Biochimica et Biophysica Acta, 941 (1988) 264–270
Elsevier

BBA 70559

BBA Report

Osmotic dependence of the lysophosphatidylcholine lytic action on liposomes in the gel state

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(Received 12 January 1988)

Key words: Liposome; Lysophosphatidylcholine; Osmolarity; Gel state; Critical lytic concentration; Critical micellar concentration

Multilamellar liposomes of dimyristoylphosphatidylcholine are susceptible to lytic action of lysophosphatidylcholine at the gel state, an effect which is not observed when liposomes are in the liquid crystalline state. The lytic action has been found to be enhanced when liposomes are dispersed in hypertonic solutions. On the contrary, hypotonic solutions decreased the effectiveness of the lysolipid. Shrunken liposomes present surface changes as detected by merocyanine 540 and 1-anilinonaphthalene-8-sulfonic acid which can be ascribed to the spontaneous curvature promoted by shrinkage.

It is known that lysophosphatidylcholines can bind to natural and model lipid membranes inducing morphological changes, cell aggregation, membrane fusion and lysis [1–10]. However, it is unknown whether some of these effects, particularly the lytic actions, are due to monomer or to micelle lysolipid interactions with the membrane and how this effect depends on the gel or liquid crystalline state of the membrane structure. Regardless the degree of fluidity, hydration and packing of bilayers is different in the gel and in the liquid crystalline state, very few attempts have been performed to relate those features to the lysolipid lytic action [6,8]. Hence, in order to get some insight into these problems, we have studied the lytic effect of monomyristoyllysophosphatidylcholine (lyso-PC) on multilamellar liposomes in the gel and in the liquid crystalline state. In addition, the effectiveness of the lysolipid was tested on liposomes suspended either in hypertonic or hypotonic solutions. On such conditions, the surface changes occurred on the liposome surface, monitored by optical and fluorescent probes, significantly affected the lytic effect of lysoPC.

Materials and methods. Dimyristoylphosphatidylcholine (DMPC) and lysoderivates were obtained from Avanti Polar Lipids Inc. 1-Anilinenaphthalene-8-sulfonic acid (ANS), octadecylrhodamine (o-Rh) and merocyanine 540 (MC540) were purchased from Molecular Probes. The purity of the lipids was checked by thin-layer chromatography.

All other chemical were of analytical grade and used without further purification. Water was twice distilled in a standard Milli Q equipment.

Abbreviations: DMPC, dimyristoylphosphatidylcholine; lyso-PC, monomyristoyllyso-phosphatidylcholine; o-Rh, octadecylrhodamine; ANS, 1-anilinonaphthalene-8-sulfonic acid; clc, critical lytic concentration; cmc, critical micellar concentration.

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Liposomes were prepared by dispersing, above the transition temperature, a film of phospholipids in 10 mM Tris-HCl buffer, at pH 7.4. This film of phospholipids was obtained by evaporating a chloroform solution under vacuum at 40 °C [15].

For trapping measurement, lipids were dispersed in buffer solutions containing nicotinamide adenine dinucleotide (NADH). The liposomes obtained on these conditions were centrifuged during 30 min at 4 °C and $33000 \times g$. The pellet was resuspended in a new buffer, without NADH, and centrifuged repeatedly until no NADH was detected in the supernatant. The presence of non-trapped NADH was controlled by UV analysis at 340 nm. The total trapping was obtained by the addition of 100 μl of a 10% Triton X-100 solution to the liposome dispersion.

The changes produced in the sample turbidity by the addition of lysoPC aliquots, were monitored in a double beam Hitachi 100-60 spectrophotometer below and above the transition temperature. Temperature was controlled by a thermostatic bath connected to the sample cuvette-holder, with a precision of ± 0.5 C°. In Fig. 1A each value represents the relative decrease of the turbidity produced by the lysolipid with respect to the absorbance of an untreated sample. After the addition of each lysolipid aliquot to the liposome dispersion, the mixture was incubated until a constant absorbance value was obtained.

All the assays below the transition temperature of DMPC (21 °C) were performed at 18 °C.

The critical micellar concentration of lysoPC (mono-14 acyl derivative) was determined in a liposome-free solution, by measuring the changes produced in the fluorescence of an ANS solution ($3.3 \cdot 10^{-5}$ M) as a function on the amount of LPC added. Swollen liposomes were obtained by dispersing liposomes containing 0.5 M KCl in KCl solutions of decreasing osmolarities as indicated in the text and figures. Shrunken liposomes were obtained by dispersing liposomes prepared in Tris-HCl buffer 10 mM, pH 7 in sucrose or KCl solutions of increasing osmolarities. The osmolarities of sucrose and KCl solutions were calculated using standard tables [11]. The values for the lysoPC critical lytic concentration (clc) were obtained from the intersection of the two-dotted lines indicated in Fig. 1A or 1B. In all cases, the lines were obtained by the least-squares method including all the points up to 0.04 mM lysoPC concentration. The assays with octadecylrhodamine were performed using liposomes prepared dispersing the film obtained by the evaporation of a chloroform solution containing octadecylrhodamine in a ratio 8% (mol/mol). On these conditions, the fluorescence is self-quenched [12,13]. Therefore, an increase in the fluorescence measured at 590 nm is expected after decreasing the octadecylrhodamine/lipid ratio. This break in the curve of the absorbance or fluorescence values plotted as a function of the lysolipid concentration, is an useful tool to measure the lytic effect in terms of the osmotic stress. A sufficient number of points below and above the intersections can be cautiously obtained and the clc can operationally be defined in a similar way as the cmc. As it will be discussed below, the clc must be considered as a range of lysolipid concentration in which a phase change can occur. It may also be considered as the point at which micelles predominate on liposomes in the overall population. However, as it also occurs in the definition of cmc [14] with monomers and micelles, liposomes and micelles may coexist around the clc point.

The MC540 experiments were performed using an Hitachi double-beam spectrophotometer, measuring the ratio of the peaks obtained at 570 and 500 nm, which indicates the partition degree of the probe within the lipid and the water phase, respectively [14]. The ANS experiments were performed using the above-mentioned fluorometer.

In both cases, the assays were done as follows. Liposomes prepared in Tris buffer were dispersed in sucrose or KCl of increasing osmolarities. Then, a merocyanine or ANS aliquot solution was added and the spectra were run.

Results. Fig. 1A shows the changes induced by lysoPC on the absorbance of a DMPC liposome dispersion, in experiments performed below and above the DMPC gel-liquid crystalline transition temperature. At the concentration range studied, lysoPC did not produce any detectable change in the parameter when tested above the DMPC transition temperature. Conversely, the addition of lysoPC to a liposome dispersion in the gel state causes, at a given concentration, a gradual decrease of the absorbance values (Fig. 1). It must

be stressed that this decrease of absorbance corresponds to the increase of octadecylrhodamine fluorescence. These dilution assays of a self-quenched membrane probe, indicate that absorbance changes are linked to a mixture of lipids. Operationally, the critical concentration at which these processes occur can be defined as the intersection of the two lines depicted in Fig. 1. Comparable clc values were obtained by absorbance and when lysoPC was added either to a sample containing two populations of liposomes, one with and another without octadecylrhodamine or to a dispersion with one population of liposomes labelled with the fluorophore (Fig. 1B).

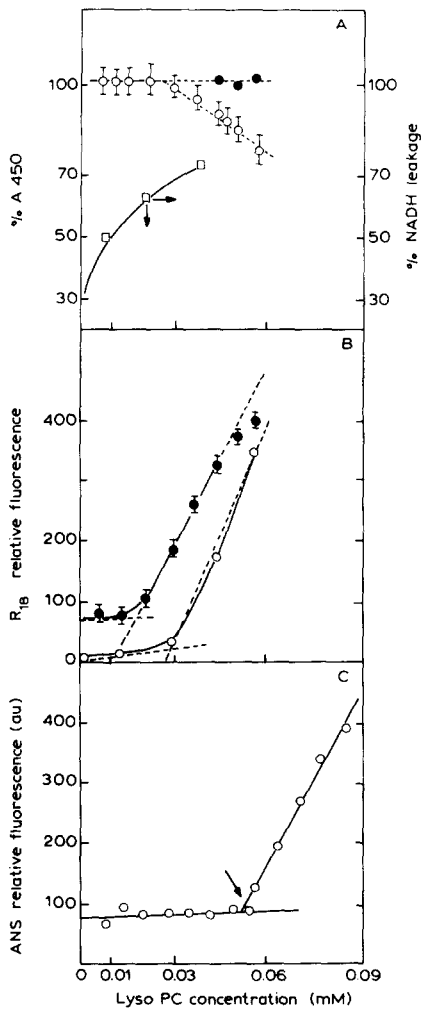

Fig. 1. Effect of monomyristoyllysophosphatidylcholine (lysoPC) on dimiristoylphosphatidylcholine liposomes. (A) Percentage of absorbance change of liposome dispersion above (●·····●) and below (○·····○) their gel-liquid crystalline transition temperature as a function of the lysoPC concentration in the medium. (□) indicates the percentage of NADH leakage from liposomes in the gel state after each lysoPC addition. (B) Changes induced by lysoPC in the relative fluorescence intensity of an homogeneous mixture of liposomes labelled with o-Rh (●) and on another dispersion constituted by two populations of liposomes one with an another without o-Rh in their membrane (○). (C) Critical micellar concentration (cmc) of lysoPC as measured by the fluorescence of an ANS solution. The arrow corresponds to a cmc value of $54 \cdot 10^{-6}$ M (See Table I and Materials and Methods).

TABLE I

EFFECT OF DIFFERENT OSMOLARITIES ON THE CRITICAL MICELLAR CONCENTRATION OF 14:0-LYSOPHOSPHATIDYLCHOLINE

	Buffer Tris (10 mM)	Sucrose (700 mM)	KCl (500 mM)
cmc (mM)	0.050	0.039	0.028

The critical micellar concentration of lysoPC, estimated as explained in Materials and Methods was obtained at the different osmolarities in sucrose, KCl and buffer Tris solutions (Fig. 1C and Table I). Different osmolarities affect the critical micellar concentration of the 14 : 0 lysophosphatidylcholine in the following order: buffer 10 mM Tris-HCl (pH 7), 0.050 mM; 0.7 M sucrose, 0.039 mM and 0.5 M KCl, 0.028 mM. Since in the experiments represented in Figs. 1A and 1B, the clc of lysoPC was below such concentrations ($\leqslant$ 0.020 mM), this compound would exert its lytic effect when in the monomeric form.

Another consequence of the lysoPC addition was the release of trapped NADH. This release took place only when liposomes were in the gel state. However, in this case, a 50% release was obtained at lower lysoPC concentration compared to those found using either fluorescence or absorbance methods. Such discrepancy could be due to an incorporation of the lysocompound to the liposomes, followed by a change in the barrier properties, without the disruption of the liposomes. Another possibility could be a NADH leakage, occurring in a liposome population composed of smaller particles which can not be detected by the optical methods. As it is well-known the coarse lipid dispersion has a wide size distribu-

TABLE II

VALUES OF THE CRITICAL LYTIC CONCENTRATION OF LYSOPHOSPHATIDYLCHOLINE ON DIMIRYSTOYLPHOSPHATIDYLCHOLINE LIPOSOMES IN THE GEL STATE OF DIFFERENT VOLUMES [a]

Volume (cm^3/mol)	2	3	11	10^4
clc (mM)	0.015	0.020	0.021	0.025

[a] Taken according to Bangham et al. [15] as a linear function of the inverse of the impermeant concentration in the outer solution.

tion [15,16]. Thus, the lysis of the smaller liposomes would liberate the NADH with a negligible contribution to the turbidity change. As it has been reported elsewhere, the resistance of bimolecular lipid membrane was significantly lowered by the introduction of lysoPC in the film forming solution [17]. These results suggest that, in addition to an increase in permeability, the liposome may also burst easily. The osmotic decompensation would earlier affect the smaller liposomes rather than the larger ones. In order to avoid bursting contributions to this complex phenomenon, the following experiments were carried out on liposomes dispersed in hypertonic solutions.

In this way, no burst by swelling should be expected and the mean size distribution of the liposome population would be displaced to lower values. According to Bangham the volume of the liposomes is directly proportional to the inverse of the impermeant concentration in the outside solution [15]. As can be observed in Table II a higher lysolipid concentration is necessary to achieve lysis when the volume of the liposome increases. Therefore, the clc values obtained by increasing the solution osmolarity would be related to the variation in the liposome size.

Fig. 2 shows that clc measured as in Fig. 1A, depends on the osmotic state of the liposomes. In the gel state, they became less resistant to the lysoPC lytic effect when dispersed in hypertonic solutions. The opposite effect was found when the liposomes were suspended in an hypotonic solution (Fig. 2A).

Figs. 2B and 2C show that the enhancement of the lysoPC lytic effect produced by the liposome shrinkage, is not significantly affected by the agent employed to modify the osmotic pressure of the suspension media.

Under these osmotic conditions, the surface properties of the shrunken liposomes were tested with merocyanine and 1-anilinonaphthalene-8-sulfonic acid. These probes are useful to detect the hydrophobicity of the membrane interface [14] because the A_{570}/A_{500} ratio and the fluorescence, respectively, increases when passing from the gel to the liquid crystalline state.

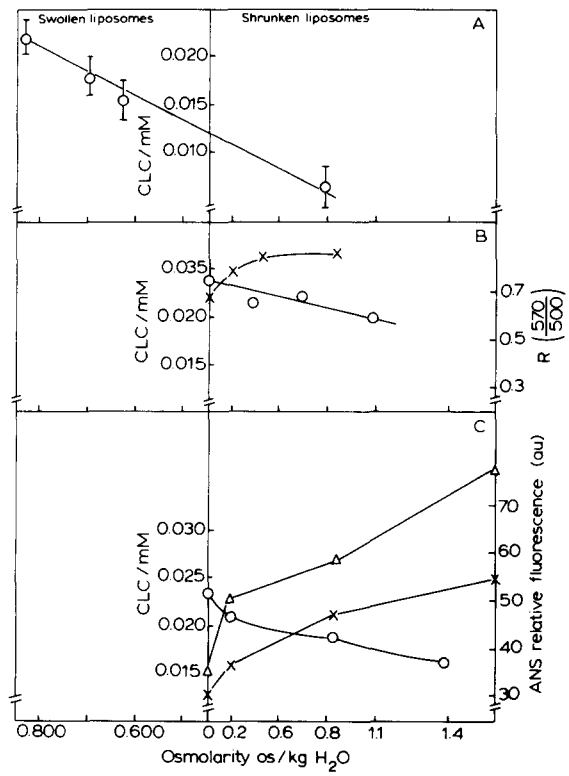

Fig. 2. Effect of the lysoPC action on DMPC liposomes in the gel state as a function of the external osmolarity. (A) lysoPC-critical lytic concentration of DMPC liposomes containing 0.1 M KCl dispersed in solutions of different osmolarities. (B) lysoPC-critical lytic concentration (○) and absorbance ratio (570/500 nm) obtained from MC540 spectra (x) for liposomes prepared in Tris-HCl buffer 10 mM at pH 7 as a function of the sucrose osmolarity of the external solution. (C) LysoPC critical lytic concentration (○) and relative fluorescence intensity of ANS obtained with DMPC liposomes dispersion of different concentrations prepared in Tris-HCl buffer 10 mm at pH 7, as a function of the KCl osmolarity of the external solution (0.13 mg/ml (△) or 0.09 mg/ml (x).

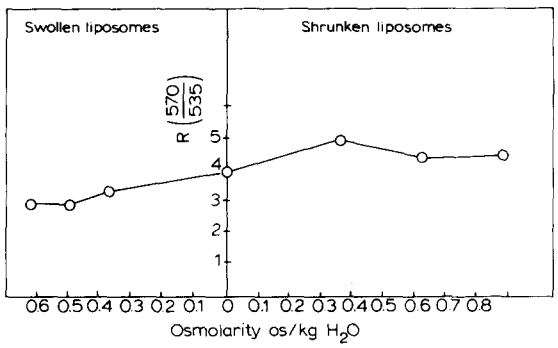

Fig. 3. Effect of the osmolarity of the external medium on the surface properties of DMPC liposomes in the liquid-crystalline state as detected by the absorbance ratio 570/535 with merocyanine 540.

Figs. 2B and 2C show that the osmotic shrinkage promotes an increase in the surface hydrophobicity, measured with both probes in the presence of neutral or charged solutes. This increase in hydrophobic region exposition is parallel to the increase of the lytic effectiveness of the lysolipid. Conversely, the swelling of liposomes makes them less sensitive to lytic action of lysoPC. This indicates that the liposome-shrinking promotes on their surface specific regions with affinity for the lysoPC. Liposomes in the liquid crystalline state, dispersed in hypertonic solutions, do not show any increase in their hydrophobicity (Fig. 3).

Discussion. According to the present results, lysoPC seems to interact with liposomes as a monomer, producing a lytic effect favored by the gel state of the bilayer. Several experimental evidences support such assumption, i.e.; (a) lysoPC induces a noticeable decrease in the turbidity and a release of trapped NADH, (b) the critical point at which absorbance starts to decrease coincides with that found using the octadecylrhodamine assays, suggesting that the changes in the turbidity are related to a mixture of lipids (Fig. 1B).

The method employing octadecylrhodamine has been successful in exploring membrane fusion [12,13]. It is based upon the fact that the fluorophore is self-quenched in a lipid bilayer and hence, an increase in fluorescence is indicative of an increase in the lipid/fluorophore ratio. In our case, a significant increment in fluorescence was obtained when liposomes, in the gel state, containing octodecylrhodamine were titrated with lysoPC. This increase could be due to a dilution of the fluorofore by the incorporation of lysoPC into the bilayer or to a formation of a new phase, for instance mixed micelles. In the first case, the NADH leakage could be ascribed to a change in the barrier properties of the bilayer. However, the dramatic permeability changes have been detected at 22 mol% of lysoPC in a concentration much higher than that assayed here [10]. A second possibility is that liberation is a consequence of the lysis of the liposome.

In another series of experiments, the method of octadecylrhodamine was employed to titrate a mixture of two populations of DMPC liposomes, one containing the fluorophore at a self-quenched concentration and another without the fluorophore. It can be observed that upon titration with lysoPC, the slope of fluorescence increase is much steeper than that found in the previous assay. However, a displacement of the clc to higher values is observed. These results suggest that lysoPC promotes the mixture of the lipids belonging to both populations. Accordingly, lysoPC might disrupt the bilayer of both types of liposomes, including the appearance of mixed micelles. Hence, the dilution of the probe is higher showing a steeper increase, being the effective concentration of lysoPC able to disrupt the probe-containing liposomes lower than in the first case. Thus, the apparent clc is displaced to higher values.

The experiment with mixed population of liposomes might also imply fusion. However, according to the titration curve shown in Fig. 1B (full symbols), the fluorescence would not be expected to increase when liposomes with the same lipid/probe ratio fuse. It seems more likely that the lysoPC is promoting a lysis of the lipid structure. This last possibility would be supported by the NADH release. It must be noticed that such leakage is detected only when the lysoPC titration is performed using liposomes in the gel state. However, the release is found below the critical point at which the decrease of absorbance and the changes in fluorescence are observed.

A similar behavior has been shown using ^{31}P-nuclear magnetic resonance and K^+ permeability [18]. An incorporation of only 1 mol% of 1-oleoyllysophosphatidylcholine into gel state dipalmitoylphosphatidylcholine is enough to destabilize

the bilayer structure making the membrane completely permeable for K^+. However, also in this case the loss of the K^+-permeability barrier occurs at lower lysophosphatidylcholine concentrations than the one needed for the start of micellization [18].

As it is well-known, multilamellar liposomes present a wide distribution in size [15,16]. Thus, a possible explanation for the NADH release below the clc detected by absorbance or fluorescence would be that lysoPC is acting on a liposome fraction no detected by optical methods. Thus, the problem of particle size (or volume) becomes relevant.

Since most of the lysolipid studies have been performed using multilamellar liposomes [6,9, 16,18], for the sake of comparison, we have studied the effect of particle size and surface properties on the lytic effect of LPC, changing the liposome volume by osmotic stress.

Our results show (Fig. 2 and Table II) that lower lysolipid concentrations are required to induce lysis when the liposome volume decreases. The liposome-NADH release appears at a lysoPC concentration lower than the one required to induce detectable changes in the suspension turbidity (clc). This apparent discrepancy could be due to a leakage of NADH induced by lysoPC without the rupture of the liposome structure, i.e., the diminution of the turbidity in Fig. 1A might represent an increment in the liposome volume consecutive to a decrease in the permeability barrier of the bilayer. However, the enhancement of the lysoPC action observed in the presence of hypertonic solutions would mitigate such possibility. The results reported in Table II show that lower lysoPC concentrations are required to produce the lysis of particles of smaller volume. Thus, the osmotic shrinkage displaces the relative size of osmotically active liposomes to lower values enhancing the lysoPC lytic effect [15,16]. As shown in Figs. 2B and 2C, hypertonic media promote a simultaneous increment both in the ANS fluorescence and in the absorbance ratio of merocyanine, which are indicative of the membrane surface changes. In addition, it is worthy to notice that the enhancement of the lysoPC-lytic effect was achieved with liposomes which have been shrunken either in hypertonic solutions of KCl or sucrose. Hence, according to these results, the influence of electrostatic charges on the dye interaction, could be discarded. The trend observed with this optical probe was similar to the one found with 1-anilinonaphthalene-8-sulfonic acid. Therefore, it is likely that besides the volume effects, the shrinkage of the liposomes affects the membrane surface, rendering the bilayer more susceptible to the lysoPC attack.

The changes in the liposome volume may influence the lysophospholipid effect either in a positive or in a negative way. Swelling of liposomes corresponds to an increase in the lysoPC clc, i.e. that the lysoPC–bilayer interaction is not favored. Conversely, shrinking of the liposomes enhances the lysoPC-lytic action. The appearance of a shift to a more hydrophobic surface upon liposome-shrinkage, might suggest that the rigid bilayer may expose fractures due to the increase in their curvature. Thus, the increase in lipid-water contact would promote a negative hydration of non polar regions which favors the hydrophobic interaction of lysoPC with PC molecules. Taking into account the conic shape of the lysoPC and the fact that it is acting as a monomer, the fracture might be a conic shape-space filled with water between the lipid molecules.

The value 0.020 mM for the 14:0 lysoPC in KCl solution is similar to that obtained by Weltzien et al. for the ether-deoxy lysoPC. However, these authors did not point out the composition of the aqueous media in which it was determined [20].

The lytic effect of lysolipids has been related to an increase in flip-flop of the lipid molecules in the bilayer, which is enhanced both by the presence of glycophorin or at the gel-liquid crystalline transition temperature [6,7].

Thus, the defect promoted by the osmotic shrinkage might be a region resembling the gel-liquid crystalline state coexistance. It is interesting to note that shrinkage of large liposomes leads to a strong positive spontaneous curvature [19] and an increase in the surface potential [15]. These hydrophobic defects are not observed when liposomes in the gel state are swollen (Fig. 2A) and thus, no lytic action is found at the concentration range in which lysophosphatidylcholine is present as a monomer. It should be noticed that, although

the KCl and sucrose osmolarities assayed to shrink the liposomes decrease the critical micellar concentration, those values were above the highest critical lytic concentration (Compare value of the cmc in Table I with those of the clc in Table II). Using liposomes containing 4% charged lipids, Inoue et al. [9] conclude that the lysophosphatidylcholine-induced liposomal damage requires certain states of fluidity in the bilayer. Below 10°C they did not find any significant damage. However, the charged lipids may in some way contribute to expand the bilayer. Thus, according to the results shown in Figs. 2 and 3, no defects would appear and no lytic action was promoted.

Notewithstanding, when the bilayer is in the liquid crystalline state, lysis may occur at concentrations at which the lysolipid is forming micelles. Expanded bilayers due to the presence of charged molecules would also be destabilized by lysophospholipids at concentrations above their cmc [8,9].

According to our results, the types of detects promoted on the bilayer by hypertonic and hypotonic media are different and hence lysolipid may act by different mechanisms.

Acknowledgements

This work was partially supported with funds provided by CONICET. The authors are indebted to Dr. Vidal for the kind provision of lysoPC, to Dr. Ertola for the facilities of spectrofluorometer measurements and to Mrs. S. Lunati for secretarial support. E.A.D. and J.J.G. are members from Carréra del Investigador CONICET.

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