence of 5 mol% myristic acid at 50 °C. Measurements of trapped K^+ in the phospholipid structures in the lower dialysis cell as described in Materials and Methods, indicated that these structures can retain K^+. The solution of the lower dialysis compartment was analyzed by sieve chromatography. Two peaks were obtained in the elution profile; one coincided with the multilayered vesicle peak and the second with the sonified vesicle peak. Similar results were obtained when vesicles were equilibrated for a week at 25 °C in the presence of 10 mol% myristic acid. These results show that phospholipids in the lower

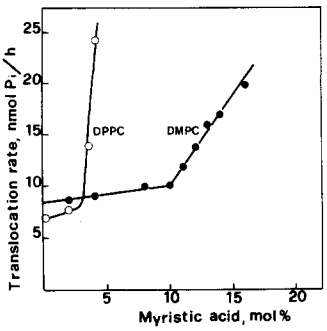

Fig. 3. Phospholipid translocation rate from vesicles with different phosphatidylcholine chain length. Variable amounts of myristic acid were added to the upper cell of the dialysis apparatus containing 2 μmol of diacyl phosphatidylcholine vesicle suspension (3 μmol P_i). The medium was the same as in Fig. 1. Temperature 50°C. Dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC).

dialysis compartment may form small closed bilayer vesicles. Likewise, Brunner et al. [20] by removing sodium cholate from lipid-detergent mixed vesicles, and Kremer et al. [21] by slowly injecting an organic solution of phospholipids at 2 mM concentration, obtained single shelled vesicles of size comparable to sonified vesicles.

B. Effect of fatty acids concentration on vesicle membrane

When myristic acid was cosonicated with dipalmitoyl phosphatidylcholine to form mixed vesicles, the phospholipid translocation rate showed a dependence on the fatty acid concentration similar to that of Fig. 2A. By checking (after sonication at regular time intervals) a single preparation of mixed vesicles maintained at 4°C, the phospholipid translocation rate was found to slow down. A parallel increase of 400 nm absorbance indicated that vesicles were transformed into more extended structures within several hours after sonication. The decrease of the translocation rate might be explained either by the formation of multilamellar vesicles not allowing the participation of the internal bilayers in the translocation process, or by the increase of curvature radius of the vesicles. Therefore experiments were performed immediately after the preparation of the mixed vesicles.

Fig. 4A shows that the abrupt increase of the translocation rate occurred at all temperatures tested, regardless of the fluid or the solid state of the vesicles. Higher 'critical' myristic acid concentrations were required at lower temperatures. Consequently, at fixed fatty acid concentration, the marked increase of the translocation rate might not occur at low temperatures, while at higher temperatures it has already occurred. Therefore, the temperature regulated the 'critical' value of myristic acid necessary for the marked increase of the translocation rate, as shown in Fig. 4B. In conclusion, at fixed temperature the abrupt increase takes place at a 'critical' myristic acid concentration, while at fixed fatty acid concentration it takes place at a 'critical' temperature.

The high myristic acid critical micellar concentration (approx. $6 \cdot 10^{-3}$ M) and the high binding of the fatty acid to the vesicle membrane [9] demonstrate that the fatty acid is present in the solution in low concentration and in

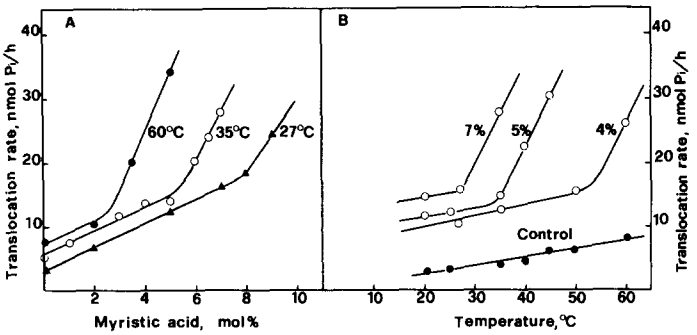

Fig. 4. A. Translocation rate dependence on the amount of externally added myristic acid. Experimental conditions as in Fig. 1. Temperatures as indicated in the figure. B. Translocation rate dependence on the temperature. Experimental conditions as in Fig. 1. The upper dialysis cell contained variable amounts of myristic acid, as indicated in the figure.

monomeric form. Therefore, membrane-bound rather than free fatty acid molecules seem to regulate the release of phospholipids into the solution in our experimental conditions. Above a 'critical' fatty acid concentration the bilayer membrane may be locally destabilized. Structural changes of the membrane were investigated by following the fluorescence response of 8-anilino-1-naphthalene sulfonic acid and pyrene. These probes are confined to the region of phospholipid polar heads and the hydrophobic core of the membrane, respectively. Moreover the permeability properties of the membrane were studied below and above the 'critical' fatty acid concentration.

Fig. 5 shows the fluorescence response of 8-anilino-1-naphthalene sulfonic acid and pyrene when a vesicle suspension is supplemented with myristic acid. The 8-anilino-1-naphthalene sulfonic acid fluorescence decreased owing to a release into the solution of bound 8-anilino-1-naphthalene sulfonic acid molecules, or to an increase of polarity or viscosity of the 8-anilino-1-naphthalene sulfonic acid microenvironment. Electrostatic repulsion between membrane-bound 8-anilino-1-naphthalene sulfonic acid molecules and negatively charged fatty acid molecules should not occur since the apparent pK_a of myristic acid bound to the membrane is approx. 8 [9,22], while the experimental pH is 5. The excimer formation of the pyrene molecule depends on the translational diffusion of the dye molecule and hence on the fluidity properties of the hydrocarbon region of the membrane [23,24]. The excimer/monomer ratio obtained, dividing the fluorescence intensity at 470 nm by the fluorescence intensity at 394 nm (excitation wavelength 340 nm), increases with the fluidity of the microenvironment. Fig. 5 shows that myristic acid induced an increase in the pyrene excimer/monomer ratio. Similar results, obtained by Usher et al. [25] using vesicles in the gel state, were explained by a pyrene clusters formation. The fluorescence response of both 8-anilino-1-naphthalene sulfonic acid and pyrene changed abruptly at the same 'critical' myristic acid concentration causing the increase of phospholipid translocation.

Fig. 6A shows the amount of K^+ trapped in the vesicles at various concentrations of externally added myristic acid after 2 h of incubation. At a 'critical' fatty acid concentration an increased K^+ efflux was observed. The 'critical' fatty acid concentration was again in the same concentration range of that

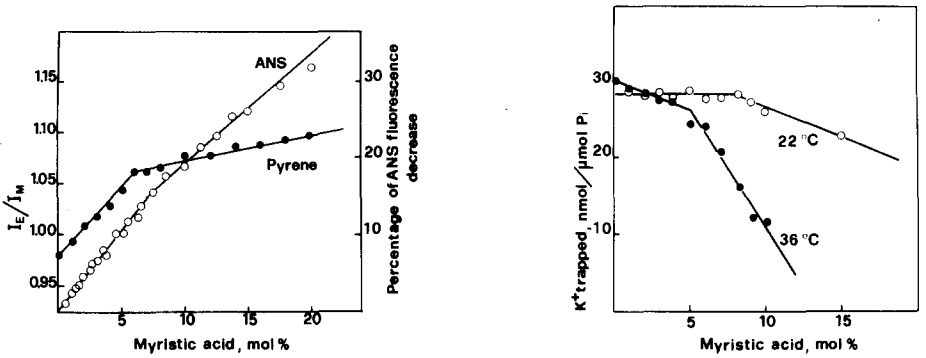

Fig. 5. Fluorescence changes of 8-anilino-1-naphthalene sulfonic acid (ANS) and pyrene induced by fatty acid. The incubation medium contained 0.1 M KCl/10^{-2} M Tris-HCl/dipalmitoyl phosphatidylcholine vesicles (0.5 μmol P_i). $4 \cdot 10^{-5}$ M 8-anilino-1-naphthalene sulfonic acid was added externally to the solution at pH 5. 2.5 mol% pyrene was cosonicated with the lipids and the experiments were performed at pH 7.2. Excitation and emission wavelengths of 8-anilino-1-naphthalene sulfonic acid were 380 and 480 nm, respectively. Excitation wavelength of pyrene was 340 nm. I_E represents the light intensity of emission of the excimer at 470 nm. I_M the light intensity of the emission of the monomer at 394 nm. Temperature 25° C.

Fig. 6. Dependence of the K^+ trapped into the vesicles on the amount of externally added myristic acid. Vesicles obtained by sonication in 0.1 M KCl, were dialyzed overnight at 4° C against a solution containing 0.1 M choline-chloride/10^{-2} M Tris-HCl/2 · 10^{-4} M EDTA-Tris pH 7.2. Dialyzed dipalmitoyl phosphatidylcholine vesicles (1 μmol P_i) were incubated for 2 h at 22 or at 36° C. The trapped K^+ was determined as described in Materials and Methods.

causing the abrupt increase of phospholipid translocation rate.

In conclusion, response of membrane-bound fluorescent probes and large electrolyte permeability changes indicate that myristic acid above a 'critical' concentration, induces structural changes in the membrane. In the next paper [22] these results will be discussed further.

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