Biochimica et Biophysica Acta, 599 (1980) 118—126

BBA 78771

II. PHOSPHOLIPID EXCHANGE AND SIZE ENLARGEMENT IN SONICATED VESICLES INDUCED BY A 'CRITICAL' FATTY ACID CONCENTRATION

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(Received July 10th, 1979)

Key words: Phospholipid exchange; Size enlargement; Fatty acid; (Dipalmitoyl phosphatidylcholine vesicle)

Summary

Size enlargement of dipalmitoyl phosphatidylcholine vesicles was greatly accelerated in the range of the phase-transition temperatures, when fatty acid concentration was above a threshold level ('critical' concentration). This 'critical' concentration varied with the length of the fatty acid chain. The size enlargement process had second-order kinetics dependent on the vesicle concentration. Alkaline pH and low ionic strength inhibited the rate of size enlargement.

Phospholipid exchange between dimyristoyl and dipalmitoyl phosphatidylcholine vesicles increased abruptly above a 'critical' fatty acid concentration. The donor vesicles were those vesicles in which fatty acids reached the 'critical' concentration. The phospholipid exchange occurred both in fluid- and in solid-state vesicles. The 'critical' fatty acid concentration accelerating the phospholipid exchange process was lower than that accelerating the size enlargement process.

The phospholipid exchange process explained in terms of a diminished hydrophobic attraction among the phospholipid molecules of the bilayer occurs via a free phospholipid molecule transfer through the aqueous phase. The size enlargement process is interpreted in terms of high fatty acid concentration in the membrane fluid domains. The membrane structure is locally perturbed inducing vesicle sticking after collision.

Introduction

The size increase of sonicated phospholipid vesicles and the phospholipid exchange between vesicles of different lipid composition were studied using

NMR [1—3], calorimetry [4,5] and light-scattering techniques [6,9]. No conclusions were drawn about the mechanism of size enlargment, the molecular interaction between fatty acids and vesicles, or the mechanism of phospholipid exchange.

As reported in the preceding paper [10], above a 'critical' concentration, myristic acid causes an abrupt increase in phospholipid release from dipalmitoyl phosphatidylcholine vesicles. In the present paper the fatty acid concentration was investigated in relation to the increase of vesicle size and the phospholipid exchange. Both processes were strongly enhanced when fatty acid was above a 'critical' concentration. This concentration was different for the two processes. Phospholipid exchange may be interpreted as a diminished hydrophobic attraction among phospholipid molecules. Vesicle size enlargement may be interpreted as a local perturbation of the bilayer structure, induced by a high fatty acid concentration in the fluid domains of the membrane. These local perturbations may induce vesicle sticking after collision.

Methods

Phospholipid vesicle preparations, dialysis and sieve chromatography were performed as described in the preceding paper [10]. Light-scattering experiments were performed with a Hitachi Perkin-Elmer spectrofluorimeter model MPF-2A at 400 nm excitation and emission wavelengths. The kinetics of vesicle size enlargement were obtained by recording the light-scattering increase. The initial rate was graphically extrapolated from recorder traces. The rate of size enlargement was evaluated from the initial rate by calculating the light-scattering change, ΔI, in 10 min. The vesicle phase transition experiments were obtained by recording the intensity of the light scattered at an angle of 90° as a function of the temperature (temperature change 1°C/min). These experiments were performed by cooling the sample from 50 to 10°C.

Results

A. Dipalmitoyl phosphatidylcholine vesicle size enlargement

Size enlargement of sonicated vesicles was observed only in the range of the phase transition temperature [1,2,4,7]. Fatty acid increased the rate of this transformation process [2,5,7]. In the absence of fatty acids, size enlargement was found to be practically negligible within several hours in our experimental conditions. Above a fatty acid 'critical' concentration the rate of size enlargement, measured by the light-scattering intensity changes, increased markedly with biphasic kinetics. Fig. 1A shows that the maximum rate of size enlargement in dipalmitoyl phosphatidylcholine vesicles occurred at 37—38°C, 2°C below the critical temperature of the sonicated vesicle suspension (superimposed curve of Fig. 1A). The temperature at which the maximum rate occurred did not depend on increasing amounts of fatty acid. The size enlarged vesicles were stable even when subjected to cooling and warming treatment, or when eluted from a Sepharose 4B chromatography column. Fig. 1B shows that the 'critical' fatty acid concentrations causing the abrupt increase of the phospholipid translocation rate across the dialysis membrane and the increase of the light-scattering response were different.

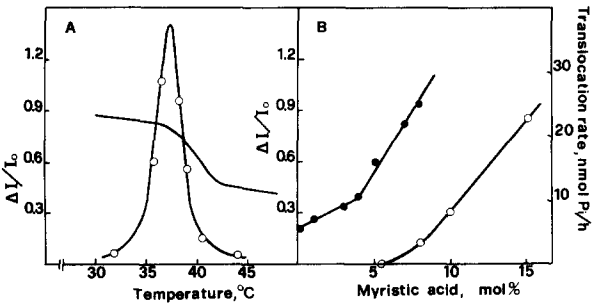

Fig. 1. A. Dependence of the light-scattering increase on the temperature. The medium contained 0.1 M KCl/10^{-2} M Tris-HCl pH 7.2/1 μmol P_i of dipalmitoyl phosphatidylcholine vesicles. The system was allowed to equilibrate 10 min, and the reaction started after addition of 20% myristic acid. In the figure the phase transition is also shown, in arbitrary units of light intensity. B. Light scattering increase and phospholipid translocation rate at various myristic acid concentrations. The medium was the same as in Fig. 1A. In the dialysis experiments (●———●) the amount of vesicles was 2 μmol P_i and the temperature was 36° C; in the light-scattering experiments (○———○) the amount of vesicles was 0.5 μmol P_i and the temperature was 38° C. ΔI represents the light intensity increase during the first 10 min taken from the initial slope of the kinetics. I_0 represents the light intensity at time zero. Excitation and emission wavelength was 400 nm.

In Fig. 2 the fatty acid dependence of the lipid translocation rate across the dialysis membrane and the light-scattering response are compared. The translocation rate at 50°C and the size enlargement at 38°C, were dependent on the fatty acid concentration regardless of the fatty acid used. The 'critical' fatty acid concentration, inducing the abrupt increase of both translocation rate and size enlargment was influenced by fatty acid acyl-chain length. A short-chain fatty acid such as lauric acid had no effect on the light-scattering response or

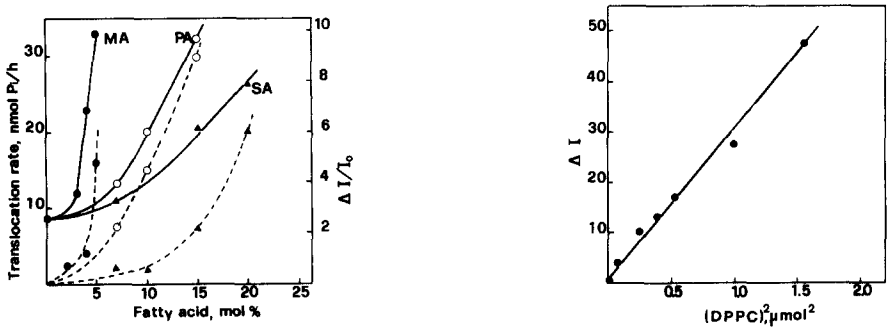

Fig. 2. Effect of fatty acid with different chain length on the phospholipid translocation rate and on the light-scattering increase. The medium was the same as in Fig. 1. The solid lines represent the phospholipid translocation rate across the dialysis membrane. The upper dialysis cell contained 2.3 μmol P_i of mixed vesicles composed of dipalmitoyl phosphatidylcholine cosonicated with variable amounts of fatty acids. Temperature was 50° C. The dashed lines represent the light-scattering increase. The amount of mixed vesicles was 1 μmol P_i. Temperature was 38° C. All experiments were performed immediately after the preparation of vesicles. ●———●, MA, myristic acid; ○———○, PA, palmitic acid; ▲———▲, SA, stearic acid.

Fig. 3. Kinetics of vesicle size enlargement. The medium was the same as in Fig. 1. Incubation temperature was 38° C. The system was allowed to equilibrate 10 min and the reaction started after addition of 20% myristic acid. DPPC, dipalmitoyl phosphatidylcholine.

on the phospholipid translocation rate. Fig. 3 shows that the rate of light-scattering response increased following second-order kinetics. A linear relationship with lipid concentration of the ratio $\Delta I/I_0$ was found, in agreement with Kremer and Wiersema [7]. Kantor and Prestegard [3] by measuring the size enlargement of dimyristoyl phosphatidylcholine vesicles containing myristic acid with the NMR technique found first-order kinetics. However, it is difficult to measure the early stages of the size enlargment process with the NMR technique.

The second-order kinetics of Fig. 3 would strongly suggest a collision process. This process is likewise expected to be dependent on the vesicle surface potential and consequently on pH and ionic strength. In fact, at alkaline pH the membrane-bound fatty acid molecules are partially dissociated, hence increasing the electrostatic repulsion between phosphatidylcholine vesicles [11], provided that the ionic strength is kept low. Fig. 4A shows that an alkaline pH inhibited the rate of size enlargement. The vesicles were incubated at neutral pH with externally added myristic acid and the light-scattering change was measured after alkalinization of the medium. Inhibition was also observed in phospholipid-fatty acid cosonicated vesicles. The rate decreased by 50% at pH 7.8, this being the value closest to the value of the apparent pK_a of myristic acid in egg lecithin vesicles [3]. Fig. 4B shows that by increasing the KCl concentration, the rate of size enlargement at pH 5 is increased. The influence of the surface potential upon the size enlargement was confirmed by the following experiments (data not shown). (1) Addition of KCl or $CaCl_2$ at pH 9 induced a progressive increase of the rate of size enlargement. (2) Phosphatidylcholine vesicles containing 2 mol% phosphatidylserine did not show size changes. (3) At pH below 3.5, the rate of size enlargement was inhibited in fact, at this pH the phosphatidylcholine molecules become positively charged. (4) Addition of low concentrations (10 mol%) of myristic acid at pH 7.6 enhanced the rate of size enlargement, whereas higher concentrations inhibited size enlargement. The progressive binding of dissociated fatty acids

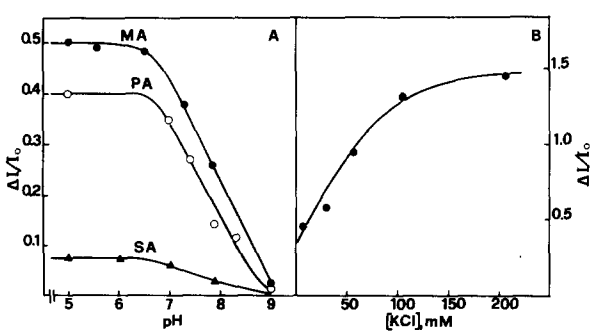

Fig. 4. Dependence of the light-scattering increase on the pH and KCl concentration. A. The medium contained 0.1 M KCl and 10^{-2} M Tris-acetate. Temperature was 38°C. 0.5 μmol P_i of vesicles were incubated with 20 mol% externally added myristic acid at pH 7 for 2 min. Then the pH was changed to the final value by addition of HCl or NaOH, and the light-scattering change was measured. B. The medium was 10^{-2} M Tris-HCl pH 7.2. Temperature was 37°C. 1 μmol P_i of vesicles were incubated with 20 mol% externally added myristic acid at various KCl concentrations. MA, myristic acid; PA, palmitic acid; SA, stearic acid.

presumably inhibited the size enlargement process due to an increase of membrane negative charges.

In myristic acid-phospholipid cosonicated vesicles the fatty acid is distributed between the two membrane layers. The fatty acid molecules localized in the external layer can be removed by adding multilayered dipalmitoyl phosphatidylcholine liposomes and spinning down the liposomes. The remaining myristic acid (measured with [^{14}C]-myristic acid) is probably localized in the internal layer. In fact, vesicles did not show any size increase and progressive additions of fatty acid restored the capacity of size enlargement. Externally bound fatty acid is necessary for the size transformation either because it increases the vesicles sticking properties or because it restricts the membrane perturbation to the external layer. Furthermore local anesthetics, at concentrations of 10^{-4}—10^{-3} M, strongly inhibited the rate of size increase (data not shown). The inhibition decreased as follows: nupercaine > butacaine > benzocaine > phenocaine > procaine. On the other hand positive hydrophobic molecules such as nonyltrimethyl ammonium increased the rate of size enlargment.

B. Phospholipid exchange

Fig. 5A shows the change of the scattered light intensity as a function of the temperature for an equimolar mixture of dimyristoyl phosphatidylcholine vesicles and dipalmitoyl phosphatidylcholine vesicles. ΔA in the figure indicates the amplitude of the light intensity change at the lipid phase transition. ΔA was found to be proportional to the phospholipid concentration up to 0.5 μmol P_i.

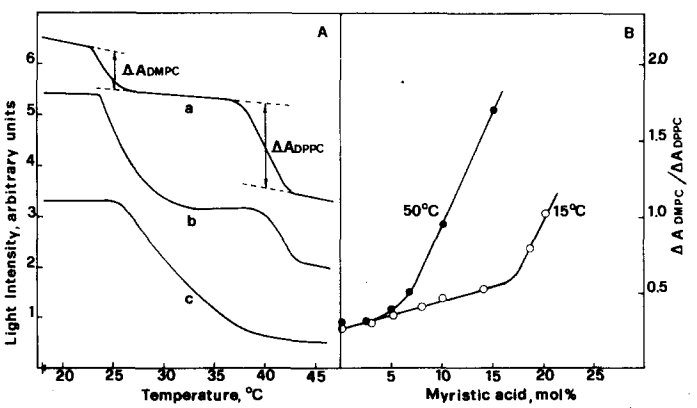

Fig. 5. A. Lipid phase transition curves of an equimolar mixture of dipalmitoyl and dimyristoyl phosphatidylcholine vesicles in the presence of myristic acid. The medium was the same as in Fig. 1. Curve a, transition curve of a mixture containing 0.25 μmol P_i of dipalmitoyl phosphatidylcholine (DPPC) vesicles and 0.25 μmol P_i of dimyristoyl phosphatidylcholine (DMPC) vesicles. Curve b, transition curve of the previous mixture incubated 30 min at 50°C in the presence of 10 mol% myristic acid. Curve c, transition curve of the same mixture incubated 90 min at 50°C in the presence of 10 mol% myristic acid. B. Effect of myristic acid on the amplitude of the lipid phase transition changes. The experimental points were calculated from the lipid phase transition curves of the mixtures used in Fig. 5A incubated 30 min at 50°C or 120 min at 15°C, at various concentrations of myristic acid. $\Delta A_{DMPC}/\Delta A_{DPPC}$ represents the ratio between the light-scattering intensity change of the lipid phase transition of dimyristoyl phosphatidylcholine vesicles and of the lipid phase transition of dipalmitoyl phosphatidylcholine vesicles as calculated in the curve a of Fig. 5A.

The ΔA of the dimyristoyl phosphatidylcholine vesicles increased, whereas the ΔA of dipalmitoyl phosphatidylcholine vesicles decreased by incubating the mixture of the two vesicles for 30 min at 50°C in the presence of 10 mol% of myristic acid (curve b). Therefore, myristic acid seems to promote an increased incorporation of dipalmitoyl phosphatidylcholine molecules in dimyristoyl phosphatidylcholine vesicles. In the absence of myristic acid (curve a) the transition temperatures were 24 and 40.2°C for dimyristoyl phosphatidylcholine vesicles and dipalmitoyl phosphatidylcholine vesicles, respectively. In the presence of 10 mol% of myristic acid, both transition temperatures were slightly increased. When the mixture of the two vesicles was incubated at 50°C, the transition temperature of dimyristoyl phosphatidylcholine vesicles progressively increased with incubation time, whereas the transition temperature of dipalmitoylphosphatidylcholine vesicles remained constant. The increase of the transition temperature of dimyristoyl phosphatidylcholine veiscles was probably due to an increased incorporation of dipalmitoyl phosphatidylcholine molecules into the membrane, while the increase of the transition temperature of dipalmitoyl phosphatidylcholine vesicles was probably due to a phospholipid immobilization induced by myristic acid [12,13]. The transition temperature changes are interpreted in terms of an unidirectional transfer of dipalmitoyl phosphatidylcholine molecules from dipalmitoyl phosphatidylcholine to dimyristoyl phosphatidylcholine vesicles. Within 2 h the transition curve of the mixture of the two vesicles overlapped with the transition curve of a suspension of 1 : 1 dimyristoyl-dipalmitoyl phosphatidylcholine cosonicated vesicles (curve c). With regard to the phospholipid translocation rate, 10 mol% of myristic acid was above the 'critical' concentration for dipalmitoyl phosphatidylcholine vesicles, but below the 'critical' concentration for dimyristoyl phosphatidylcholine vesicles [10]. Therefore, the fatty acid may induce a release of phospholipid molecules more pronounced in dipalmitoyl phosphatidylcholine than in dimyristoyl phosphatidylcholine vesicles. Papahadjopoulos et al. [4] by using differential scanning calorimetry, showed that myristic acid caused a gradual shift of the two endothermic peaks toward intermediate temperatures. The discrepancy between our results and those of Papahadjopoulos [4] may be explained by the presence of large multilayered dipalmitoyl phosphatidylcholine vesicles consequent to the high lipid concentration used by these authors in the course of sonication. In multilayered vesicles the surface available for lipid exchange is smaller than in monolayered vesicles having the same phospholipid concentration. Therefore, the rate of phospholipid release from multilayered dipalmitoyl phosphatidylcholine vesicles is comparable to the rate of phospholipid release from dimyristoyl phosphatidylcholine vesicles.

Lipid exchange between the two vesicle populations is analyzed in terms of changes of the ratio between the amplitudes of the light intensity change at the lipid phase transition ($\Delta A_{DMPC}/\Delta A_{DPPC}$). This ratio is dependent on myristic acid concentration, as shown in Fig. 5B. When the vesicles were incubated at 50°C for 30 min or at 15°C for 120 min, the lipid exchange abruptly increased at a 'critical' fatty acid concentration. Therefore, the lipid exchange occurs not only in fluid-state vesicles as previously reported [4,6—8,12], but also in solid-state vesicles.

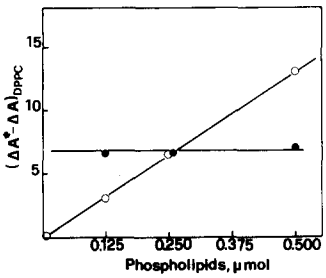

Fig. 6. Rate of disappearance of phospholipid molecules from donor vesicles. The medium was the same as in Fig. 1. The vesicle mixture was incubated 30 min at 50°C in the presence of 10 mol% of myristic acid. ○———○, the amount of dimyristoyl phosphatidylcholine vesicles was 0.25 μmol P_i, the amount of dipalmitoyl phosphatidylcholine vesicles varied as shown in the abscissa. ●———●, the amount of dipalmitoyl phosphatidylcholine vesicles was 0.25 μmol P_i, the amount of dimyristoyl phosphatidylcholine vesicles varied as shown in abscissa. ΔA^* and ΔA are the amplitudes of the light-scattering changes at the phase transition of dipalmitoyl phosphatidylcholine vesicles in the absence (ΔA^*) and presence (ΔA) of myristic acid.

Lipid exchange may occur: (1) by free lipid molecule diffusion [6,14]; (2) by vesicle collision [8]; (3) by vesicle merging [3]. In the collision or merging mechanisms the rate of phospholipid exchange is expected to show a dependence on the concentration of both donor and acceptor vesicles. In the case of diffusion of free molecules, the rate-limiting step in the process is presumably the release of phospholipids from donor vesicles. Actually, in the absence of fatty acids a negligible release of phospholipid from vesicles is measured by the dialysis method. We might conclude that, in the presence of a 'critical' concentration of fatty acids the rate of phospholipid exchange depends on the concentration of donor vesicles, and not on the concentration of acceptor vesicles. The rate of disappearance of dipalmitoyl phosphatidylcholine molecules from donor vesicles, measured as ΔA^* (myristic acid absent) minus ΔA (myristic acid present), is given in Fig. 6. When the concentration of acceptor vesicles was kept constant, the rate of disappearance increased linearly with the concentration of donor vesicles. When the concentration of donor vesicles was kept constant and the concentration of acceptor vesicles was variable, the rate of disappearance was constant. Furthermore, in the collision or merging mechanisms the rate of phospholipid exchange should decrease with the increasing amount of negatively charged phospholipids in the membrane and it should remain constant in phospholipid diffusion mechanism. In agreement with the results obtained with other negatively charged vesicles [4,14], vesicles containing 2 mol% of phosphatidylserine, although not showing any size enlargement at 38°C, fully retained their capacity for lipid exchange. These experiments pointed out that the lipid exchange process presumably occurs by diffusion of free lipid molecules. Kremer et al. [8], by measuring the decrease of the scattered light as a function of the total lipid concentration, found second-order kinetics and they concluded that the lipid exchange occurred through collision between vesicles of unequal radii. However, the decrease of the scattered light intensity is related to an average variation in size of the vesicle populations, that is only a part of the lipid exchange process.

Discussion

In sonicated vesicles formed by chain-saturated lecithins, phospholipid exchange, size enlargement and fusion occur spontaneously at a very slow rate. Fatty acids above a threshold concentration induce a marked increase in the rate of these processes. Low concentrations, 3—5 mol%, of fatty acid, are proper for inducing vesicle transformation processes. On the other hand, relatively high concentrations of fatty acids have been found in heart, and brain and, consequent to phospholipid turnover, in many other cells. Fatty acids may therefore trigger important biological reactions in the cell membrane, related to cell-cell adhesion and recognition, fusion and changes in the permeability properties. These reactions may be also controlled by proteins, Ca^+, different classes of phospholipid and regions of different curvature in the membrane. The interaction mechanism of fatty acid at critical concentration with membrane phospholipid is briefly examined.

(1) Vesicle size enlargement. Solid and fluid domains coexist in the vesicle membrane when the temperature is in the range of the solid-fluid phase transition, the dimension and the number of these domains being dependent on the temperature itself [15]. Fatty acids distribute preferentially in the fluid domains [16,17] and they could modify the bilayer structure of these domains when they reach a 'critical' concentration. In fact, calorimetric and light-scattering experiments indicate that fatty acids, above a threshold concentration, induce a destabilization of the membrane structure [7,13]. In the destabilized areas micellar-like structures may be formed by fatty acids, increasing the probability of vesicle sticking after collision. At the temperature 2—3 °C below the vesicle transition temperature, where the maximum rate of size enlargement occurs, the number and the dimension of the fluid domains permit the highest local concentration of fatty acid. The inhibitory effect of local anesthetics strongly supports the latter hypothesis. Local anesthetics, by increasing membrane fluidity [18,19], allow a more homogeneous partitioning of fatty acids on the bilayer membrane and they decrease the fatty acid concentration in the fluid domains. In agreement with Kantor and Prestegard [3], separate phases of distinct fatty acid-phospholipid composition are involved in promoting vesicle transformation. However, the fatty acid lateral separation hypothesis may be replaced by assuming that fatty acids localize preferentially in fluid rather than in solid membrane domains.

(2) Phospholipid exchange. Fatty acids above a 'critical' concentration greatly accelerate the rate of translocation of phospholipids across a dialysis membrane and the rate of phospholipid exchange between vesicles. Fluorescent probes and permeability changes of the membrane indicate that the bilayer structure is perturbed when fatty acids are above their 'critical' concentration. The data presently available in literature bring out two tentative hypotheses about the real nature of the structural changes occurring in the membrane. According to Hauser et al. [20] fatty acids appear to be clustered within the plane of the bilayer. Podo and Blasie [21] on the other hand, proposed that fatty acids induce a chain shortening of phospholipid molecules compensated for by a local thinning of the membrane. In our view both phenomena could occur only when fatty acids reach a 'critical' concentration. A decrease of the

hydrophobic interaction between phospholipid molecules may take place either at the boundary of the fatty acid clusters or as a consequence of the membrane thinning which reduces the area occupied by the hydrocarbon-chain. The consequent diminished attraction among phospholipid molecules and an increase of water solubility of the phosphatidylcholine-fatty acid complex may considerably increase the concentration of phospholipid molecules or phospholipid-fatty acid complexes free in the aqueous phase.

Acknowledgement

The authors would like to thank Mr. B. Ziche for his technical assistance.

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