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Calcium and magnesium induced changes in the relative fluidity of phosphatidylcholine liposomes

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Abstract. The effect of Ca^{2+} and Mg^{2+} on relative fluidity of phosphatidylcholine liposomes was studied by measuring the degree of chlorophyll fluorescence polarization. An increase in the degree of fluorescence polarization was observed on incubation of liposomes with different concentrations of Ca^{2+} or Mg^{2+} . The results have been interpreted on the basis of increase in the size of liposomes which could be brought about by calcium or magnesium induced fusion of small unilamellar liposomes to form larger vesicles. Fusion of liposomes has also been confirmed by the experiments on efficiency of energy transfer from chlorophyll b to chlorophyll a, and transmission electron microscopy of liposomes before and after incubation with Ca^{2+} and Mg^{2+} .

Keywords. Liposomes; fluidity; fluorescence polarization; phosphatidylcholine.

Introduction

Fluidity of the lipid bilayer plays a role in the membrane fusion. Small sonicated vesicles, because of their large curvature are known to exhibit different physical properties from large bilayer vesicles and multilamellar liposomes (Scheetz and Chan, 1972). In particular, small sonicated vesicles have intrinsically greater tendency to fuse as compared to the larger vesicles (Prestegard and Fellmeth, 1974; Miller and Racker, 1976; Liao and Prestegard, 1979). Fusion of vesicles of pure phospholipids incubated above their phase transition temperatures is considerably less due to the repulsive forces at the interface of the membranes of neutral phospholipids (LeNeveu *et al*., 1978, Parsegian and Rand 1983).

However, rapid fusion of phosphatidylcholine vesicles occurs at the phase transition temperatures of these lipids (Prestegard and Fellmeth, 1974; Taupin and McConnel, 1972). Fusion between mixed (unsaturated) vesicles (Maeda and Ohnishi, 1974) and heterofusion between saturated and unsaturated phosphatidyl-choline vesicles have also been reported.

The purpose of the present work was to study the effect of Ca^{2+} and Mg^{2+} on the fluidity of small unilamellar vesicles of phosphatidylcholine, in acidic and neutral environment. Based on our results, we suggest that the decrease in the relative fluidity of phosphatidylcholine liposomes, after incubation with Ca^{2+} /Mg^{2+} , is due to the formation of large multilamellar vesicles.

Materials and methods

L-α-phosphatidylcholine (from egg yolk) was purchased from CSIR Centre for Biochemicals, Delhi. $CaCl_2$ and $MgCl_2$ were from E. Merck, Germany and Glaxo, India respectively. Chlorophylls were extracted from fresh spinach leaves in methanol-petroleum ether (2 : 1 v/v) and were transferred into diethyl ether. The concentration of chlorophylls was determined according to Arnon (1949).

Small unilamellar vesicles of phosphatidylcholine were prepared in 0·1 M NaCl, 0·1 mM EDTA and 0·5 mM Tris (hydroxymethyl) methylamine buffer, pH 7·4 and 5·5. The limpid (10mM) was dispersed in the buffer and was sonicated in a bath-type sonicator for 75 min. The preparation was subsequently ultracentrifuged at 115,000g for 1 h in Beckman SW 50·1 rotor to remove the large vesicles and/or aggregates.

Vesicles with chlorophylls within the limpid bilayer were prepared as described by Mishra *et al.* (1988). The ratio of lipid: chlorophylls was always kept constant at 50:1 (w/w). The suspension of liposomes after sonication and ultracentrifugation was passed through a Sephadex G-25 column (25 × 1·5 cm) in order to remove the non-incorporated chlorophyll molecules.

Solutions of $CaCl_2$ or $MgCl_2$ were added to the liposomal suspension to give different concentrations. The fusion mixture was incubated at room temperature for 3 h and then excess of EDTA was added to chelate the metal ions. The mixture was again incubated for 1 h at room temperature before measuring the fluorescence emission and degree of fluorescence polarization. The samples were excited at 468 nm and the emission was measured at 658, 677 and 700 nm respectively on a Shimadzu RF-450 spectrofluorometer (Japan). The efficiently of energy transfer from Chl b to Chl a was given by $(F_{677}–F_{700})/(F_{658}–F_{700})$ (Gad and Eytan, 1983).

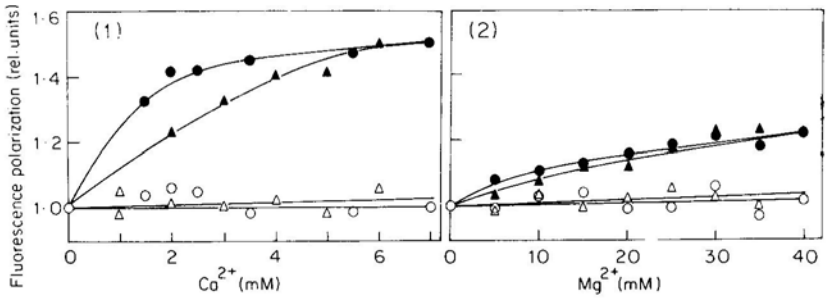
The measurement of steady-state fluorescence polarization was done by setting the excitation and emission wavelengths at 468 and 677 nm. The relative intensities of the 4 combinations of vertical and horizontal excitation and emission beams were recorded on a Shimadzu RF-540 spectrofluorometer (Japan). The steady-state fluorescence polarization was calculated as reported earlier (Dale and Eisinger, 1975; Dale *et al*., 1977). All the experiments were carried out at two different pH *i.e*., one at neutral pH 7·4 and the other at acidic pH 5·5.

The liposomes were negatively stained with 2% phosphotungstic acid for transmission electron microscopy. The pH of the stain solution was adjusted to 7·4 using 1 M NaOH. The liposomal suspension was taken on a copper grid (300 mesh size), coated with carbon-formvar and stained directly by putting a drop of 2% phosphotungstic acid solution on the grid (Munn, 1974). Staining was done for 3–4 min and the grid was blotted from side with a filter paper. After air drying the grid was mounted and viewed at a magnification of 135000 X in a transmission electron microscope (CM 10, Philips, The Netherlands).

Results

On incubation of liposomes with Ca^{2+} at pH 5·5 at room temperature, a sharp increase in the degree of fluorescence polarization was observed as the concentration of Ca^{2+} was raised from 0–2 mM. It maintained almost the same level between the Ca^{2+} concentration of 2 and 7 mM (figure 1). Results of experiments at pH 7·4 also showed a similar pattern but the increase in fluorescence polarization was slower (figure 1). No significant change was observed on incubation of liposomes without Ca^{2+} either at pH 5·5 or 7·4. Incubation of liposomes with Mg^{2+} also resulted in an increase in the fluorescence polarization value both at pH 5·5 and 7·4. The increase was, however, slower at pH 7·4 than that at pH 5·5 (figure 2).

The fusion of pigmented liposomes with the non-pigmented ones was monitored



Figures 1 and 2. Changes in the degree of fluorescence polarization of the mixture of pigmented and non-pigmented liposomes of phosphatidylcholine before (O, Δ) and after (●, ▲)incubation with Ca^{2+} (**1**) and Mg^{2+} (**2**) at pH 5·5 (O, Δ) and 7·4 (●, ▲)The samples were excited at 468 nm and the fluorescence emission was detected at 677 nm.

by the changes in efficiency of energy transfer from Chl b to Chl a as described by Gad and Eytan (1983) and Mishra *et al.* (1988). Table 1 shows the changes induced

Table 1. Efficiently of energy transfer from Chl b to Chl a in the mixture of pigmented and non-pigmented liposomes of phosphatidyl-choline before and after incubation with different concentrations of Ca^{2+} and Mg^{2+} at pH 5·5 and 7·4 (see 'materials and methods' for details).

Ca^{2+}	pH 5·5		Ca^{2+}	pH 7·4		Mg^{2+}	pH 5·5		pH 7·4	
[mM]	Before	After	[mM]	Before	After	[mM]	Before	After	Before	After
0·0	1·64	1·45	0·0	2·68	1·64	0	1·59	1·50	1·14	1·12
1·5	1·65	1·24	1·0	1·66	1·49	5	1·57	1·38	1·22	1·08
2·0	1·55	1·17	2·0	1·55	1·52	10	1·66	1·35	1·15	1·09
2·5	1·55	1·17	3·0	1·61	1·49	15	1·52	1·35	1·13	1·09
3·5	1·51	1·19	4·0	1·60	1·49	20	1·50	1·30	1·13	1·09
5·5	1·55	1·25	5·0	1·65	1·47	25	1·55	1·28	1·17	1·08
7·0	1·50	1·21	6·0	1·63	1·43	30	1·59	1·23	1·15	1·06
			7·0	1·63	1·56	35	1·59	1·27	1·14	1·06

by various concentrations of Ca^{2+} at pH 5·5 and 7·4 respectively. It is apparent from the table that reduction in the efficiently of energy transfer is more at pH 5·5 than that at pH 7·4. The changes were less when liposomes were incubated with Mg^{2+}. However, acidic pH still favoured the reduction in energy transfer at all concentrations of Mg^{2+} (table 1). The fusion of liposomes induced by Ca^{2+} and Mg^{2+} were also confirmed by electron microscopy before and after incubation with Ca^{2+} or Mg^{2+}. Figure 3 shows the increase in the size of phosphatidylcholine vesicles after incubation with 5mM $CaCl_2$ and 35 mM $MgCl_2$.

Discussion

Our results show that Ca^{2+} brings about increase in the degree of chlorophyll fluorescence polarization more than Mg^{2+}. The degree of fluorescence polarization is considered to be a measure of mobility of the fluorophore, the chlorophyll molecule, within the lipid bilayer. Mobility of the fluorophore is affected by 3 major

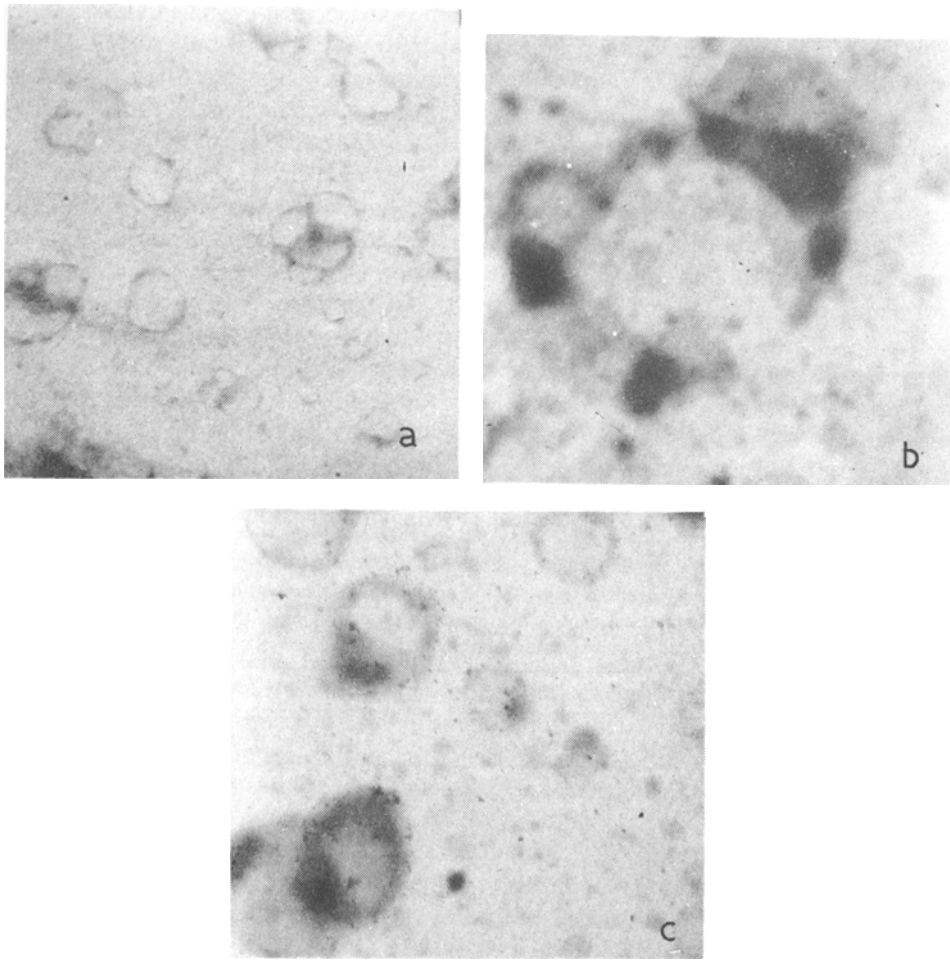


Figure 3. Electron micrographs of negatively stained liposomes before incubation (a) and after incubation with 5 mM $CaCl_2$ (b) and 35 mM $MgCl_2$ (c) at 405,000 X magnification (see 'materials and methods' for details).

factors *viz*., temperature, viscosity of the medium and, size and shape of the matrix containing the fluorophore. Considering the experimental conditions, changes in fluorescence polarization could only be expected in the lipid bilayers containing the chlorophyll molecules.

The results could be interpreted on the basis of an increase in the size of liposomes after incubation with Ca^{2+} or Mg^{2+}, which could happen only by fusion of small sonicated liposomes to form large multilamellar vesicles. It is known, that small unilamellar vesicles form larger structures in a period of several hours when incubated at appropriate temperatures (Taupin and McConnel, 1972). Taupin and McConnell (1972) were the first to suggest that this process involves liposome-liposome fusion which was further substantiated by other reports (Prestegard and Fellmeth, 1974; Papahadjopoulos *et al*., 1972; Kantor and Prestegard, 1975). The mechanism of fusion, however, is indirect and not well understood and can be

interpreted in terms of simple molecular exchange of the type described earlier (Pagano and Huang, 1975; Lawaczek *et al*., 1975, 1976).

The greater ability of Ca^{2+} over Mg^{2+} in bringing about changes in the relative fluidity of liposomes has not been understood well and it needs further investigation. Reasons do exist for divalent cations induced fusion of liposomes of charged phospholipids but so far there is no conclusive evidence to show the binding of Ca^{2+} or Mg^{2+} to phosphatidylcholine liposomes. Thus the mechanism of Ca^{2+} /Mg^{2+} induced decrease in the bilayer fluidity and liposome fusion seems to be indirect to give apparent results presented here; but this mechanism appears to be sensitive to its ionic environment as the effects are more pronounced in the acidic medium.

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