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Cholesterol affects divalent cation-induced fusion and isothermal phase transitions of phospholipid membranes

Sergei A. Shavnin [1,*], Maria C. Pedroso de Lima [1,*], Jane Fedor [1,*], Periann Wood [1,*], Joe Bentz [2,3] and Nejat Düzgüneş [1,2]

[1] *Cancer Research Institute, School of Medicine, and Departments of* [2] *Pharmaceutical Chemistry and* [3] *Pharmacy, School of Pharmacy, University of California, San Francisco, CA (U.S.A.)*

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The influence of cholesterol on divalent cation-induced fusion and isothermal phase transitions of large unilamellar vesicles composed of phosphatidylserine (PS) was investigated. Vesicle fusion was monitored by the terbium/dipicolinic acid assay for the intermixing of internal aqueous contents, in the temperature range 10–40 °C. The fusogenic activity of the cations decreases in the sequence $Ca^{2+} > Ba^{2+} > Sr^{2+} \gg Mg^{2+}$ for cholesterol concentrations in the range 20–40 mol%, and at all temperatures. Increasing the cholesterol concentration decreases the initial rate of fusion in the presence of Ca^{2+} and Ba^{2+} at 25 °C, reaching about 50% of the rate for pure PS at a mole fraction of 0.4. From 10 to 25 °C, Mg^{2+} is ineffective in causing fusion at all cholesterol concentrations. However, at 30 °C, Mg^{2+}-induced fusion is observed with vesicles containing cholesterol. At 40 °C, Mg^{2+} induces slow fusion of pure PS vesicles, which is enhanced by the presence of cholesterol. Increasing the temperature also causes a monotonic increase in the rate of fusion induced by Ca^{2+}, Ba^{2+} and Sr^{2+}. The enhancement of the effect of cholesterol at high temperatures suggests that changes in hydrogen bonding and interbilayer hydration forces may be involved in the modulation of fusion by cholesterol. The phase behavior of PS/cholesterol membranes in the presence of Na^+ and divalent cations was studied by differential scanning calorimetry. The temperature of the gel–liquid crystalline transition (T_m) in Na^+ is lowered as the cholesterol content is increased, and the endotherm is broadened. Addition of divalent cations shifts the T_m upward, with a sequence of effectiveness $Ba^{2+} > Sr^{2+} > Mg^{2+}$. The T_m of these complexes decreases as the cholesterol content is increased. Although the transition is not detectable for cholesterol concentrations of 40 and 50 mol% in the presence of Na^+, Sr^{2+} or

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* Present addresses:
S.A. Shavnin, Department of Biophysics, Ural Timber Technology Institute, Sverdlovsk, 620032 U.S.S.R.
M.C. Pedroso de Lima, Department of Chemistry, University of Coimbra, 3049 Coimbra, Portugal.
J. Fedor, Department of Pharmacy, University of California, San Francisco, CA 94143, U.S.A.
P. Wood, Dominican College, San Rafael, CA 94901, U.S.A.

Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; T_m, temperature of the gel–liquid crystalline transition; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; DPA, dipicolinic acid; Tes, *N*-tris(hydroxymethyl)methylaminoethanesulfonic acid; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)PE; Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)PE.

Correspondence: N. Düzgüneş, Cancer Research Institute, University of California, San Francisco, CA 94143-0128, U.S.A.

Mg^{2+}, the addition of Ba^{2+} reveals endotherms with T_m progressively lower than that observed at 30 mol%. Although the presence of cholesterol appears to induce an isothermal gel–liquid crystalline transition by decreasing the T_m, this change in membrane fluidity does not enhance the rate of fusion, but rather decreases it. The effect of cholesterol on the fusion of PS/phosphatidylethanolamine (PE) vesicles was investigated by utilizing a resonance energy transfer assay for lipid mixing. The initial rate of fusion of PS/PE and PS/PE/cholesterol vesicles is saturated at high Mg^{2+} concentrations. With Ca^{2+}, saturation is not observed for cholesterol-containing vesicles. The highest rate of fusion for both Ca^{2+}- and Mg^{2+}-induced fusion is observed with vesicles containing 30 mol% cholesterol.

Introduction

Cholesterol is a major component of cellular membranes [1,2], and it appears to influence membrane fusion in various experimental systems, including virus-liposome fusion, virus-induced cell-cell fusion, and myoblast fusion [3]. Phospholipid vesicles have been used extensively as a model system to understand the role of individual membrane components and environmental factors in membrane fusion [4–7], to develop biophysical theories of the mechanisms of fusion [7–12], and to analyze fusion assays [13]. Previous studies have documented in detail how phospholipid headgroups and acyl chains, glycolipids, membrane fluidity, cation binding and membrane surface dehydration affect membrane fusion [4–7,14]. The effect of cholesterol on the Ca^{2+}-induced fusion of different phospholipid vesicle systems is also an area of active investigation [3,15–17].

Early studies had indicated that the incorporation of equimolar cholesterol into dipalmitoylphosphatidylglycerol vesicles inhibits the fusion of small vesicles with large vesicles in the presence of Ca^{2+} over a time-course of 2 h [18]. However, Braun et al. [15] have shown recently that small unilamellar vesicles (SUV) composed of phosphatidylserine (PS)/cholesterol display a higher fusion rate constant than pure PS vesicles in the presence of Ca^{2+} and 660 mM NaCl. Here, the relatively high salt concentration was used to reduce the rate constant sufficiently [19] to allow for an accurate determination. The fusion rate constant of large unilamellar vesicles (LUV) fusing the presence of Ca^{2+} is considerably lower than that of SUV [8,9]. Thus, Bental et al. [16] were able to determine the effect of cholesterol even at 100 mM NaCl. The rate constant of fusion in this system also increases with cholesterol content; however, the overall rate of fusion (which is a combination of the aggregation and fusion reactions) decreases.

Here we have examined the effect of cholesterol on membrane fusion induced by various divalent cations, using LUV composed of PS or its 1:1 mixture with phosphatidylethanolamine (PE). Fusion of PS LUV induced by Ba^{2+} or Sr^{2+} constitutes a convenient experimental system to monitor fusion below and above the gel–liquid crystalline phase transition temperature (T_m) of the divalent cation-PS complex [20,21]. We have therefore investigated the effect of cholesterol on the phase behavior of PS/cholesterol membranes in the presence of Sr^{2+} and Ba^{2+}, as well as of Na^+ and Mg^{2+}, using differential scanning calorimetry. Increasing the cholesterol content lowers the T_m of the divalent cation-PS complex. Thus, at 25 °C, the Ba^{2+}-PS complex transforms from the gel to the liquid crystalline state by the inclusion of 30 mol% cholesterol in the membrane. Nevertheless, the presence of cholesterol in PS vesicles inhibits the initial rate of fusion induced by Sr^{2+} or Ba^{2+}. In contrast, the inclusion of cholesterol in PS membranes enhances the effect of Mg^{2+} on fusion at temperatures above 25 °C. Our preliminary results have been presented previously [22].

Materials and Methods

Bovine brain phosphatidylserine (PS), phosphatidylethanolamine (PE) transphosphatidylated from egg phosphatidylcholine, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)PE (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)PE (Rh-PE) were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesterol was from Behring Diagnostics (La Jolla, CA), and was recrystallized twice from methanol. Lipids were stored under argon in sealed ampoules at −70 °C. $TbCl_3$ was obtained from

Ventron (Danvers, MA). Dipicolinic acid (DPA) and *N*-tris-(hydroxymethyl)methylaminoethanesulfonic acid (Tes) were purchased from Sigma. $C_{12}E_8$ (Behring Diagnostics) was recrystallized twice. $CaCl_2$, $MgCl_2$, $BaCl_2$ and $SrCl_2$ were from Fisher, and NaCl and sodium citrate were from Mallinckrodt. Water was twice distilled, the second time in an all-glass apparatus, and further purified in a Barnstead Nanopure system.

Large unilamellar vesicles (LUV, approx. 120 nm in diameter) were prepared by reverse-phase evaporation followed by sequential extrusion through polycarbonate membranes of 200 and 100 nm diameter [23–25]. Vesicles containing 30 mol% cholesterol or more in their membranes were extruded twice through the 100 nm filter. In some experiments, the vesicles were extruded in a Lipex Biomembranes (Vancouver, B.C.) high-pressure extrusion device.

Two populations of vesicles were prepared for the fusion assay. One population contained 2.5 mM $TbCl_3$/50 mM citrate (Na salt)/5 mM Tes, (pH 7.4) in the internal aqueous space. The other population contained 50 mM DPA (Na salt)/20 mM NaCl/5 mM Tes (pH 7.4). Nonencapsulated material was removed by chromatography on Sephadex G-75 equilibrated with 100 mM NaCl/5 mM Tes/1 mM EDTA (pH 7.4). The size distribution of the vesicles was ascertained by dynamic light scattering in a Coulter N4 MD sub-micron particle analyzer. The mean diameter of the liposomes varied between 118 and 126 nm, with a standard deviation of 30–38 nm.

The fusion assay was performed as described previously [16,24]. Maximal fluorescence (100%) was determined by lysing the appropriate concentration of Tb-containing vesicles (freed of external EDTA by gel-filtration on a second Sephadex G-75 column with 100 mM NaCl/5 mM Tes (pH 7.4) as the elution buffer) with 0.8 mM $C_{12}E_8$ in the presence of 20 μM DPA, and sonicating for 5 min under argon in a bath-type sonicator to ensure the complete reaction of Tb and DPA. In experiments where the effect of temperature was investigated, 100% fluorescence was set for each temperature, since the fluorescence intensity of the Tb-DPA complex changes with temperature. The temperature was maintained within 0.2 C° using a circulating water bath (Neslab Endocal RTE-8DD). The pH of the solution was corrected for the effects of temperature. The assay was carried out in a quartz fluorometer cell in a total volume of 1 ml of 100 mM NaCl/5 mM Tes/0.1 mM EDTA (pH 7.4) and containing equimolar amounts of Tb- and DPA-containing vesicles at a total lipid concentration of 50 μM (determined by lipid phosphorus [26]).

Fluorescence was measured at wavelengths above 530 nm by using a Corning 3-68 cutoff filter and an excitation wavelength of 276 nm in an SLM 4000 fluorometer. 90° light scattering at 276 nm was measured simultaneously in the second channel of the fluorometer.

For a given phospholipid concentration, the inclusion of cholesterol in the membrane increases the number of vesicles, since the total lipid concentration is increased. This effect was corrected by diluting the vesicle suspension by a factor of K_p to obtain the same vesicle concentration for the preparations containing different mole fractions of cholesterol. K_p was calculated by keeping the total area of membrane per volume constant. Thus $K_p = 1 + ap/(1-p)$, where p = the mole fraction of cholesterol, and a = (area per cholesterol)/(area per PS). The molecular areas of PS and cholesterol were taken as 70 [27] and 35 Å^2, respectively. According to these values, $K_p = 1$, 1.125, 1.214 and 1.333, for cholesterol mole fractions of 0, 0.2, 0.3 and 0.4, respectively. The area per molecule of pure cholesterol, determined from surface pressure measurements, indicates a limiting value of about 38 Å^2 [28]. Because of the condensing effect in PS/cholesterol monolayers [28], the average molecular area of cholesterol was taken as 35 Å^2 for all the cholesterol mole fractions used in our study. The area per molecule in a monolayer of 60 mol% PS and 40 mol% cholesterol is approximately 53 Å^2 [28], whereas our assumption would give a value of 56 Å^2. At lower cholesterol mole fractions this discrepancy disappears completely. We should also note that these values for the area per molecule are for monolayers at the air/water interface, and thus may not correspond exactly to bilayer membranes. Our approximation is very close to experimental values, and would be within the error introduced by assuming an average diameter for the vesicles, which actually have a size distribution.

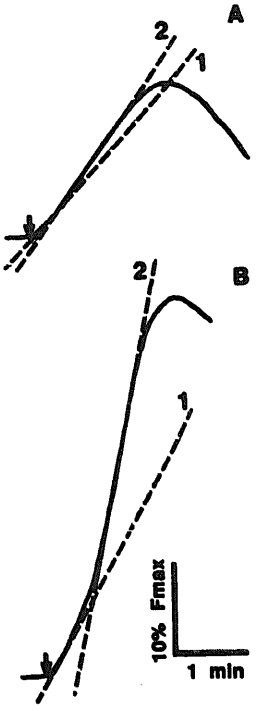

Fig. 1. Time courses of Tb/DPA fluorescence during fusion of 50 μM (A) PS and (B) PS/30 mol% cholesterol LUV at 25°C, induced by 10 mM Sr^{2+} added at the arrows. The initial rate of fusion (r_i) was measured as the tangent to the curve at 3–6 s after initiation of fusion (line 1). The maximal rate (r_m) was measured as the tangent at the region of the steepest increase in fluorescence (line 2).

Fusion was also monitored by the 'probe-dilution' assay for lipid mixing [29–31]. NBD-PE and Rh-PE were incorporated into the 'labeled' vesicles at 0.6 mol% each. For the assay, these vesicles were mixed with 'unlabeled' vesicles at a 1:1 ratio. The 100% fluorescence level was set using vesicles containing 0.3 mol% of each probe at the same total lipid concentration used for the assay (50 μM phospholipid). The concentration of vesicles was not corrected. The excitation wavelength was 450 nm, and the emission was recorded at 520 nm.

In the case of the Tb/DPA assay, two fusion rates were measured, as shown in Fig. 1. The initial rate, r_i, was taken as the slope of the tangent to the fluorescence intensity curve 3–6 s after the injection of divalent cations. The maximal rate, r_m, was determined from the slope of the steepest part of the fluorescence curve. The results presented are the average of two or three runs under each condition. Several liposome preparations were made at different times for each membrane composition. Typical standard deviations are given in the text for each set of experimental conditions.

Differential scanning calorimetry was performed as described by Düzgüneş et al. [20]. LUV were prepared in 100 mM NaCl/5 mM Tes/0.1 mM EDTA. The divalent cations were added to the vesicles (1 μmol of lipid/ml) at 35°C and incubated for 30 min. The vesicles were collected by centrifugation (aggregated vesicles at 10000 × *g* for 20 min; dispersed vesicles at 150000 × *g* for 3 h at 25°C) and transferred to aluminum calorimeter pans, which were then sealed hermetically. Thermograms were obtained in a Perkin-Elmer DSC-2 calorimeter at a scan rate of 5 C°/min and a sensitivity setting of 1 mcal/s.

Results

Fusion of PS/cholesterol vesicles induced by divalent cations

We have investigated the kinetics of fusion of PS/cholesterol vesicles in the presence of Ca^{2+}, Ba^{2+}, Sr^{2+} or Mg^{2+} in the temperature range 10–40°C. The rate of fusion in the presence of Ca^{2+} was higher than that with Ba^{2+} by a factor of 1.2–1.3, but the dependence of fusion on the cholesterol mole fraction and on temperature was similar for both ions. Here we will focus our description on Ba^{2+} rather than Ca^{2+}.

The initial rate of fusion (r_i) for 10 mM Ba^{2+} and 50 μM PS vesicles at 25°C was 1.45 ± 0.12% maximal fluorescence (F_{max})/s. The maximal rate, r_m, was 2.48 ± 0.11 F_{max}/s. The corresponding rates of fusion in the presence of 10 mM Sr^{2+} were 0.15 ± 0.02 and 0.19 ± 0.02% F_{max}/s. Under the same conditions, Mg^{2+} did not induce any fusion, consistent with earlier results [32]. The standard deviations given above for the initial and maximal rates of fusion are representative of those obtained at different cholesterol mole fractions and at different temperatures.

The influence of cholesterol concentration in the membrane on r_i and r_m for Sr^{2+} and Ba^{2+} at 25°C is shown in Fig. 2A. Increasing the mole fraction of cholesterol caused a monotonic decrease in both r_i and r_m for Ba^{2+}, reaching about 50% of the pure PS rate at a mole fraction of 0.4.

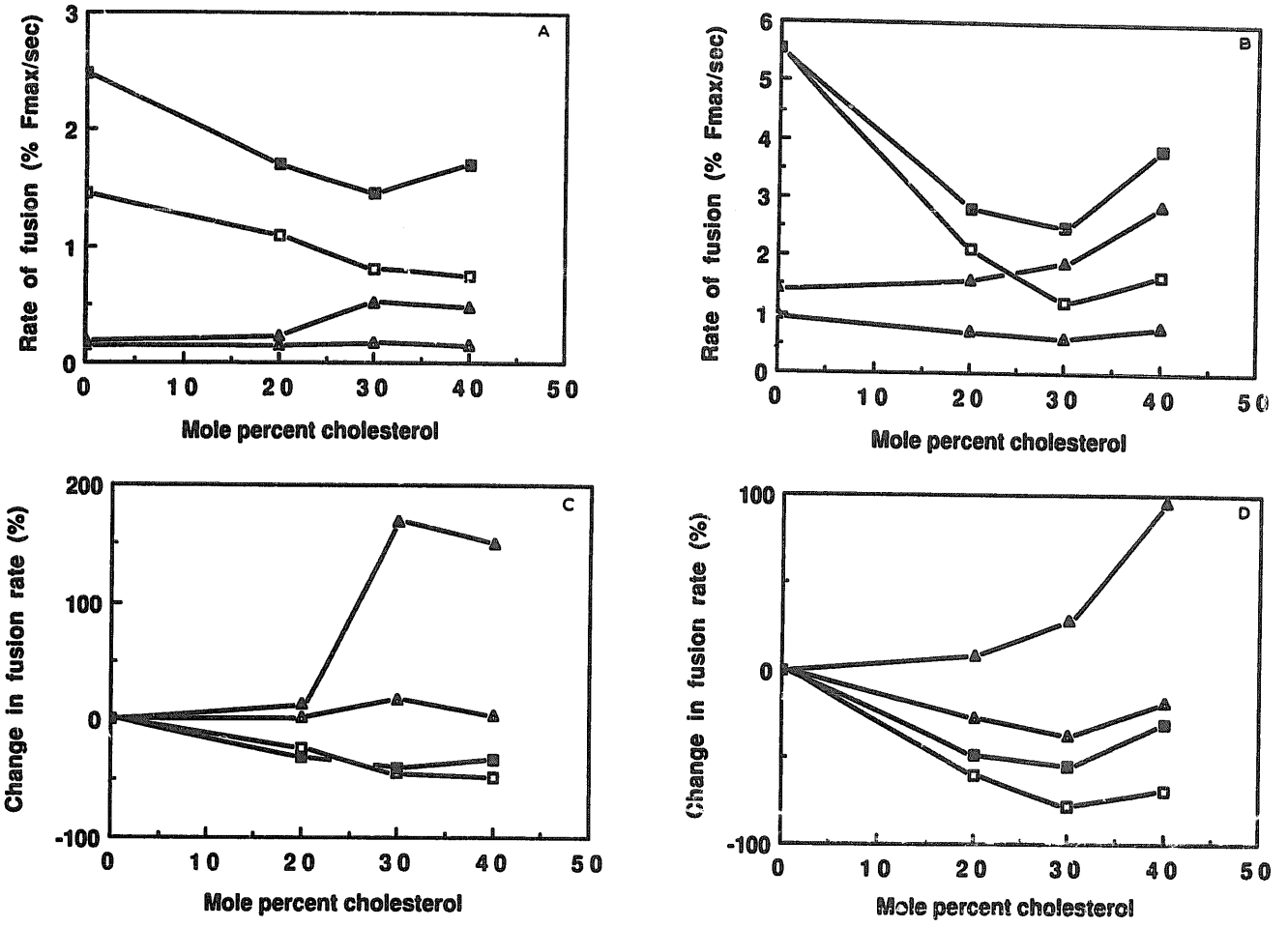

Fig. 2. The dependence on cholesterol concentration of the initial (r_i) and maximal (r_m) rates of fusion of PS vesicles induced by 10 mM Ba^{2+} or Sr^{2+} at 25 °C (A) and 40 °C (B). △ and ▲ represent r_i and r_m of Sr^{2+}-induced fusion, respectively. □ and ■ represent r_i and r_m of Ba^{2+}-induced fusion, respectively. The vesicle concentrations were adjusted in the case of cholesterol-containing vesicles, as described in Materials and Methods. The ordinates in A and B have different scales. Figs. 2C and 2D show the percent change in the fusion rate with mol% cholesterol, and correspond to A and B, respectively.

In contrast, the r_i values for Sr^{2+} did not change significantly with increasing cholesterol content. However, the r_m increased by a factor of 2.5–2.7 at mole fractions of 0.3 and 0.4 (Fig. 2C). The time-course of Sr^{2+}-induced fusion of vesicles composed of PS or PS/30 mol% cholesterol at 25 °C is shown in Fig. 1. Here the effect of cholesterol on r_m can be seen clearly. This difference between the cholesterol dependence of r_m and r_i stresses the importance of distinguishing these two parameters. These results also point out the different mechanism of action of Ba^{2+} and Sr^{2+} on cholesterol-containing PS vesicles. Although the relative change in r_m at 30 mol% cholesterol is higher for Sr^{2+} than Ba^{2+}, the absolute value of the rate (r_m) is still 2-times smaller than that for Ba^{2+}. We have found that for all the cholesterol mole fractions tested, the sequence of effectiveness of the divalent cations was $Ca^{2+} > Ba^{2+} > Sr^{2+} \gg Mg^{2+}$.

The cholesterol dependence of Ba^{2+}- or Sr^{2+}-induced fusion at 40 °C is shown in Fig. 2B. For Ba^{2+}-induced fusion of pure PS vesicles, r_i was 5.54 ± 0.34% F_{max}/s, and was identical to r_m. The r_i and r_m for Sr^{2+}-induced fusion were 1.00 ± 0.05 and 1.46 ± 0.08% F_{max}/s, respectively. Increasing the cholesterol content at 40 °C caused a greater decline in r_i for Ba^{2+} than at 25 °C. The decrease at 30 mol% cholesterol was about 4-fold compared to pure PS (Fig. 2D). The decline in r_m with cholesterol was less than that of r_i. Increasing the mole fraction of cholesterol from 0.3 to 0.4

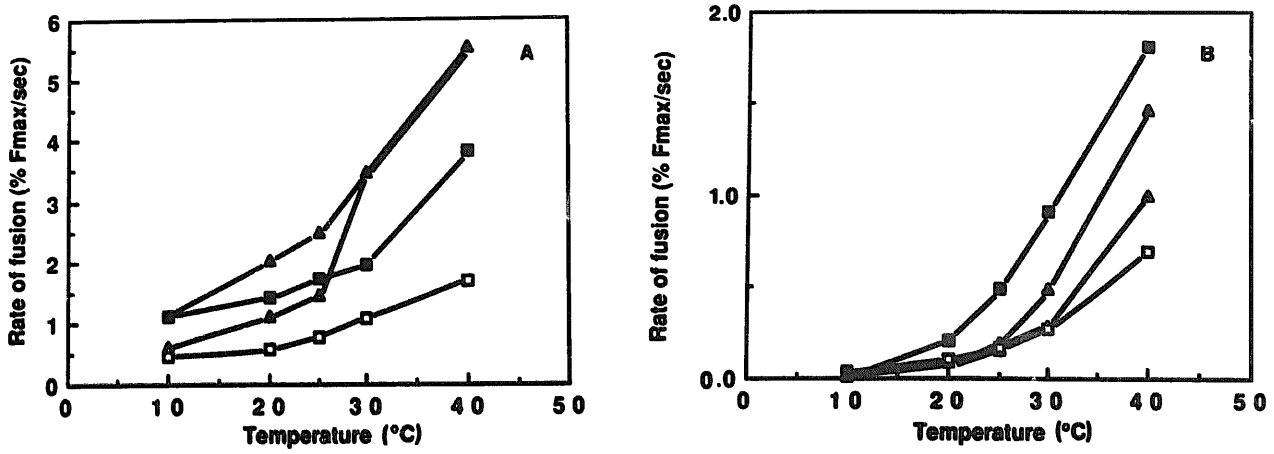

Fig. 3. Temperature dependence of the initial (r_i) and maximal (r_m) rates of fusion of PS (50 μM) and PS/40 mol% cholesterol vesicles in the presence of 10 mM Ba^{2+} (A) or 10 mM Sr^{2+} (B). △ and ▲ are the r_i and r_m of pure PS vesicles, respectively. □ and ■ are the r_i and r_m of PS/40 mol% cholesterol vesicles. The ordinates in A and B have different scales.

induced a distinct increase in r_m. In the case of Sr^{2+}-induced fusion, increasing the cholesterol mole fraction up to 0.3 decreased r_i, in contrast to the results at 25°C. At a cholesterol mole fraction of 0.4, r_i increased to 80% of the PS value from 60% at a mole fraction of 0.3 (Fig. 2D). The r_m for Sr^{2+} gradually increased with the cholesterol concentration in the membrane. This observation also points to the significance of analyzing separately r_i and r_m.

Temperature dependence of the fusogenic activity of divalent cations

The rates of divalent cation-induced fusion of pure PS and PS/cholesterol LUV increased monotonically with temperature in the range 10–40°C for all the cholesterol concentrations tested. The largest difference in fusion rates was between vesicles composed of pure PS and PS/40 mol% cholesterol. The difference in fusion rate between vesicles containing ≤ 30 mol% cholesterol and pure PS was considerably smaller. In Fig. 3 are shown the fusion rates r_i and r_m of PS and PS/40 mol% cholesterol vesicles. Both r_m and r_i for Ba^{2+}-induced fusion of pure PS were larger than those for PS/cholesterol for temperatures above 10°C (Fig. 3A). For pure PS, the values of r_m and r_i were different below 30°C and indistinguishable above this temperature, whereas for PS/cholesterol vesicles these parameters were different at all temperatures. The temperature dependence of fusion of pure PS vesicles was significantly more pronounced than that of PS/cholesterol vesicles in the temperature range 25–40°C. At 40°C the r_i for PS/cholesterol vesicles was about 3-times lower than that of pure PS vesicles.

The temperature dependence of Sr^{2+}-induced fusion of PS and PS/40 mol% cholesterol vesicles is shown in Fig. 3B. At 10°C, the r_i and r_m for pure PS vesicles were equal ($0.012 \pm 0.004\%$ F_{max}/s), and for PS/cholesterol they were 0.033 ± 0.004 and $0.043 \pm 0.003\%$ F_{max}/s, respectively. Increasing the temperature resulted in an increase of both parameters, for both types of vesicles. Above 30°C, r_i and r_m for PS vesicles became distinguishable. The initial rate of fusion (r_i) of PS vesicles was significantly higher than that of PS/cholesterol vesicles at 40°C. However, r_m was higher for PS/cholesterol vesicles at all temperatures, in contrast to the case with Ba^{2+}-induced fusion (Fig. 3A), where the r_m for PS/cholesterol vesicles was lower than that for PS vesicles at temperatures above 10°C.

Perhaps the most significant effect of cholesterol was observed in experiments on the temperature dependence of Mg^{2+}-induced fusion. At 25°C and below, essentially no fusion was observed with 10 mM Mg^{2+} with any of the liposome compositions studied. At 30°C, however, a significant initial rate of fusion was observed with vesicles composed of 30 or 40 mol% cholesterol (Fig. 4A). The r_i for 30 mol% cholesterol was about 0.04% F_{max}/s, and for 40% cholesterol, 0.02% F_{max}/s. At 40°C, the time-course of Mg^{2+}-induced fusion was dramatically different than at lower temperatures (Fig. 4B). Here it was possible

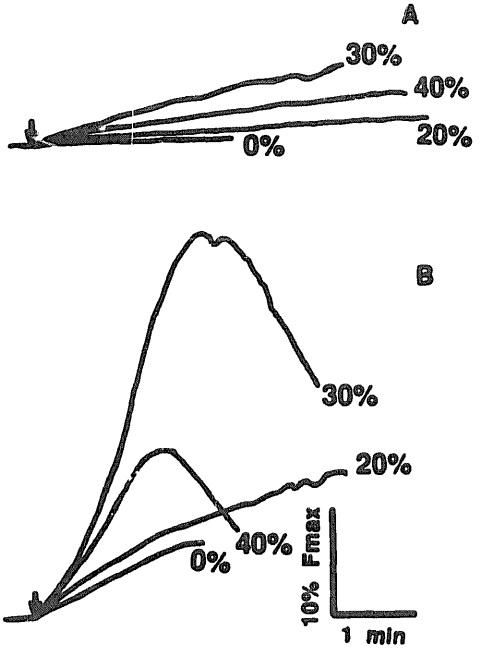

Fig. 4. The effect of 10 mM Mg^{2+} on the time course of Tb/DPA fluorescence of PS and PS/cholesterol LUV at 30 °C (A) and 40 °C (B). The mol-percentages of cholesterol are indicated on the traces.

to distinguish an initial rate and a maximal rate of fusion. At 30 mol% cholesterol, r_i was 0.18 ± 0.02, and r_m was $0.55 \pm 0.03\%$ F_{max}/s. Increasing the concentration of cholesterol to 40 mol% reduced r_i and r_m to 0.11 ± 0.01 and $0.20 \pm 0.02\%$ F_{max}/s, respectively. However, in comparison with the values for Sr^{2+}-induced fusion, r_i and r_m were still 3- and 5-times lower, respectively, in the case of Mg^{2+}. Similar results were obtained in experiments utilizing 50 μM phospholipid throughout the cholesterol concentration range, where the total vesicle concentration would be higher at higher cholesterol mole fractions [3]. Bental et al. [16] have also mentioned their results on the enhanced capacity of PS/33 mol% cholesterol vesicles to fuse in the presence of Mg^{2+}. However, in contrast to our results, they have observed fusion at 25 °C. This observation may be a reflection of the difference in the bovine brain PS preparations used in the two studies.

Role of cholesterol in the fusion of PS/PE vesicles

Earlier studies have indicated that the inclusion of PE in PS vesicles imparted fusion susceptibility to the vesicles in the presence of Mg^{2+} at 25 °C [4,33]. Furthermore, PE did not significantly inhibit Ca^{2+}-induced fusion, contrary to the effect of PC. It was of interest to determine if cholesterol modulated the ability of Mg^{2+} to fuse PS/PE vesicles, perhaps by locally phase separating pure PS domains via a preferential interaction with PE, or separating PS/cholesterol domains by preferentially interacting with PS. For these experiments, a resonance energy transfer assay for the intermixing of lipids was utilized [29–31]. This assay was chosen primarily for the convenience, since column chromatography of the vesicle preparations is not necessary, unlike the Tb/DPA assay. The fluorescence probes NBD-PE and Rh-PE were incorporated in one population of vesicles, and their dilution into unlabeled target membranes was monitored. The assay used in this 'probe dilution' mode has been shown to be insensitive to the mere aggregation of the vesicles [31,34]. However, the initial rates of fusion are usually higher than the contents-mixing assays, presumably because the intermixing of lipids proceeds faster than the destabilization of the membrane and communication of the internal aqueous contents [31,34,35]. Ca^{2+} does not affect NBD-PE fluorescence directly [31,36], although the interaction of Ca^{2+} with the PS molecules in the membrane may affect the fluorescence intensity of NBD-PE [31]. We have shown previously that the fluorescence of vesicles containing 0.6 mol% of each probe is changed only by a few percent when Ca^{2+} is added to pure PS vesicles [31]. This change would not affect appreciably the measurement of the initial rate of fusion.

The initial rates of lipid mixing for PS/PE (1 : 1) vesicles containing various mole fractions of cholesterol as a function of Ca^{2+} or Mg^{2+} are shown in Fig. 5. The rates displayed the highest dependence on Ca^{2+} in the concentration range 4–5 mM. For PS/PE vesicles, the initial rate of lipid mixing saturated above 5 mM Ca^{2+}; however, for vesicles containing cholesterol, the rate continued to increase with increasing Ca^{2+} concentration. The initial rate was highest for vesicles containing 30 mol% cholesterol, throughout the range of Ca^{2+} concentration tested. The cholesterol dependence of fusion was rather complex and depended on the Ca^{2+} concentration. For example, at 5 mM Ca^{2+}, the rate of fusion decreased in the sequence 30% > 0% > 40% > 50% > 20%,

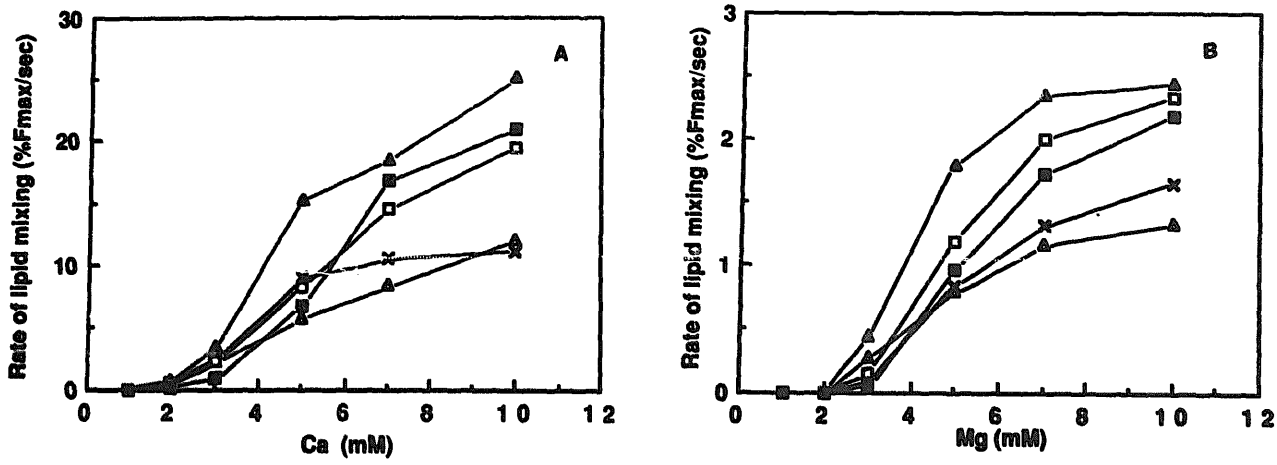

Fig. 5. Dependence of the initial rate of lipid mixing (measured as the increase in NBD fluorescence) of PS/PE (1:1) LUV containing various amounts of cholesterol on the Ca^{2+} (A) or Mg^{2+} (B) concentration. The mol% of cholesterol were 0% (×), 20% (△), 30% (▲), 40% (□) and 50% (■).

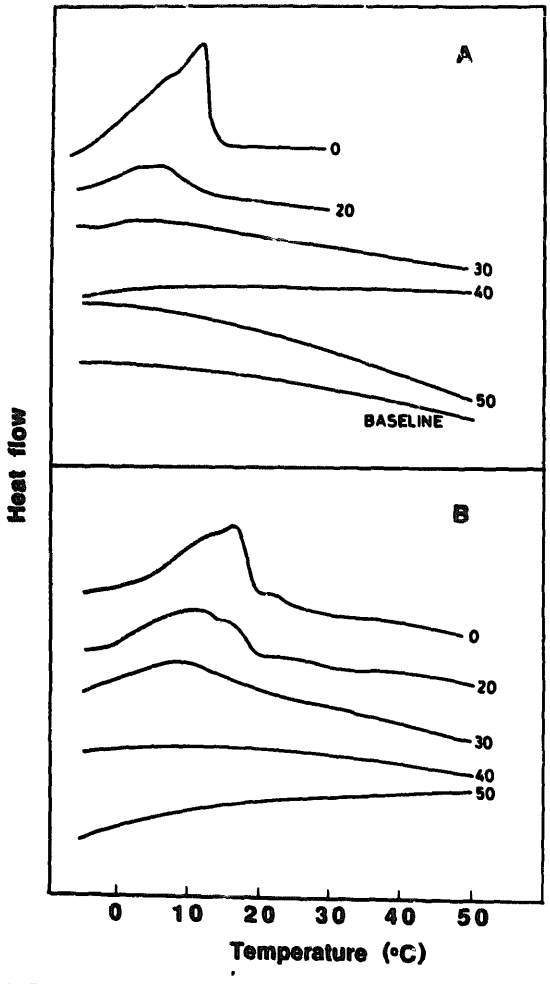

Fig. 6. Differential scanning calorimetry thermograms of PS LUV containing the indicated mol% of cholesterol, and suspended in 100 mM NaCl/5 mM Tes (pH 7.4) and 0.1 mM EDTA (A). In (B) the vesicles were incubated with 10 mM $MgCl_2$. The heating scans are shown.

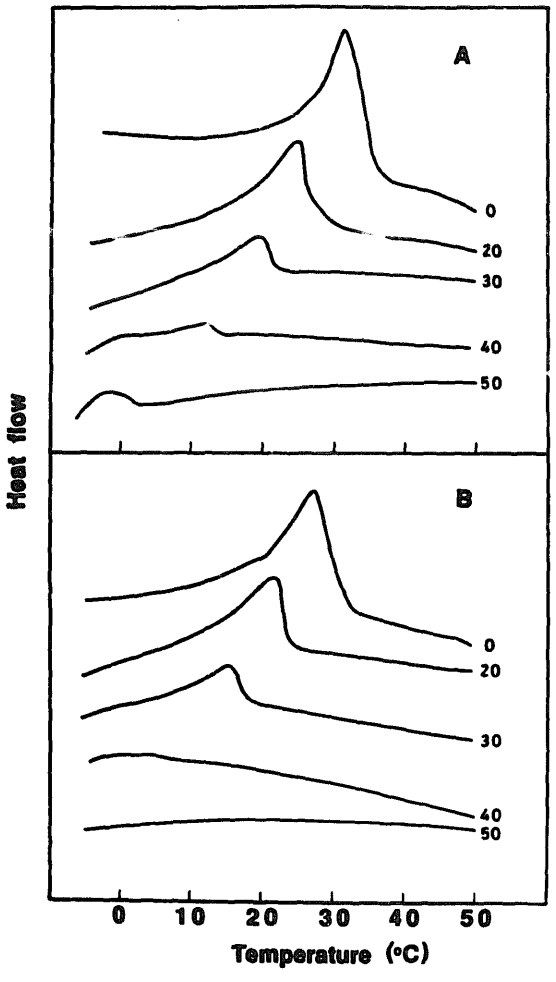

Fig. 7. Differential scanning calorimetry thermograms of LUV composed of PS and the indicated mol% of cholesterol, prepared in NaCl and incubated with 10 mM $BaCl_2$ (A) or $SrCl_2$ (B). The traces are of the heating scans.

whereas at 10 mM Ca^{2+} it decreased in the order 30% > 50% > 40% > 20% ≥ 0%. The initial rates of lipid mixing in the presence of Mg^{2+} were about an order of magnitude lower than those with Ca^{2+}. The highest rates were again obtained with 30 mol% cholesterol, and the cholesterol dependence of the rates was complex. The initial rates appeared to saturate with Mg^{2+} concentrations above 7 mM.

Ionotropic phase transitions in PS membranes containing cholesterol

Large unilamellar PS vesicles in 100 mM NaCl/5 mM Tes (pH 7.4) exhibited a broad gel–liquid crystalline transition endotherm with a peak temperature, T_m, of 11.5 °C. The inclusion of 20 mol% cholesterol in the membrane lowered the peak of the transition endotherm by about 6 C° (Fig. 6A). Increasing the cholesterol content reduced the enthalpy of transition to a non-detectable value at 40 and 50 mol%. The addition of Mg^{2+} to PS LUV caused a shift in the T_m to 16.5 °C (Fig. 6B). The inclusion of cholesterol lowered the T_m to levels expected from the lower T_m of the corresponding Na^+ complexes. The T_m of PS LUV is shifted to higher temperatures in the presence of Ba^{2+} or Sr^{2+} [20]. When cholesterol was included in the membrane the T_m shifted to lower temperatures (Fig. 7A and B). A distinct transition endotherm became apparent even at 30 mol% cholesterol, although the Na^+ complex of PS/cholesterol had a barely detectable endotherm (Fig. 6A). Remarkably, Ba^{2+}, but not Sr^{2+}, induced a transition endotherm at 40 and 50 mol% cholesterol.

Discussion

Our studies indicate that the sequence of effectiveness of divalent cations on the fusion of PS LUV containing cholesterol is $Ca^{2+} > Ba^{2+} > Sr^{2+} \gg Mg^{2+}$, and hence the same as that obtained earlier with pure PS vesicles for the fusion rate constant [19,21,37]. This sequence did not change with temperature in the range 10–40 °C. The difference between the fusogenic activities of Ca^{2+} and Mg^{2+} have been attributed to the difference in the ability of these cations to dehydrate the surface of PS membranes [38–40]. Since we have obtained the same sequence of effectiveness of divalent cations in PS/cholesterol vesicles, it is likely that the addition of cholesterol has not altered the fusion mechanism. Ba^{2+} and Sr^{2+} probably cause intermediate extents of dehydration between Ca^{2+} and Mg^{2+}. However, factors other than dehydration may also be involved in the fusion event [4,6].

As the mole fraction of cholesterol in PS LUV was increased, the initial rate of Ba^{2+}-induced fusion decreased. A similar decrease in the overall rate of fusion with increasing cholesterol content has been obtained for the Ca^{2+}-induced fusion of PS LUV [16]. It should be noted that the rates of fusion reported in our study refer to the overall rates of the aggregation step and the fusion step. Bental et al. [16] have reported a 2-fold decrease in the aggregation rate constant and a 2.5-fold increase in the fusion rate constant by increasing the cholesterol concentration from 0 to 33 mol%.

The initial rate of fusion induced by Sr^{2+} did not change significantly with increasing cholesterol content, while the maximal rate of fusion increased. The maximal rate presumably reflects the increased probability of fusion of a single vesicle when in a multimeric aggregate, compared to a dimeric aggregate. Sr^{2+} is expected to induce extensive aggregation before substantial fusion takes place, because of its low fusogenic capacity compared to Ba^{2+} [19,21]. In this regard, the dependence of the maximal fusion rate on the cholesterol content or temperature may well reflect the destabilization processes incumbent to large areas of apposed membranes, due here to multiple aggregates [41,42]. The initial fusion rates are better indicators of the interaction between two vesicles, and our studies indicate that they are less sensitive to the alteration of cholesterol levels.

The difference in fusion rates between PS and PS/cholesterol vesicles was enhanced with increasing temperatures, especially in the range 30–40 °C. We have previously observed that the overall fusion rates and the fusion rate constants of PS LUV in the presence of Ca^{2+}, Ba^{2+} or Sr^{2+} increase with temperature [20,21,43]. This increase may be related to the greater mobility of the phospholipid and cholesterol molecules at higher temperatures, as evidenced by measurements of microviscosity and lateral mobility in the fluid

state [44–46], but probably not related to changes in binding constants of the ions to PS. The Ca^{2+}/PS binding constant is essentially independent of temperature [47]. The increase in fusion at high temperatures may also be connected to the changes in water structure at these temperatures. Raman spectroscopic studies of water structure as a function of temperature have shown that the mole fraction of water molecules involved in 4-bonded interactions decreases and that involved in 3-bonded interaction increases considerably between 0 and 50°C. Moreover, at temperatures above 30°C, the mole fraction of 2-bonded water molecules begins to increase [50]. These changes in hydrogen bonding between water molecules may thus contribute to the rearrangement of water molecules around the phospholipid headgroups. These changes may then influence the repulsive hydration forces between interacting membranes [6,48,49]. Further studies on hydration forces between PS bilayers containing cholesterol, in the presence of Na^+ or various divalent cations, and at different temperatures, are necessary to clarify some of the possibilities raised above.

The decrease in the initial rate of fusion with cholesterol content in the case of Ba^{2+} (and, to a limited extent, of Sr^{2+} at high temperatures) may be related to several factors. The first is the possibility of the increased hydration of the membrane surface with increasing cholesterol. Studies on PC/cholesterol vesicles indicate that the presence of cholesterol increases the degree of hydration of the membrane surface [51,52]. The second is the dilution of PS molecules by the inclusion of cholesterol. As a result, the possibility of formation of intermembrane contact leading to fusion may be lowered. A third factor is the reduced 'fluidity' of membranes containing cholesterol, as measured by fluorescence anisotropy [46], which implies a reduced molecular mobility.

For lipids with saturated acyl chains, cholesterol primarily broadens the transition endotherm with little change in T_m [53–56]. However, in the case of monounsaturated or diunsaturated phosphatidylcholines, a significant decrease in the T_m has been observed with increasing cholesterol content [57,58]. Bach [59] has reported that multilamellar vesicles composed of mixtures of spinal cord PS and cholesterol reveal significant enthalpies of transition even above 50 mol% cholesterol. In addition, a separate peak at about 37°C was observed, and was interpreted as being due to pure cholesterol. The latter results are rather puzzling and have not been observed with any other phospholipid/cholesterol system, including ours.

The limited shift of the T_m of the PS LUV in the presence of Mg^{2+}, compared to that of multilamellar vesicles of PS, has been attributed to the interaction of Mg^{2+} with only one monolayer of LUV, while the inner monolayer exerts a fluidizing effect [60,61]. The observation that Ba^{2+} induces a transition endotherm at 40 and 50 mol% cholesterol suggests that this ion partially segregates PS molecules, perhaps those with particular acyl chains, to form a Ba^{2+}-PS complex surrounded by cholesterol. This complex is apparently not sufficiently segregated to form a pure domain (which would have produced a transition endotherm at 31.5°C), but is only partially freed from the transition-broadening effect of cholesterol.

With bovine brain PS, cholesterol causes a decrease of T_m. For example, at 25°C, the Ba^{2+} complex of PS transforms from the gel state to the liquid crystalline state when 30 mol% cholesterol is present in the membrane (Fig. 7A). This alteration of the membrane-phase state by cholesterol does not seem to affect the fusion rate in the direction one would expect, since the rate of fusion decreases with increasing cholesterol content. Membranes in the fluid state are, typically, considerably more prone to fusion than those in the solid phase [43]. Since the rate of fusion of PS/cholesterol vesicles is lower than that of pure PS vesicles even at 40°C (Fig. 3), where both the PS and PS/cholesterol complexes with Ba^{2+} are in the fluid phase, we have suggested that the reduction in the 'fluidity' of the membrane by cholesterol [46] may contribute to the lower fusion rate. Since the rate of fusion increases steeply above the T_m for PS-divalent cation complexes [20,21,43], lowering the T_m by the inclusion of cholesterol would be expected to increase the rate of fusion at a particular temperature. This is clearly not the case. Thus, the gel–liquid crystalline transition of the PS/cholesterol-divalent cation complex per se is not a significant determinant of the modulation of fusion by cholesterol.

Although at temperatures below 30°C, Mg^{2+} had no fusogenic activity against PS or

PS/cholesterol vesicles, it induced significant fusion of the latter at 30 °C, and particularly at 40 °C. The effect was maximal at 30 mol% cholesterol. In contrast to Sr^{2+}, both the initial and maximal rates of fusion increased with cholesterol concentration in the presence of Mg^{2+}. Fusion of PS LUV by Mg^{2+} at high temperatures (≥ 40 °C), detected by the Tb/DPA assay, has been reported earlier [43], whereas lipid mixing has been observed at lower temperatures (25 °C) in some studies [62], but not in others [31]. This difference is probably due to the different acyl chain compositions of the PS preparations. Fusion at higher temperatures may be attributed to the temperature dependence of the structure of the Mg^{2+}-PS coordination complex. It appears, therefore, that the presence of cholesterol may exert a further effect on the coordination complex by altering the spacing between PS molecules. This spacing may be particularly crucial in the case of Mg^{2+}, since this ion has rigid requirements for the coordination bonds [63].

The enhancement of the rate of fusion at 30 mol% cholesterol was also observed in the case of PS/PE vesicles fusing in the presence of Ca^{2+} or Mg^{2+} at 25 °C. It is possible that this effect of cholesterol is related to the formation of specific types of phospholipid/cholesterol clusters conducive to participation in fusion. Such an effect of cholesterol was also observed by Stamatatos and Silvius [17] in the case of PS/PE (1 : 3) vesicles containing 33 mol% cholesterol, undergoing Mg^{2+}-induced fusion as monitored by lipid mixing. However, these investigators noted a decrease in the rate of both Mg^{2+}- and Ca^{2+}-induced contents mixing in the presence of cholesterol.

In summary, cholesterol modulates the fusion of PS or PS/PE vesicles in the presence of Ca^{2+}, Ba^{2+}, Sr^{2+}, and Mg^{2+}, although this effect is not very large. Probably the most significant effect of cholesterol is observed in the case of Mg^{2+}-induced fusion of PS/cholesterol vesicles at relatively high, but physiologically relevant, temperatures.

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