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Effect of the asymmetric Ca^{2+} distribution on the bilayer properties of phosphatidylcholine-sonicated vesicles

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The incorporation of Ca^{2+} in the inner volume of egg phosphatidylcholine vesicles increases the fluorescence anisotropy of a diphenylhexatriene probe. This increase is higher than for Na^{+} at the same normality. An effect of the same magnitude is induced by Ca^{2+} when using binary lipid mixture (dioleoylphosphatidylcholine and dipalmitoylphosphatidylcholine) as long as the mixture is maintained below the phase-transition temperature of the saturated specie. The influence of Ca^{2+} may be explained by an asymmetric distribution of the saturated and unsaturated lipids between the internal and the external monolayers.

Introduction

Previous evidence has shown that asymmetric Ca^{2+} distribution in egg PC sonicated vesicles induces leakage of the vesicle contents [1]. Also, internal Ca^{2+} modulates anion adsorption on the outer interface and vesicle aggregation in the presence of high-density negative ions [2,3].

These effects have been related to the perturbation of transmembrane inner Ca^{2+} observed by means of ^{31}P-NMR in the sense that adsorption of Ca^{2+} on the outer interface is enhanced by the Ca^{2+} concentration in the internal solution [4,5].

In liposome and vesicle bilayers, asymmetric properties can be obtained by increase in curvature, even in the case of membranes composed of neutral phospholipids. Hence, it may be expected that the stabilization of small vesicles occurring upon sonication would result in a bilayer with different packing constraints, and probably surface properties, on the inner and the outer monolayers. The adsorption at each side may follow different mechanisms, that is, the Ca^{2+} adsorption at the convex and the concave surface may cause different structural rearrangements in the bilayer [6].

One of the reasons for which Ca^{2+} binding on phosphatidylcholine bilayers is controversial comes from the fact that metal binding studies have been performed with either single-shelled vesicles or coarse lipid dispersions [7–10]. This gives a variety of values for the stoichiometry and binding constants [11]. In addition, no study has clearly demonstrated the influence of the asymmetric Ca^{2+} distribution between the two sides of the bilayer on the binding constant magnitudes and its structural consequences.

The specific point that we want to consider in this paper is the Ca^{2+} distribution and the adsorption at each side of a sonicated vesicle bilayer and its consequence on the bilayer properties.

The purpose is to establish the effect on the hydrocarbon regions when Ca^{2+} adsorbs on the outer and/or the inner monolayers of the bilayer using two fluorescent probes which solubilize in the nonpolar region of the membrane. One of them, DPH, interacts mainly with the regions of the terminal methylene groups. On the other hand, TMA-DPH, due to its polar group, reports changes corresponding to methylenes near the polar head [12]. The outer surface properties were studied by an interfacial dye probe such as Merocyanine 540 [13].

This study may illustrate the extent of the action of Ca^{2+} on the overall membrane properties according to the side at which it interacts.

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluene sulfonate; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine.

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Materials and methods

Egg PC was obtained from egg yolks by standard methods and purified in a silica gel column. A single spot was apparent by thin-layer chromatography. The peroxidation index of the final fractions was checked by ultraviolet spectroscopy determining the ratio between the absorbances at 234 and 210 nm, respectively, of a PC solution in absolute ethanol.

DMPC, DPPC and DOPC were obtained from Avanti Polar Lipids and used as received. Purity of the lipids was checked by chromatography using chloroform/methanol/ammonia/water (90 : 54 : 5.5 : 5.5, v/v).

Liposomes were prepared by drying a chloroform solution of the lipid in a round-bottom flask under vacuum at 40 °C and suspending the film in a volume of the desired solution, enough to obtain a final concentration of 2 mg/ml. The solutions were buffered with 10 mM Tris-HCl (pH 7).

After the liposomes were prepared, a 1.5 ml sample of the dispersion was sealed in a glass tube under N_2 and sonicated in a bath sonicator. The final point (lowest turbidity and highest anisotropy) was obtained after at least 100 min of sonication. The data of Fig. 1 correspond to a typical curve of the evolution of the turbidity and anisotropy during the sonication time. When the sonication was done using a tip sonicator, the procedure was complete after 20 min. In this case, the sonication was performed at 90–100 min at 0 °C under N_2 and at intervals of 30 s. After the sonication, the solution was centrifuged at $33000 \times g$ at 4 °C for 30 min using a SS34 rotor in a Sorval centrifuge.

The mean radius of the vesicles obtained with both procedures was approx. 190 Å as determined by dynamic light scattering and Sepharose 2B exclusion chromatography [1]. After the sonication, the absorbance increase of the peak corresponding to peroxides (234 nm) was in all cases less than 15% in comparison to that of unoxidized PC (210 nm), taken as 100%. DPH and TMA-DPH were from Molecular Probes. All others chemicals were of analytical grade.

Fluorescence determinations were carried out in an Aminco Bowman spectrofluorometer with DPH and TMA-DPH at an excitation wavelength of 360 nm.

The anisotropy fluorescence parameter was calculated by [14]

$$r = \frac{I_{\parallel} - I_{\perp} G}{I_{\parallel} + 2 I_{\perp} G}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the emission at 444 nm obtained with the analyser parallel or normal, respectively, to the direction of polarization of the excitation beam. The molar ratio of probes to PC was 1 : 200. In all experiments corrections for light-scattering blank were made measuring the excitation/emission ratio in the absence of the fluorophore. This value is negligible for vesicle suspensions. $I_{\parallel}$ and $I_{\perp}$ values were corrected with the phototube sensitivity by the geometrical factors (G). The temperature of the sample chamber was controlled by means of a Lauda thermoregulated bath. A thermistor probe connected to a digital ohmmeter was used to measure the temperature of the samples in the cuvette to within ± 0.1 °C.

Vesicles prepared in different Ca^{2+}/Na^{+} ratios were dialyzed overnight against isotonic Na^{+} solutions. The absence of Ca^{2+} in the outer solution was checked by the formation of the Ca^{2+}-murexide complex at 480 nm. Dialysis was stopped when the absorbance for the external vesicle solution was equal to that corresponding to the Na^{+}-murexide complex. Absorbances were obtained using 1 μM murexide concentration. The isosbestic point at 507 nm between murexide and murexide-Ca^{2+} complex was taken as a reference to avoid differences in absorbances of the dispersion.

After the dialysis, external Ca^{2+} concentration was adjusted by adding aliquots of isotonic Ca^{2+} solutions to a vesicle dispersion.

After the vesicles were prepared with the chosen internal and external Ca^{2+} concentrations, aliquots of a 2 mM DPH solution in tetrahydrofuran or TMA-DPH in acetonitrile were added in order to achieve a 1 : 200 probe : lipid ratio.

The solutions were then gently swirled for at least 1 h in the darkness to permit equilibration of the dye with the vesicle bilayers. The other way to incorporate the probes was to include them in the lipid/chloroform solutions. Both procedures gave comparable results.

The turbidities of the dispersions were measured as absorbances at 450 nm and 25 °C in a PMQ3 Zeiss spectrophotometer. To study the properties of the inner and the outer interfaces it is important to establish equilibrium conditions between the inside and the outside solutions of the vesicle. There are two possibilities. One is to prepare vesicles in a salt solution and to disperse them in a medium iso-osmotic with the inside solution. However, with salts of ions of different valencies this procedure promotes an uneven distribution of charges at the two sides. The other way is to balance the outer and the inner solutions with respect to charges, although the number of particles inside and outside may produce an osmotic imbalance. However, it must be taken into account that vesicles are stable also in hypotonic media [15]. Therefore, we decided to balance the outer and the inner solutions by charges even in the cases in which the vesicle could be in a hypotonic medium. The cases in which the vesicles are in a hypertonic solution were specifically studied (see below).

The equivalent concentrations (C^{*}) of the salt solutions inside and outside the vesicles were prepared taking into account the relation

$$C^{*} = Z_{+} \nu_{+} C = Z_{-} \nu_{-} C$$

where Z_+ and Z_- are the charge numbers of the ionic constituents and ν_+ and r_- the stoichiometric coefficients. C is the molar concentration of the electrolyte [16]. With this procedure equivalent distribution of charges in the outer and the inner media was achieved.

The Merocyanine 540 (MC540) experiments were performed in a Hitachi double-beam spectrophotometer, measuring the absorbance ratio at 570 and 500 nm peaks. This ratio indicates the partition degree of the probe between the lipid and the water phase. The relative magnitudes of the 500 and 570 nm denote if the bilayer is in the fluid or in the crystal state. After the gel-liquid crystalline transition temperature the peak at 500 nm disappears at the expense of an increase in the peak at 570 nm. This is interpreted as an increase in the partition of MC540 in the bilayer, because the same peak is formed for MC540 in non-polar solvents [13].

Results

The sonication of a coarse lipid dispersion results in an increase in the rigidity of the bilayer of the dispersed particles. The data of Fig. 1 show that the decrease in the absorbance during sonication occurs with a concomitant increase in the anisotropy parameter as measured with DPH as a probe. These results are useful as a criterion for the vesicle formation. When anisotropy

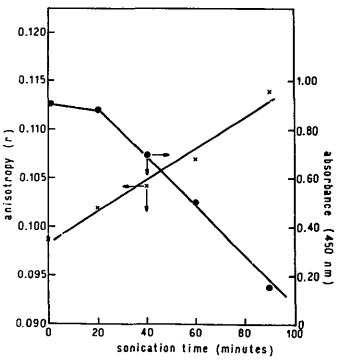

Fig. 1. Effect of sonication on the absorbance and anisotropy of a coarse dispersion of egg PC. Each point corresponds to different samples sonicated during the time indicated in the abscissa. Dye incubation after the sonication was made according to the procedure described in the Materials and Methods. Absorbance at 450 nm (●) was determined in the sonicated samples without centrifugation. All experiments were done in buffer 10 mM Tris-HCl (pH 7.4) and at 25°C.

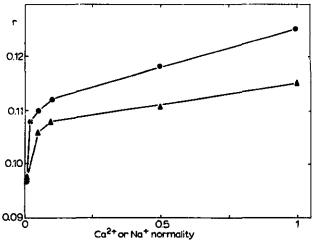

Fig. 2. Effect of the ionic concentration on the anisotropy parameter of egg PC vesicles. $CaCl_2$ (●) or NaCl (▲) concentrations at pH 7.4 and at 25°C using DPH as the fluorescent probe. The salt normality was equal inside and outside the vesicles for the chosen cation, but differed from one sample to another. Each point corresponds to the mean of two different preparations measured in duplicate. In all cases the S.D. was between ±0.002 and ±0.004.

increases nearly 20% with respect to the liposome dispersion the turbidity is near zero.

In these conditions the lipid dispersion is composed mainly of small unilamellar vesicles of approx. 150 Å radius as determined by dynamic light scattering and Sepharose 2B column chromatography [1]. This anisotropy value for the vesicles is independent of the way in which sonication is performed.

It must be noticed that these results were obtained in buffer 10 mM Tris-HCl (pH 7.4) without Na^+ or Ca^{2+} in the dispersing solution. However, significant differences in rigidity were obtained when vesicles were prepared in the presence of Ca^{2+} or Na^+. It can be observed in Fig. 2 that the final anisotropy parameter is a function of the ion type and concentration. Vesicles prepared in Ca^{2+} solutions show a more packed bilayer than those prepared in Na^+ in the range of concentrations assayed.

An increase in rigidity is also observed for increasing Ca^{2+}/Na^+ ratios using TMA-DPH as a fluorescent probe.

In the assays of Fig. 2 inner and outer solutions of the vesicles were of the same composition and concentration. Fig. 3A shows a discontinuity in the curve r versus temperature around 25–30°C for Ca^{2+}-containing vesicles, using DPH as a probe. However, no transition is noticed in Na^+ vesicles using DPH or TMA-DPH in both types of vesicles (Fig. 3B). It must be observed that the discontinuity in Fig. 3A for Ca^{2+} vesicles is much higher than the standard deviation mentioned in Fig. 2.

The increase in rigidity observed in the presence of Ca^{2+} may be ascribed either to a peculiar packing of the lipid molecules due to Ca^{2+} present during sonication or to a specific adsorption after the vesicle is formed.

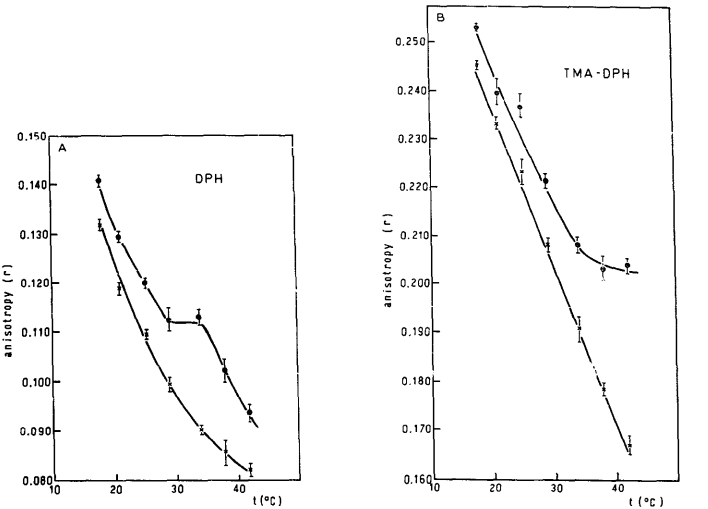

Fig. 3. Effect of temperature on the anisotropy parameter of egg PC vesicles prepared in Na^+ or Ca^{2+} solutions. (A) $CaCl_2$ (●) and NaCl (×) vesicles using DPH. (B) $CaCl_2$ (●) and NaCl (×) vesicles using TMA-DPH as the probe. Ca^{2+} and Na^+ concentrations inside the vesicles were 1 N in both cases.

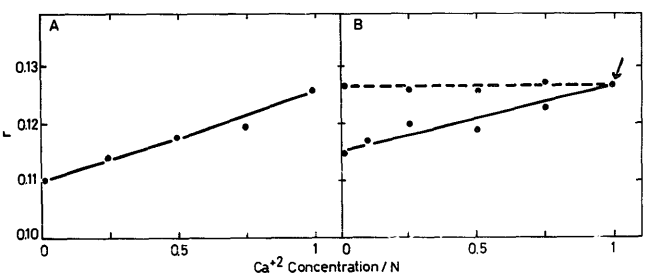

Fig. 4. Effect of the anisotropy parameter of egg PC vesicles of the symmetric and asymmetric Ca^{2+} distribution between the inner and the outer solutions. (A) Ca^{2+} concentration was varied inside and outside maintaining the total salt normality at 1 N with NaCl. Abscissa represents the Ca^{2+} variations in the inner and the outer vesicle solutions. (B) Ca^{2+} concentration was varied only inside (full line) or only outside (dotted line) maintaining in both cases the total normality at each side equal to 1 N. In these cases, the abscissa stands for Ca^{2+} variations inside (full line) or Ca^{2+} variations outside (dotted line). For calculations of normalities see Materials and Methods. The arrow corresponds to a vesicle with symmetric Ca^{2+} distribution.

To gain an insight into this point we must compare two situations. In one of them, packing increases when vesicles are prepared in the presence of Ca^{2+}.

The second situation arises when the Ca^{2+} concentration in the vesicle interior is changed after the vesicle has been formed. In this case two possibilities can be assessed: to decrease the inner Ca^{2+} concentration by adding a Ca^{2+} ionophore or to increase the Ca^{2+} inner concentration by dispersing the Ca^{2+} vesicles in an hypertonic solution. The first possibility was discarded because the addition of the ionophore A 23187 caused, by itself, an increase in anisotropy even in the absence of Ca^{2+}. No effort was made to clarify this point.

The increase of the inner Ca^{2+} concentration was achieved by increasing the concentration of an impermeant solute such as glucose or NaCl in the external solution.

In Fig. 4 we present the results obtained with vesicles formed at different inner Ca^{2+} concentrations. In Fig. 5, inner Ca^{2+} concentration was increased by osmosis after the vesicle was formed.

Vesicles for the experiments of Fig. 4A were prepared in solutions with different Ca^{2+}/Na^{+} ratios maintaining a total concentration of 1 N and dispersed in a solution of identical concentration and composition. For the experiments shown in Fig. 4B, vesicles prepared with the same procedure and solutions as for Fig. 4A were diluted in a 1 N Ca^{2+} solution. Each point in Fig. 4B (full line) represents different batches of vesicles with asymmetric Ca^{2+} distribution between the inner and the outer solution. They contain a 1 N solution of increasing Ca^{2+}/Na^{+} ratios inside and 1 N Ca^{2+} concentration (without Na^{+}) outside. Inner Ca^{2+} concentration is always lower than outside. The maximum is achieved when Ca^{2+} concentration is equal to 1 N at both sides. The increase of inside Ca^{2+} promotes an increase in bilayer rigidity.

The results shown by the dotted line in Fig. 4B correspond to vesicles prepared in a 1 N Ca^{2+} concentration and dispersed in decreasing Ca^{2+}/Na^{+} ratios for a total concentration of 1 N. In this case the asymmetric distribution is given, because Ca^{2+} concentration is higher inside than outside.

At constant Ca^{2+} in the inner solution, the decrease in Ca^{2+} in the external solution does not promote variations in the anisotropy parameter.

One reason for this result could be a preferential location of the fluorescence probe, DPH, on the inner monolayer, and thus no effect of the outer Ca^{2+} is found. To clarify this point, control assays were performed in two ways. In one of them, the fluorophore was mixed with the lipids in the chloroformic solution used to prepare the dry film and the liposomes. In the other, the probe was added after the sonicated vesicles have been obtained following the procedures described in Materials and Methods. In both cases, similar results were obtained. In case the miscibility of DPH is higher in the inner monolayer it would not be unusual to obtain similar results in both types of assay. To avoid this uncertainty, experiments were repeated using TMA-DPH. Due to its polar groups, this probe will be fixed near the polar headgroup regions of the bilayer. Moreover, on the inner monolayer, electrostatic repulsions

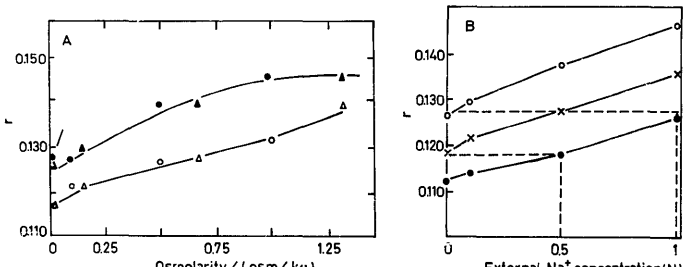

Fig. 5. Effect of the anisotropy parameter of Ca^{2+} and Na^{+} vesicle of the osmotic stress. (A) vesicles corresponding to those indicated by the arrow in Fig. 4B were dispersed in NaCl (▲) or glucose (●) solutions of increasing osmolarities. (△) and (○) correspond to vesicles containing 1 N NaCl, and dispersed in Na^{+} and glucose solutions, respectively. (B) Anisotropy increase of vesicles containing 0.1 N (●); 0.5 N (×) and 1 N (○) $CaCl_2$ and dispersed in increasing osmolarities of NaCl. Dotted lines connect the values of r obtained when vesicles are prepared in increasing Ca^{2+} concentrations (points on the ordinate axis) and when internal Ca^{2+} concentration of a formed vesicle was increased by osmosis (points at 0.5 N and 1 N external Na^{+} concentration) (see text). In all cases, outer osmolarities were increased by adding to the external solution of the Ca^{2+}-containing vesicle the Na^{+} concentrations indicated in the figure.

are large; hence, more dye should be found on the outside.

The results with this probe were consistent with those obtained with DPH. The anisotropy values were much higher than those obtained with the latter probe due to the localization of the TMA-DPH near the polar headgroups.

It is clear that a decrease in the Ca^{2+} concentration of the outer solution does not affect the anisotropy in vesicles containing 1 N Ca^{2+} inside ($r = 0.128$, see arrow in Fig. 4B). However, a net increase is observed when they are dispersed in solutions of increasing hypertonicity (Fig. 5A).

An increase in anisotropy was also found when Na^{+}-containing vesicles (those corresponding to points at zero Ca^{2+} concentration in Fig. 4A and B) were osmotically shrunken. Fig. 5B summarizes the data obtained with vesicles formed with different concentrations and with vesicles whose inner Ca^{2+} concentration was concentrated by osmosis.

The anisotropy values obtained with vesicles prepared in increasing Ca^{2+} concentrations (points on the ordinate axis) are close to those found with a vesicle whose inner Ca^{2+} concentration was increased by osmosis (follow dotted lines).

However, it can be observed in Fig. 5A that the anisotropy increase obtained by osmosis is steeper when vesicles contain Ca^{2+} in comparison to those filled with Na^{+}.

The comparison of Fig. 2A with Fig. 4A indicates that, in addition to the increase in rigidity produced by the vesicle formation, Ca^{2+} makes a specific contribution to the increase in such rigidity. This can be inferred from the fact that the anisotropy values increase when Na^{+} is replaced by Ca^{2+} maintaining the total normality at a constant value.

The slope of the anisotropy as a function of Ca^{2+} concentration for a symmetric Ca^{2+} distribution (Fig. 4A) is slightly higher than that obtained with an asymmetric distribution (Fig. 4B, full line). However, the main effect of Ca^{2+} on bilayer rigidity seems to be due to inner Ca^{2+}. Fig. 5B indicates that this effect is comparable when the vesicle is formed with a given Ca^{2+} concentration or when Ca^{2+} is concentrated inside after the vesicle has been formed.

It may be possible that, as a consequence of the heterogeneous mixture of saturated and non-saturated phospholipids of the egg PC batches employed, the preferential effects of Ca^{2+} on the inner side would be due to an uneven distribution of those species between the inner and the outer monolayer. This distribution would be induced by the sonication.

In order to gain an insight into this, Ca^{2+} effect was studied in vesicles composed of DMPC/DOPC and DPPC/DOPC. Anisotropy values similar to those obtained with egg PC are found for vesicles composed of

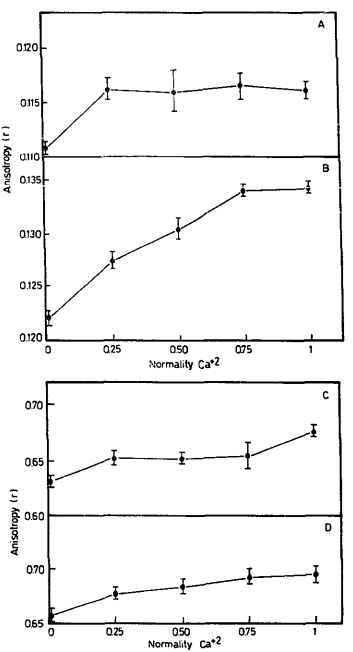

Fig. 6. Anisotropy parameters of DOPC/DMPC vesicles and DOPC/DPPC vesicles prepared at different Ca^{2+} concentrations. Vesicles composed of (A) 46:54 mol/mol DOPC/DMPC at 25°C; (B) 63:37 mol/mol DOPC/DPPC at 25°C; (C) 46:54 mol/mol DOPC/DMPC at 45°C; (D) 64:37 mol/mol DOPC/DMPC at 45°C. Anisotropy was measured using DPH as a probe.

54:46 mol/mol DOPC/DMPC or 64:36 mol/mol DOPC/DPPC.

In Fig. 6 the anisotropy of vesicles with the abovementioned composition were studied as a function of Ca^{2+}. It can be observed that the anisotropy increase at 25°C for vesicles containing DPPC (Fig. 6B) is twice that obtained for the same vesicles at 45°C (Fig. 6D) and those containing DMPC at 25 and 45°C (Fig. 6A and C).

A similar behavior was observed with vesicles composed of 80:20 mol/mol DOPC/DPPC or DMPC.

Finally, the physical state of the phospholipids in the outer monolayer of Ca^{2+}-containing vesicles of different composition was studied using Merocyanine 540 as a membrane probe. It can be observed that DOPC/DMPC, DOPC/DPPC and egg PC vesicles present the same 570/500 absorbance ratio as liposomes in the fluid state [13]. In contrast, the 570/500 nm absorbance ratio is much lower for liposomes in the gel state. This observation indicates that the outer monolayer of the vesicle bilayers used in these assays are in the fluid state.

Discussion

It is well known that Ca^{2+} adsorption on PC membranes is enhanced when, at constant temperature, the lateral pressure is increased or when, at constant lateral pressure, temperature is decreased below the gel-liquid cystalline transition [17,18].

The first case has been accomplished with monolayers of DMPC. The increase in the surface potential is a function of the lateral pressure when Ca^{2+} is present in the subphase [18,20].

The second possibility has been observed measuring the electrophoretic mobility of liposomes in the gel state. The surface potential increases as a consequence of the Ca^{2+} adsorption on the external interface [18].

Figs. 1 and 2 show that a similar situation can be achieved when planar bilayers of egg PC liposomes are forced to pack into curved bilayers of small vesicles.

At different stages of the sonication, the anisotropy increases while turbidity decreases. This fact indicates that the multilamellar population is transformed to vesicles with a higher lateral pressure.

The results in Figs. 2 and 3 strongly suggest that Ca^{2+} has a specific effect on the bilayer rigidity of sonicated vesicles. The anisotropy increases when Na^+ is replaced by Ca^{2+} in the solutions of equal normalities.

According to the data in Figs. 4B, 5A and B the Ca^{2+} action is preferentially located on the inner side of the vesicle.

A close inspection of Fig. 5B shows that a vesicle formed in 0.1 N Ca^{2+} can reach the rigidity of a vesicle formed in 0.5 N Ca^{2+} when it is dispersed in 0.5 N NaCl. This means that the dispersion in 0.5 N NaCl would increase the Ca^{2+} concentration inside up to the new 0.5 N $CaCl_2$. Similar conclusions can be derived for vesicles prepared in 0.1 N and dispersed in 1 N NaCl. However, osmosis can not affect the inner Ca^{2+} concentration without changing the vesicle volume.

On the basis of the results in Fig. 5A, both Na^+ and Ca^{2+} vesicles increase their rigidity by osmotic stress. The increase in anisotropy in these assays is steeper when vesicles contain Ca^{2+}. That is, for a given lateral compression, and hence a given state of rigidity, Ca^{2+} can adsorb preferentially, increasing further the rigidity.

TABLE I

Phase state of the outer monolayer of vesicles and liposomes of different composition as detected by the 570/500 nm absorbance ratio with Merocyanine 540

For details see Materials and methods and Ref. 31. Temperature was 25 °C.

Vesicle composition	Vesicle content (all salts 1 N)	Absorbance ratio
DOPC/DMPC	NaCl	4.7
DOPC/DMPC	$CaCl_2$	4.2
DOPC/DPPC	NaCl	4.0
DOPC/DPPC	$CaCl_2$	4.3
Egg yolk PC	NaCl	4.1
Egg yolk PC	$CaCl_2$	3.6
DPPC multilamellar liposomes	Na^+ or Ca^{2+}	1.1

The increase of the bilayer rigidity obtained by vesicle formation suggests that, at constant temperature, an increase in lateral pressure has occurred. Taking into account that Ca^{2+} affects the anisotropy when it is on the inner side and that, according to Table I, the outer interface is in the fluid state, the lateral pressure increase would take place in the inner monolayer. Therefore, the break observed in Fig. 3A for Ca^{2+} vesicles would correspond to the inner monolayer phase transition.

The formation of egg PC vesicles promotes changes in the bilayer phase state which are not involved in planar liposomes bilayers. As it is known, egg PC bilayers have a transition temperature at −20 °C. It can be observed in Fig. 3A that a break is apparent in the plot r vs. T which can be interpreted as a transition between two states at 30 °C. It is also important to notice that this break is a function of Ca^{2+} concentration.

This transition might be ascribed, based on the arguments given above, as taking place in the inner monolayer of the bilayer, probably at the inner membrane core of the bilayer.

This last inference is based on the observation that the transition is clearly noticed using DPH with Ca^{2+} vesicles but it is absent when TMA-DPH is used as a probe.

Therefore, the presence of Ca^{2+} during sonication promotes an organization of the bilayer structure different from that obtained with Na^+.

There are at least two possibilities for which it can occur. One is a direct consequence that egg yolk PC is a mixture of saturated and non-saturated phospholipids. Then, a redistribution of saturated and non-saturated species between the inner and the outer monolayer would be forced by the sonication. The other explanation is that a change in the free volume of the unsaturated species is enhanced by Ca^{2+} during sonication.

The explanation that sonication in the presence of Ca^{2+} imposes a distribution of the phospholipids of the egg yolk lipid species between the inner and the outer monolayer is supported by the experiments performed with defined mixtures of DOPC, DMPC and DPPC.

In Fig. 6 it is clear that the action of Ca^{2+} takes place when one of the components of the mixture is below its transition temperature. One possible interpretation of the data in Figs. 4 and 5 is that, upon sonication, saturated phospholipids would be oriented towards the vesicle interior, while the dioleoyl would be in the outer monolayer. Thus, adsorption would be enhanced, at a temperature below the gel-liquid crystalline transition, on the inner monolayer. The outer monolayer would remain in the fluid state as shown in Table I.

The possibility that during sonication two populations of vesicles are formed, one composed only of saturated lipids and the other with the non-saturated ones, is unlikely. Control assays showed that when saturated DMPC or DPPC vesicles were put in Ca^{2+} solution below the gel-liquid crystalline transition temperature, they rapidly underwent dispersion, increasing the turbidity [21]. No turbidity changes above the values observed for the longest times in Fig. 1 were obtained in experiments shown in Fig. 6.

Moreover, it has been reported that although those lipid species do not mix ideally, they do not segregate into separate bilayers when dispersed as large liposomes [22]. However-, the same lipids seem to mix ideally up to a ratio of 50% when they stabilize in small vesicles [23].

One may also argue that, according to geometrical restrictions, DOPC would be located in the inner monolayer. The packing of the headgroups would allow a radial orientation (as in an inverted micelle) giving more space for the non-saturated fatty acid chains. The saturated species would be packed in the external monolayer stacking side by side the hydrocarbon chain, but allowing the polar headgroups to be separated as in the fluid state.

With this argument, the increase of rigidity in Na^{+} vesicles due to the osmotic shrinkage can be explained by a decrease in the free volume of the non-saturated phospholipids.

If this is the case for Ca^{2+} vesicles, it is difficult to explain the Ca^{2+} action on the inner side. With this picture, as the saturated species would be located in the outer monolayer, the Ca^{2+} adsorption should be thought of as taking place on the unsaturated lipids packed in the inner side.

The DOPC monolayer should be packed, at 25 °C, in the same way as a gel-like structure. Taking into account that the gel-liquid crystalline transition temperature is below zero, this is highly improbable.

The transition points observed experimentally in Fig. 3 are close to those corresponding to the saturated phospholipids employed in the mixture.

It is important to notice the effect of temperature in Fig. 6 and compare it with data in Fig. 3 and Table I. As shown in Table I, in all the compositions assayed, the outer monolayer of the vesicle is in the fluid state, even when it is composed of a lipid such as DPPC, whose transition temperature is well above 25 °C. At this temperature, a well-defined Ca^{2+} effect is noticed in DPPC/DOPC vesicles (Fig. 6B). However, when the temperature is raised above that of the DPPC transition temperature, no effect of Ca^{2+} on the anisotropy is observed (Fig. 6C). Therefore, it can be concluded that the effect of Ca^{2+} on the inner monolayers is related to its phase state. In this case, it corresponds to a monolayer composed mainly of DPPC, according to the response with temperature. The effect of Ca^{2+} is not observed in DMPC/DOPC vesicles at temperatures a few degrees above the transition temperature of the saturated phospholipid.

Previous results have shown, following geometrical considerations, that the packing arrangements of the phospholipid molecules in small vesicles are somewhat less regular than in multibilayers [15]. Some authors have pointed out, that this is mainly a consequence of the variations in the area per phospholipid molecule at each headgroup and the constraints of the hydrocarbon chains [15,24,25]. This would indicate that a decrease in the free volume of the lipid molecules is inherent to vesicle formation. Thus, the bulk modulus of unilamellar lipid bilayers is smaller than in multilamellar liposome, i.e., the membrane becomes hardened [26]. However, this argument has been used to explain packing in vesicles composed of a single phospholipid species.

Lipid distribution between the two monolayers are assumed to obey Boltzmann's law with the addition requirement for a constant density of the two layers. However, lipid asymmetry induced by packing restrictions in curved membranes can be the result of a departure of the ideal mixing in the membrane plane [23,27].

The structural cause for which vesicles undergo a phase transition has been reported to be due to changes in the inner monolayer. Then, at constant temperature, the two monolayers of the vesicle bilayer have different physicochemical properties. Therefore, it is not unexpected that inner and outer monolayers of a sonicated vesicle have different adsorptive properties for Ca^{2+}.

This finding is in the same direction to those previously reported [23]. Egg yolk PC sonicated vesicles are more fragile and leaky when prepared in Ca^{2+} solutions [1] and the diameters corresponding to Ca^{2+} and Na^{+} vesicles are 380 and 200 Å, respectively for 0.1 N concentrations (Disalvo, Bakás and Ohki, unpublished data).

These observations can be explained by egg PC ves-

icle bilayers having attained properties similar to those found in saturated PC bilayers.

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