Membrane Fusion and the Lamellar-to-Inverted-Hexagonal Phase Transition in Cardiolipin Vesicle Systems Induced by Divalent Cations

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ABSTRACT The polymorphic phase behavior of bovine heart cardiolipin (CL) in the presence of different divalent cations and the kinetics of CL vesicle fusion induced by these cations have been investigated. ^{31}P-NMR measurements of equilibrium cation-CL complexes showed the lamellar-to-hexagonal (L_α-H_{II}) transition temperature (T_H) to be 20–25°C for the Sr^{2+} and Ba^{2+} complexes, whereas in the presence of Ca^{2+} or Mg^{2+} the T_H was below 0°C. In the presence of Sr^{2+} or Ba^{2+}, CL large unilamellar vesicles (LUVs) (0.1 μm diameter) showed kinetics of destabilization, as assessed by determination of the release of an aqueous fluorescent dye, which strongly correlated with the L_α-H_{II} transition of the final complex: at temperatures above the T_H, fast and extensive leakage, mediated by vesicle-vesicle contact, was observed. On the other hand, mixing of vesicle contents was limited and of a highly transient nature. A different behavior was observed with Ca^{2+} or Mg^{2+}: in the temperature range of 0–50°C, where the H_{II} configuration is the thermodynamically favored phase, relatively nonleaky fusion of the vesicles occurred. Furthermore, with increasing temperature the rate and extent of leakage decreased, with a concomitant increase in fusion. Fluorescence measurements, involving incorporation of *N*-NBD-phosphatidylethanolamine in the vesicle bilayer, demonstrated a relative delay in the L_α-H_{II} phase transition of the CL vesicle system in the presence of Ca^{2+}. Freeze-fracture electron microscopy of CL LUV interaction products revealed the exclusive formation of H_{II} tubes in the case of Sr^{2+}, whereas with Ca^{2+} large fused vesicles next to H_{II} tubes were seen. The extent of binding of Ca^{2+} to CL in the lamellar phase, saturating at a binding ratio of 0.35 Ca^{2+} per CL, was close to that observed for Sr^{2+} and Ba^{2+}. It is concluded that CL LUVs in the presence of Ca^{2+} undergo a transition that favors nonleaky fusion of the vesicles over rapid collapse into H_{II} structures, despite the fact that the equilibrium Ca^{2+}-CL complex is in the H_{II} phase. On the other hand, in the presence of Sr^{2+} or Ba^{2+} at temperatures above the T_H of the respective cation-CL complexes, CL LUVs rapidly convert to H_{II} structures with a concomitant loss of vesicular integrity. This suggests that the nature of the final cation-lipid complex does not primarily determine whether CL vesicles exposed to the cation will initially undergo a nonleaky fusion event or collapse into nonvesicular structures.

INTRODUCTION

It is well established that biological membranes contain significant amounts of lipids that, in isolation, do not adopt a lamellar organization but rather prefer an inverted configuration, such as the hexagonal (H_{II}) phase (for reviews, see Cullis et al., 1986, 1990; Gruner et al., 1985). Prominent examples of nonbilayer lipids are cardiolipin (CL) in the presence of Ca^{2+} or other divalent cations (Rand and Sengupta, 1972; Cullis et al., 1978; De Kruijff et al., 1982) and unsaturated phosphatidylethanolamine (PE) (Cullis and De Kruijff, 1978). Among the possible functional roles of nonbilayer lipids in membrane structure and function, the involvement of inverted structures in membrane fusion processes has received a good deal of attention. Early experimental evidence for a role of inverted structures as fusion intermediates has been obtained from freeze-fracture electron-microscopic observations on model systems (for a review, see Verkleij, 1984). In these studies, "lipidic particles," seen at the interface of interacting vesicles, were taken to represent inverted micellar structures functioning as intermediates in bilayer fusion (Verkleij et al., 1979a,b, 1980).

The discussion on the role of inverted structures in membrane fusion processes gained considerable impetus through the contributions of Siegel, Bentz, Ellens, and co-workers (for reviews, see Siegel, 1987; Siegel et al., 1988; Bentz and Ellens, 1988). Early theoretical work of Siegel (1986a,b) suggested that vesicle systems that have a tendency to undergo a lamellar-to-hexagonal (L_α-H_{II}) phase transition rapidly develop inverted micellar intermediates (IMIs) between the apposed bilayers at the sites of vesicle contact. At temperatures above the L_α-H_{II} phase transition temperature of the lipid at equilibrium (T_H), abundant formation of IMIs would induce a lateral aggregation of IMIs in the apposed bilayers to form inverted hexagonal tubes or their precursors, with the concomitant loss of vesicular integrity. Only under specific conditions would the IMIs break into an interlamellar attachment (ILA) site, which in effect corresponds to the formation of a fused vesicular structure. Using vesicles consisting of *N*-methylated dioleoylphosphatidylethanolamine (DOPE-Me), Ellens et al. (1989) have presented evidence for fusion occurring only at tempera-

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tures just below the T_H of the lipid, under conditions where the lipid at equilibrium exhibits an isotropic ^{31}P-NMR signal and the x-ray diffraction pattern of the lipid corresponds to an inverted cubic phase (Siegel, 1986c; Gruner et al., 1988; Shyamsunder et al., 1988; Siegel and Banschbach, 1990).

More recent theoretical considerations suggest that the IMI in fact is not a likely intermediate structure during lipid bilayer fusion. The formation of the IMI appears to require considerably more energy than the formation of an alternative intermediate, the so-called stalk (Siegel, 1993). A "stalk" mechanism for lipid bilayer fusion was originally proposed by Markin et al. (1984) and Chernomordik et al. (1985, 1987) and elaborated afterward (Siegel, 1993; Zimmerberg et al., 1993; Chernomordik and Zimmerberg, 1995). The stalk model gained further support from elegant work showing that lysophosphatidylcholine and free fatty acids inhibit or promote stalk formation and fusion, respectively, as a result of the dynamic shape of these molecules (Chernomordik et al., 1995a). Recent studies (Siegel et al., 1994; Siegel and Epand, 1997) have presented cryo-transmission electron microscopy evidence to indicate that in various phosphatidylethanolamine systems the L_α-H_{II} transition occurs via a mechanism involving stalks rather than IMIs. The stalks would rapidly evolve into *trans* monolayer contacts (TMCs), which would proceed to form either ILAs or H_{II} phase precursors (Siegel and Epand, 1997). Stalks and TMCs in this model in fact represent hemifusion intermediates, while ILAs correspond to pores and complete bilayer merging.

While the relationship between lipid polymorphism and fusion has been examined in considerable detail in PE-containing systems, the situation is much less clear for vesicles containing CL, a lipid that can also adopt an inverted hexagonal configuration under certain conditions. In the absence of divalent cations, CL is organized in a lamellar arrangement, but in the presence of Ca^{2+} or Mg^{2+} it prefers the H_{II} configuration (Rand and Sengupta, 1972; Cullis et al., 1978; De Kruijff et al., 1982; Vasilenko et al., 1982). Ca^{2+}-induced fusion of liposomes composed of mixtures of CL and phosphatidylcholine (PC) has been studied extensively by application of kinetic fluorescence assays, and it appears that these vesicles fuse in a largely nonleaky manner (Wilschut et al., 1982, 1985). However, despite extensive morphological examination of the CL system (Cullis et al., 1978; Verkleij et al., 1979a; De Kruijff et al., 1982; Vasilenko et al., 1982; Lin et al., 1982; Frederik et al., 1989), it remains to be established which kind of intermediate participates in divalent-cation-induced fusion of CL-containing vesicles.

In the present paper we report on the fusion of CL LUVs induced by various divalent cations. Fusion was studied by monitoring mixing and leakage of aqueous vesicle contents using the terbium/dipicolinic acid (Tb/DPA) assay (Wilschut et al., 1980, 1981). The fusion characteristics are related to the polymorphic behavior of the different cation-CL complexes, as examined by ^{31}P-NMR and freeze-fracture electron microscopy. The following divalent cations were investigated: Ca^{2+}, Ba^{2+}, Sr^{2+}, and Mg^{2+}. It has been established that the T_H of the Ca^{2+}-CL and Mg^{2+}-CL complexes at equilibrium is below 0°C, while the T_H of the the Ba^{2+}-CL complex is ~25°C (Vasilenko et al., 1982). Thus in the case of Ba^{2+} (and for Sr^{2+}) fusion characteristics can be examined at low temperatures, where the lipid remains lamellar, or at higher temperatures, where the lipid is induced to undergo a lamellar-to-H_{II} phase transition, while with Ca^{2+} and Mg^{2+} a lamellar-to-H_{II} transition is always induced. It is concluded that the nature of the final cation-lipid complex is not the primary determinant of whether, initially, nonleaky fusion between the vesicles will occur.

MATERIALS AND METHODS

Materials

Bovine heart CL and *N*-(7-nitro-2,1,3-benz-oxadiazol-4-yl) phosphatidylethanolamine (*N*-NBD-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). $TbCl_3 \cdot 6H_2O$ was from Aldrich (Brussels, Belgium), and dipicolinic acid (DPA) was from Sigma Chemical Co. (St. Louis, MO). 5,6-Carboxyfluorescein (CF) was from Eastman Kodak (Rochester, NY) and was purified according to the method of Ralston et al. (1980). [^{14}C]Sucrose, $^{45}CaCl_2$ (2 mCi/ml, 21 mCi/mg Ca), $^{90}SrCl_2$, and $^{133}BaCl_2$ were obtained from Amersham International (Amersham, England). The Ca^{2+} ionophore A23187 was from Boehringer (Mannheim, Germany). All of the other reagents were of the highest purity available.

Vesicle preparation

LUVs were prepared from CL by reverse-phase evaporation (Szoka and Papahadjopoulos, 1978) and extrusion (Hope et al., 1985) through 0.1-μm-pore Unipore polycarbonate filters (Nuclepore, Pleasanton, CA), essentially as described before (Wilschut et al., 1980, 1983). The trapped volume of the vesicles was ~1.9 l/mol, as determined by the encapsulation of 1 mM [^{14}C]sucrose (1 μCi/ml) and that of Tb^{3+}.

Vesicles to be used in the Tb/DPA assay were prepared in one of the following aqueous media: 1) 5 mM $TbCl_3$, 50 mM sodium citrate (Tb vesicles); 2) 50 mM sodium dipicolinate, 20 mM NaCl (DPA vesicles); or 3) 2.5 mM $TbCl_3$, 25 mM sodium dipicolinate, 10 mM NaCl (Tb/DPA vesicles). All of the above media contained 5 mM HEPES adjusted to a final pH of 7.4. Vesicles to be used in the CF assay were prepared in a medium containing 50 mM CF and 5 mM HEPES adjusted to a pH of 7.4. Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-75, using 100 mM NaCl, 1.0 mM EDTA, 5 mM HEPES (pH 7.4) as elution buffer.

To follow the kinetics of the lamellar-to-hexagonal phase transition in vesicle systems (Bentz et al., 1987; Hong et al., 1988), *N*-NBD-PE was incorporated into the vesicle membrane to a concentration of 0.1 mol% (relative to lipid phosphorus), and the vesicles were prepared in 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES (pH 7.4).

Vesicle concentrations were determined on the basis of lipid phosphorus, according to the method of Böttcher et al. (1961).

Vesicle aggregation and fusion

Aggregation was followed by turbidity measurements at a wavelength of 450 nm in a Beckman DU-7 spectrophotometer. The lipid concentration was 25 μM.

Fusion was followed on the basis of mixing of aqueous vesicle contents, as assessed by the Tb/DPA assay (Wilschut et al., 1980, 1981, 1983). A

small aliquot (100 μl) of a concentrated 1:1 mixture of Tb and DPA vesicles was injected into a cuvette containing a final volume of 2 ml of 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES (final concentrations) and $CaCl_2$, $MgCl_2$, $SrCl_2$, or $BaCl_2$ at the desired final concentrations. The medium in the cuvette was stirred continuously and maintained at the desired temperature. Fluorescence was recorded continuously with an SLM 8000 fluorometer equipped with a double excitation monochromator (SLM/Aminco, Urbana, IL). Excitation and emission wavelengths were 276 and 545 nm, respectively, and a cutoff filter (<530 nm) was placed between the sample and the emission monochromator to eliminate interference from light scattering. The fluorescence scale was calibrated in the presence of 20 μM DPA in the medium, in the absence of EDTA, by releasing the Tb from an appropriate concentration of Tb vesicles with cholate (0.5% w/v). Thus the 100% value corresponded to all of the Tb present being complexed to DPA (Wilschut et al., 1980, 1981). It has been reported that this calibration procedure cannot be used at high temperatures because of a difference in the extent of dissociation of the diluted Tb/DPA complex after release of Tb from the vesicles upon the addition of detergent and that of the complex trapped at a high concentration inside the vesicles (Ellens et al., 1989). We have observed that this effect becomes significant only above 50°C. At this temperature, under the conditions of our experiments, the difference between the fluorescence of the Tb/DPA complex encapsulated in the vesicles and that of the equivalent amount of Tb after release from the vesicles in the presence of 20 μM DPA was still only as little as 8%.

Stopped-flow fluorescence measurements of mixing of aqueous vesicle contents were made in a modular spectrofluorometer from HiTech (Salisbury, England). The excitation monochromator was set at 276 nm, and the emission was followed with a HiTech OG530 cutoff filter (530 nm).

Leakage of vesicle contents

Leakage of preencapsulated Tb/DPA complex was measured by following its fluorescence quenching (Bentz et al., 1983). Measurements were carried out in the same way as the fusion measurements, except that one population of Tb/DPA vesicles (at the same total phospholipid concentration as in the corresponding fusion measurements) was used. The fluorescence scale was calibrated in the same way as in the corresponding fusion measurements.

Release of CF was measured in the same buffer as that used in the Tb/DPA experiments. Excitation and emission wavelengths were 430 and 520 nm, respectively. For calibration of the fluorescence scale, maximum release was induced by lysing the vesicles with 1% (v/v) Triton X-100.

Lamellar-to-hexagonal transition

An assay based on the increase in *N*-NBD-PE fluorescence incorporated into the vesicle bilayer was employed to monitor the kinetics of cation-induced L_α-H_{II} transitions in CL LUV systems and to estimate the T_H (Bentz et al., 1987; Hong et al., 1988). A small aliquot (100 μl) of a concentrated vesicle suspension was injected into a cuvette with the NaCl/HEPES buffer used in the fusion and leakage assays containing the desired concentration of Ca^{2+}, Sr^{2+}, or Ba^{2+}, and the relative increase in fluorescence was continuously monitored at excitation and emission wavelengths of 465 and 530 nm, respectively, with a cutoff filter (<520 nm) between the sample and the emission monochromator. The initial rate of the fluorescence change was taken as a measure of the kinetics of the L_α-H_{II} transition in the system.

^{31}P-NMR measurements

Lipid samples for ^{31}P-NMR were prepared by dispersing 35 μmol (lipid phosphorus) of CL, dried from chloroform under high vacuum as a thin film in the bottom of a glass tube, in 5 ml 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES (pH 7.4) containing 10 mM divalent cation, at 0°C. The samples were freeze-thawed three times in liquid N_2, and the CL salts were collected by centrifugation at 0°C. A new aliquot of buffer containing 10 mM of the divalent cation was added, the lipid was dispersed, and the dispersion was freeze-thawed three times. After centrifugation, this procedure was repeated two more times, and the pellets were finally resuspended in 1 ml of buffer containing 10 mM divalent cation and kept on ice until the NMR spectra were collected. High-power, proton noise-decoupled, ^{31}P-NMR spectra were obtained as described before (Chupin et al., 1987); 600 scans were collected for each spectrum, with a 2-s interpulse time and a 5-min equilibration between temperatures.

Electron microscopy

Freeze-fracture electron microscopy was performed according to established procedures. Equilibrium samples of CL in the presence of divalent cations were prepared in the same way as the samples used for ^{31}P-NMR and quenched with the jet-freezing technique, using a KF 80 Reichert Jung in the absence of cryoprotectant. Alternatively, CL LUVs were examined after exposure to divalent cations under conditions as applied in the fusion assay. Briefly, 50 ml of a CL LUV suspension at a concentration of 50 μM (lipid phosphorus) was incubated at 50°C for 10 min in the presence of either 10 mM $CaCl_2$ or $SrCl_2$. Vesicle aggregates were collected by centrifugation at 25,000 $\times$ *g* at 37°C and jet-frozen from room temperature for freeze-fracturing.

Cation binding

Binding of Ca^{2+}, Sr^{2+}, and Ba^{2+} to CL LUVs was determined by equilibrium dialysis using $^{45}Ca^{2+}$, $^{90}Sr^{2+}$, and $^{133}Ba^{2+}$. The experiments were carried out in a Dianorm dialysis device (Diachema, Zürich, Switzerland), using 2-ml Teflon cells separated into two 1-ml compartments by a Spectrapor-2 membrane. CL LUVs were prepared as described above. In the case of Ca^{2+}, the Ca^{2+} ionophore A23187 was incorporated into a portion of the vesicle suspension by the addition of a concentrated ethanolic solution to a final ionophore-to-lipid molar ratio of 1:1000. One compartment of each cell was loaded with 1 ml of a 1.5 mM CL LUV suspension, and the other with 1 ml of $CaCl_2$, $SrCl_2$, or $BaCl_2$ solutions of different concentrations, in 100 mM NaCl, 0.1 mM EDTA, 5 mM HEPES (pH 7.4), containing an appropriate amount of the radioactive cation. The cells were rotated at 10 rpm for 4 h at 20–25°C, after which samples from each of the two compartments were collected and analyzed for lipid content by phosphorus determination (Böttcher et al., 1961) and cation concentration by radioactivity measurements. The relative binding of the cation to the lipid was calculated by dividing the difference in the cation concentration between the two compartments by the measured lipid concentration, while the cation concentration in the compartment without CL was taken as the final free cation concentration. Turbidity (A_{450}) was also determined for each liposome suspension immediately after the dialysis experiment.

RESULTS

Characterization of final equilibrium states

First we determined the bilayer-to-hexagonal (H_{II}) transition temperature (T_H) of the four different cation-CL complexes in equilibrium with 10 mM of the cation in the medium (this particular cation concentration was chosen for reasons outlined below). Samples were prepared at 0–4°C. ^{31}P-NMR spectra were taken starting at 5°C and subsequently at higher temperatures at 5° intervals.

As an example, Fig. 1 shows the ^{31}P-NMR spectra obtained with the Sr^{2+}-CL complex. Below 20°C, the complex exhibited an NMR spectrum with a high-field peak and a low-field shoulder, consistent with a lamellar lipid arrange-

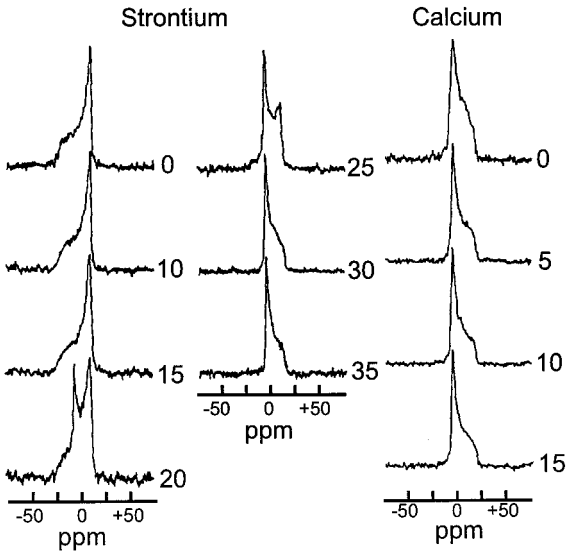


FIGURE 1 ^{31}P-NMR spectra for dispersions of CL in final equilibrium with 10 mM Sr^{2+} or 10 mM Ca^{2+} at the temperatures (°C) indicated.

ment. On the other hand, at 30°C and above, the spectrum revealed an inversed symmetry and a twofold reduced width, which is consistent with the hexagonal H_{II} phase. At intermediate temperatures, features of either lipid organization can be recognized in the NMR spectrum. The Ba^{2+}-CL complex showed behavior very similar to that of the Sr^{2+}-CL complex, with an estimated T_H value of 20°C (results not shown). The Ca^{2+}-CL (Fig. 1) and Mg^{2+}-CL (not shown) complexes were hexagonal throughout the entire temperature range examined. Importantly, no isotropic signal was observed in any of the spectra at any temperature, indicating that the transition intermediates are short-lived on the NMR time scale and that, at equilibrium, only bilayer and/or H_{II} structures are present.

Vesicle aggregation

Fig. 2 shows the dependence of the aggregation of CL LUVs on the concentration of divalent cations in the medium. Aggregation was monitored at room temperature as an increase in the apparent absorbance at 450 nm (turbidity) at a lipid concentration of 25 μM. For each of the cations used, the threshold concentration for aggregation was in the range of 4–6 mM; above this value aggregation rates increased sharply. A similar sharp increase in the rate of vesicle aggregation and fusion has been noted before in studies on CL/PC vesicle systems in the presence of Ca^{2+} (Wilschut et al., 1982, 1985). In all subsequent fusion experiments a cation concentration of 10 mM was used. This concentration is well above the threshold value for all four cations and lies above the concentration range in which the rate of vesicle aggregation is steeply dependent on the cation concentration.

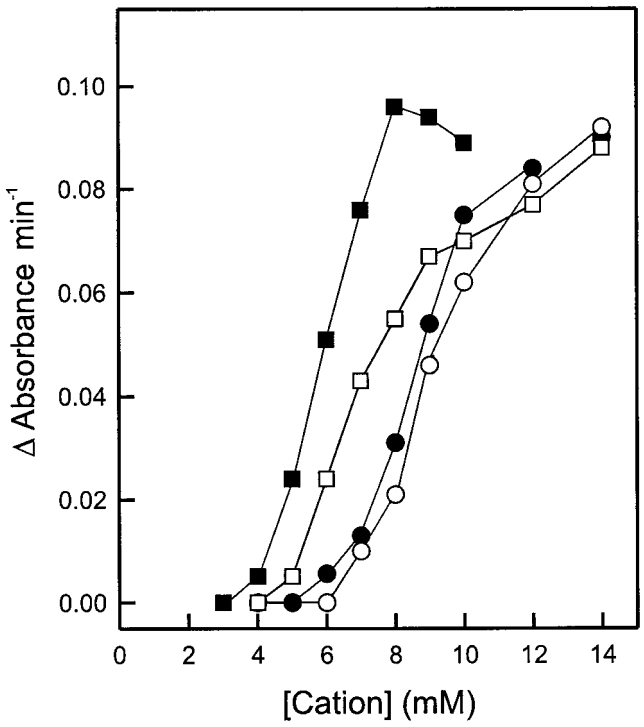


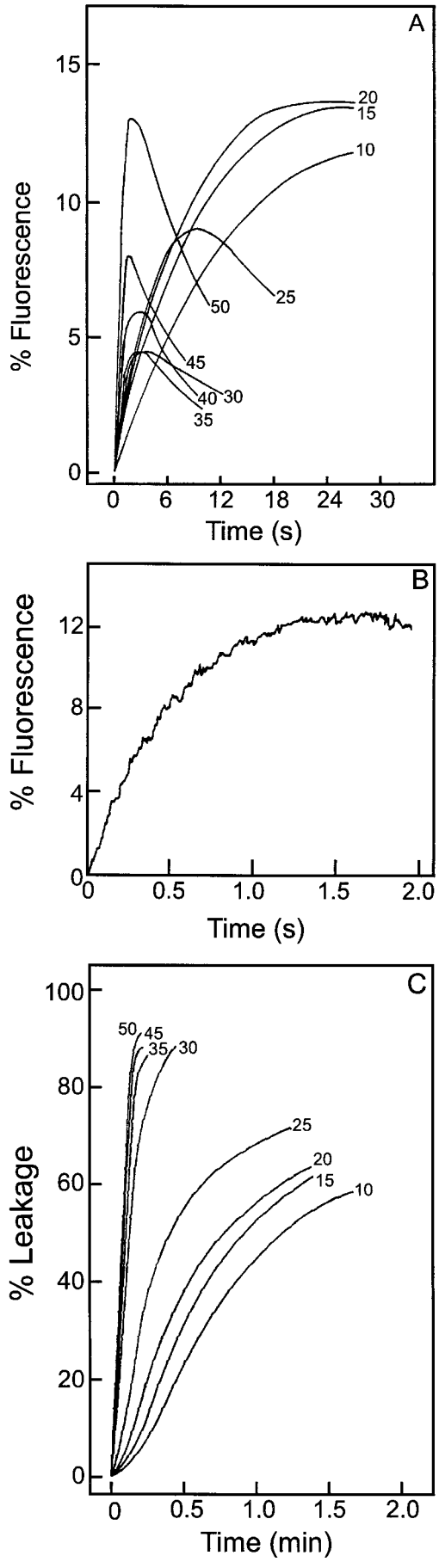
FIGURE 2 Divalent ion concentration dependence of the initial rate of increase of turbidity (A_{450}) of CL LUVs at 25°C. The lipid concentration was 25 μM. ○, Mg^{2+}; ●, Ca^{2+}; □, Sr^{2+}; ■, Ba^{2+}.

Sr^{2+}- and Ba^{2+}-induced mixing and release of vesicle contents

Fig. 3 *A* shows the fluorescence development curves upon injection of a 1:1 mixture of Tb- and DPA-containing LUVs, at a lipid concentration of 25 μM, into a medium containing 10 mM $SrCl_2$ at different temperatures. An initial rapid fluorescence increase was observed, due to mixing of internal contents during fusion, followed by a slow decrease in fluorescence due to the release of vesicle contents to the external medium (Wilschut and Papahadjopoulos, 1979; Wilschut et al., 1980, 1981, 1982, 1983). The initial rate of fusion increased with increasing temperature. However, importantly, the shape of the curves changed markedly when the temperature was raised through the T_H of the final Sr^{2+}-CL complex (25°C). Specifically, at and above 25°C, the secondary decrease in the fluorescence intensity, representing the release of vesicle contents, became prominent, indicating a rapid collapse of the vesicles.

The fast contents mixing at 50°C in the presence of 10 mM Sr^{2+} was investigated in further detail by means of stopped-flow fluorescence spectroscopy. Fig. 3 *B* shows that a time-resolved increase in Tb fluorescence was observed, reaching a maximum of 13% after 2 s. This experiment clearly indicates that fast mixing of aqueous vesicle contents does occur before leakage becomes prominent.

Leakage of aqueous vesicle contents to the external medium was also monitored directly in separate experiments. Tb/DPA complex was encapsulated within the vesicles, and



the decrease in fluorescence was followed under conditions identical to those in the fusion assay. Fig. 3 *C* shows the results for the case of Sr^{2+} at 10 mM. Again, the rate of leakage of vesicle contents increased steeply at temperatures above the T_H of the final Sr^{2+}-CL complex. Similar fusion and leakage characteristics were observed for CL LUVs in the presence of 10 mM $BaCl_2$ (results not shown).

Ca^{2+}- and Mg^{2+}-induced mixing and release of vesicle contents

Fig. 4 shows the curves for Ca^{2+}-induced mixing and leakage of contents in the CL LUV system at different temperatures. The initial rate of fusion (Fig. 4 *A*) appeared to increase with temperature in a more gradual manner than in the presence of Sr^{2+} (cf. Fig. 3). Remarkably, during the period of time shown in Fig. 4 *A*, there was only a marginal secondary decrease in the fluorescence intensity. This is indicative of a relatively slow rate of release of vesicle contents during the fusion process.

These results were confirmed by direct leakage measurements (Fig. 4 *B*). Clearly, release rates were relatively slow, and, moreover, the rates decreased with increasing temperature. This latter observation is consistent with the corresponding fusion experiments, where very high sustained levels of fluorescence intensity were attained at the higher temperatures, reaching values of ~80% at 50°C (Fig. 4 *A*). On the other hand, the results are in marked contrast to those obtained with Sr^{2+} or Ba^{2+}, where very high rates of leakage were observed at elevated temperatures. With Mg^{2+} we observed fusion characteristics qualitatively similar to those seen with Ca^{2+} (not shown). Release in the presence of Mg^{2+} was significantly higher than with Ca^{2+}, and it did not decrease with increasing temperatures; rather the rate of release in the presence of Mg^{2+} remained almost constant throughout the temperature range studied (release curves not shown, but see Fig. 5).

Fig. 5 presents a survey of the initial rates of fusion (Fig. 5 *A*) and leakage (Fig. 5 *B*). Clearly, the rates of Ca^{2+}- and Mg^{2+}-induced fusion increased in a gradual manner with increasing temperature. By contrast, for Sr^{2+} and Ba^{2+} the fusion rates started to increase in the temperature range of the L_α-H_{II} transition. With these latter ions, concomitant increases in the rate of release of vesicle contents were observed (Fig. 5 *B*). Fig. 5 *B* clearly shows the deviating behavior of the Ca^{2+}-CL and Mg^{2+}-CL systems in this

FIGURE 3 (*A*) Fluorescence development during Sr^{2+}-induced fusion of CL LUV as monitored by the Tb/DPA assay. The temperature (°C) is indicated. A 1:1 mixture of Tb and DPA vesicles was injected into a medium containing 10 mM $SrCl_2$ (final concentration) at pH 7.4. The final lipid concentration was 25 μM. The increase in Tb fluorescence was monitored continuously. (*B*) Stopped-flow time-resolved fluorescence curve for the system described in *A* at 50°C. CL LUV suspensions and cation solutions were mixed at a 1:2 (v/v) ratio. (*C*) Leakage of contents for the system described in *A*, measured by the release of Tb/DPA complex (decrease in fluorescence intensity).

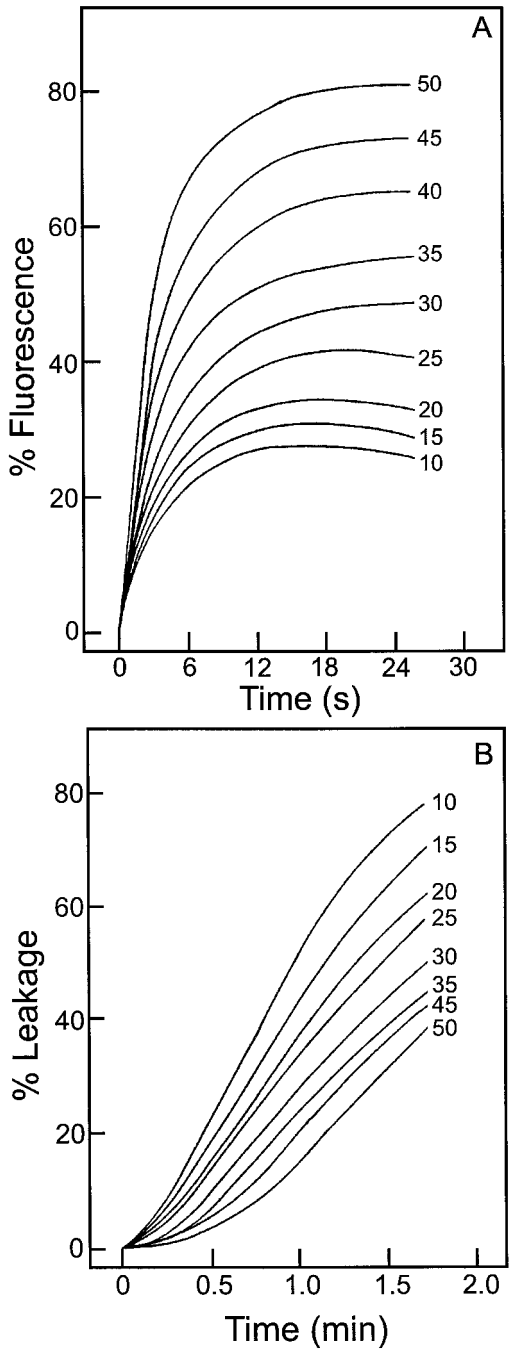


FIGURE 4 (*A*) Fluorescence development during Ca^{2+}-induced fusion of CL LUVs as monitored by the Tb/DPA assay. The temperature (°C) is indicated on the curves. A 1:1 mixture of Tb and DPA vesicles was injected into a medium containing 10 mM $CaCl_2$ (final concentration) at pH 7.4. The final lipid concentration was 25 μM. The increase in Tb fluorescence was monitored continuously. (*B*) Leakage of contents for the same systems as in *A*, measured by release of Tb/DPA complex (decrease in fluorescence intensity).

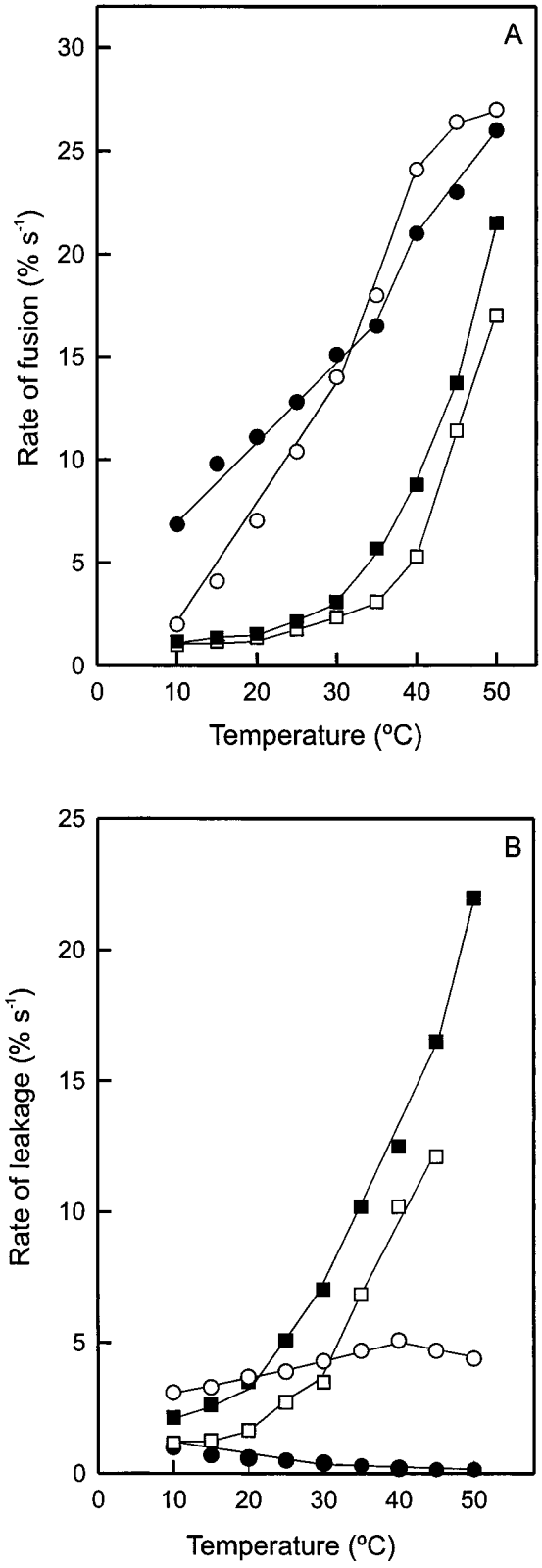


FIGURE 5 (*A*) Temperature dependence of the initial rate of divalent cation-induced fusion of CL LUVs as monitored by the Tb/DPA assay. (*B*) Temperature dependence of the rate of divalent cation-induced leakage of CL LUVs as monitored by the Tb/DPA release assay. Rates were determined from the tangents to the steepest parts of the curves. ○, Mg^{2+}; ●, Ca^{2+}; □, Sr^{2+}; ■, Ba^{2+}. The concentration of the ions was 10 mM, and the lipid concentration was 25 μM.

respect. Even though in the entire temperature range studied the final complexes of these ions with CL are in the H_{II} configuration, the rates of release, determined from the tangents to the steepest parts of the curves, remained constant or even decreased with increasing temperature. Indeed,

with Ca^{2+} the rate of leakage decreased to a relatively very low value of 0.6%/s at 50°C.

To ascertain that the remarkable leakage characteristics of the Ca^{2+}-CL system were not due to a peculiarity of the Tb/DPA complex, release was also measured utilizing the relief of fluorescence self-quenching of carboxyfluorescein (CF). The pattern obtained for the four cations studied was very similar to that obtained with the Tb/DPA leakage assay (results not shown). When measured with CF, the decrease in the rate of release with increasing temperature in the Ca^{2+}-CL system was even more prominent than with the Tb/DPA leakage assay.

Leakage is mediated by vesicle-vesicle contact

From the above results it is evident that the release of vesicle contents in the Sr^{2+}-CL or Ba^{2+}-CL systems is highly dependent on the system being competent to undergo an L_α-H_{II} phase transition upon exposure of bilayer vesicles to the particular cation. Within this context, it was of interest to investigate whether the release of vesicle contents occurring above the T_H of the final cation-CL complexes is dependent on vesicle-vesicle contact (Ellens et al., 1984, 1986). When a double-logarithmic plot of the initial rates of CF leakage as a function of the vesicle concentration at 40°C in the presence of 10 mM Sr^{2+} or Ba^{2+} was made, for both ions straight lines were obtained with a slope very close to 2 (results not shown). Thus the release process is of second order with respect to the vesicle concentration, indicating that it is dependent on vesicle-vesicle interaction. This in turn demonstrates a requirement for vesicle-vesicle contact in the formation of H_{II}-phase precursors in vesicular systems.

Kinetics of the lamellar-to-hexagonal transition

The fast and extensive release of aqueous contents from CL vesicles in the presence of Sr^{2+} or Ba^{2+}, specifically at temperatures above the T_H of the final cation-CL complexes, suggests that the vesicular lamellar phase is rapidly converted to H_{II} phase precursors upon exposure of the vesicles to the cation. Because in the Ca^{2+}-CL system the rate of release was slow and decreased with increasing temperature, it would appear that, upon addition of the cation to CL vesicles, the formation of H_{II} phase precursors is retarded. Fig. 6 presents the results of an experiment in which the kinetics of the L_α-H_{II} phase transition were determined in the various cation-CL vesicle systems studied. It has been shown previously that the fluorescence quantum yield of *N*-NBD-PE incorporated into a phospholipid bilayer system increases when the system undergoes a bilayer-to-hexagonal phase transition (Bentz et al., 1987; Hong et al., 1988). This increase in fluorescence intensity was exploited to determine the kinetics of the L_α-H_{II} transition in CL LUVs upon exposure to 10 mM $SrCl_2$, $BaCl_2$, or $CaCl_2$.

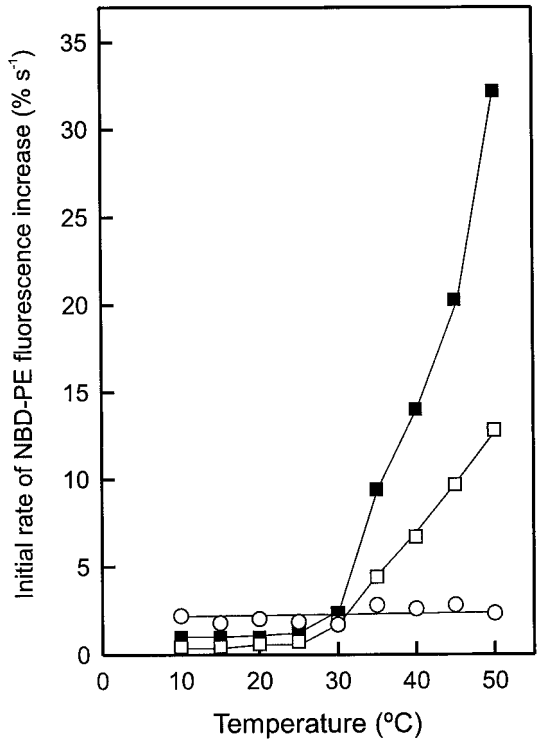


FIGURE 6 Initial rates of the Ca^{2+}- (○), Sr^{2+}- (□), and Ba^{2+}- (■) induced increase in the fluorescence of *N*-NBD-PE (0.1 mol% relative to total lipid) containing heart CL LUVs as a function of temperature. The concentration of the ions was 10 mM, and the lipid concentration was 25 μM.

With Sr^{2+} or Ba^{2+} an abrupt increase in the rate of *N*-NBD-PE fluorescence increase was observed at temperatures above 25°C (Fig. 6), corresponding to the T_H of the final cation-CL complexes (cf. Fig. 2). This indicates a rapid conversion of the bilayer system to H_{II} phase (precursors). On the other hand, with Ca^{2+} the rate of fluorescence increase remained constant and low in the entire temperature range studied, indicating that indeed in this system the formation of H_{II} phase (precursors) is delayed. This retardation apparently favors the relatively nonleaky fusion of the vesicles (cf. Fig. 4).

Electron microscopy

Samples corresponding to mixtures of CL LUVs with Ca^{2+} or Sr^{2+} were examined using freeze-fracture electron microscopy (Fig. 7). Fig. 7 *A* shows the Ca^{2+} salt of CL obtained after repeated equilibration with 10 mM Ca^{2+} at 4°C, to ensure formation of 1:1 complexes, as was done for the preparation of the ^{31}P-NMR samples. Only the H_{II} phase was seen in this case, establishing that this is the thermodynamically favored phase for the Ca^{2+}-CL complex at 4°C and higher. On the other hand, a different structure was obtained after exposure of a dilute suspension of CL LUVs to 10 mM Ca^{2+} at 50°C. Fig. 7, *B* and *C*, shows that this condition did not result in the formation of a pure H_{II} phase.

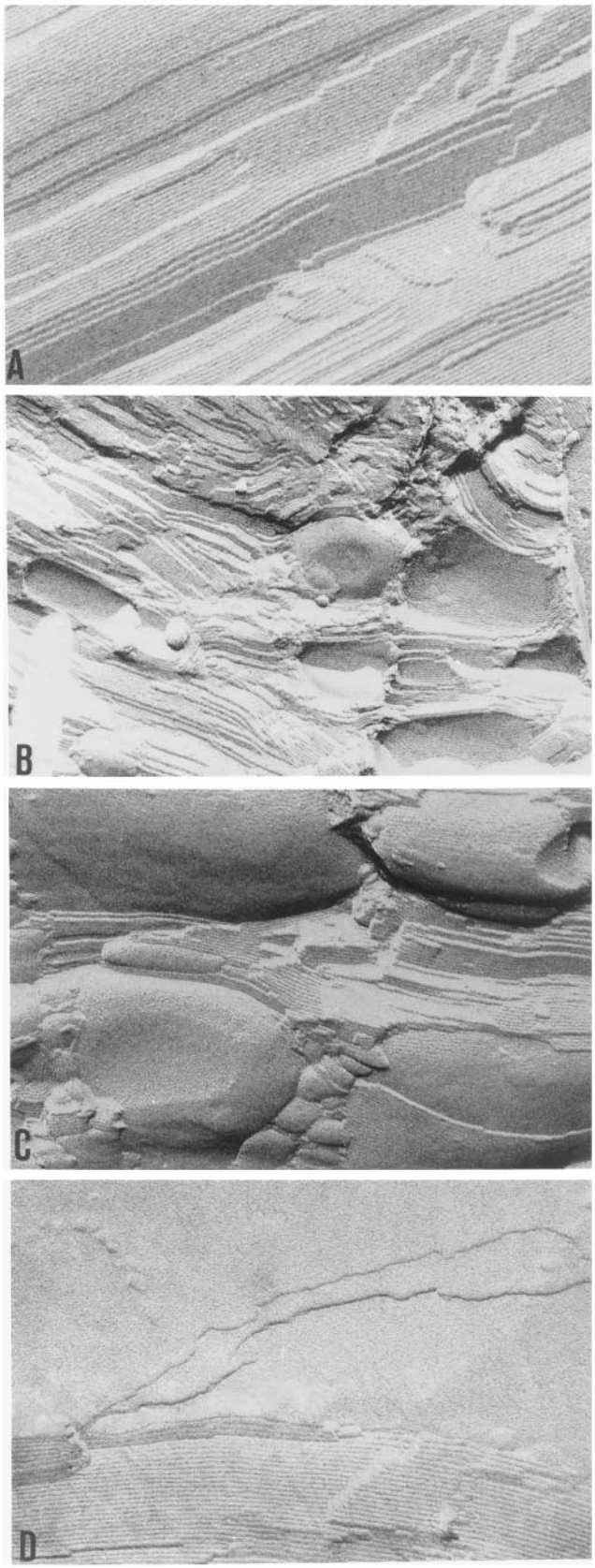


FIGURE 7 Freeze-fracture electron micrographs of various Ca^{2+}-CL and Sr^{2+}-CL systems. (*A*) Equilibrium Ca^{2+}-CL complex in the presence of 10 mM Ca^{2+} (for details of the procedure see Materials and Methods). (*B, C*) Ca^{2+}-CL complexes obtained after the addition of a concentrated CL LUV suspension to a medium containing a final concentration of 10 mM $CaCl_2$ (final lipid concentration 50 μM), followed after 10 min at 50°C by sedimentation of the aggregates at 20,000 × *g* and freezing of the samples from room temperature (see Materials and Methods). (*D*) Sr^{2+}-CL complexes obtained as in *B* and *C*. Magnifications: *A, C,* and *D,* 100,000×; *B,* 50,000×.

Rather a mixture of aggregated vesicles larger than the starting vesicles and a H_{II} phase were observed. Even though these electron micrographs, obtained after a 10-min exposure of the vesicles to Ca^{2+} and subsequent collection of the vesicle aggregates by centrifugation, do not reveal dynamic fusion intermediates, the results do indicate that the formation of the H_{II} phase in the Ca^{2+}-CL LUV system is kinetically retarded in favor of the formation of larger fused vesicles.

In Fig. 7 *D* the structures obtained in a similar experiment with Sr^{2+} ions are shown. Hexagonal tubes were observed, in some areas along with lipid in the lamellar phase. No vesicular structures remained whatsoever. The presence of the lamellar phase may be due to the fact that the sample was frozen from a temperature just around the T_H of the Sr^{2+}-CL complex.

Cation binding to CL LUVs

In an attempt to find an explanation for the relative retardation of H_{II} phase precursor formation in the Ca^{2+}-CL system, we considered a possible effect of the extent of Ca^{2+} binding to CL in the lamellar state versus the extent of binding to CL in the H_{II} phase, as compared to the binding of the other cations. It has been reported that binding of Ca^{2+} to CL bilayers saturates at a ratio of ~0.35 Ca^{2+} per CL, while in the H_{II} phase the binding ratio is 1:1 (De Kruijff et al., 1982). The limited degree of Ca^{2+} binding to CL bilayers could be a reason for the retardation of H_{II} formation in vesicular systems. Inasmuch as De Kruijff et al. (1982) performed their binding studies with multilamellar CL vesicles, we determined the extent of Ca^{2+} binding to CL LUVs, using $^{45}Ca^{2+}$.

Fig. 8 *A* shows that in the 0.4–2.2 mM free Ca^{2+} range the apparent Ca^{2+}/CL binding ratio approached a value of ~0.2. Despite a small increase in turbidity (Fig. 8 *B*), under these conditions the system remained lamellar and vesicular integrity was maintained, as addition of the Ca^{2+}-ionophore A23187 resulted in practically a doubling of the amount of Ca^{2+} bound per CL (Smaal et al., 1987), indicating that in the absence of the ionophore the vesicles are largely impermeable to Ca^{2+}. At higher free Ca^{2+} concentrations the amount of Ca^{2+} bound per CL increased (Fig. 8 *A*), along with a large increase in the turbidity of the suspension (Fig. 8 *B*). At ~5 mM free Ca^{2+} the binding ratio reached a value of 1:1. Accordingly, the lipid was present in the form of relatively few large aggregates, impeding turbidity measurements, while also under these conditions the binding ratios in the absence and presence of the ionophore were the

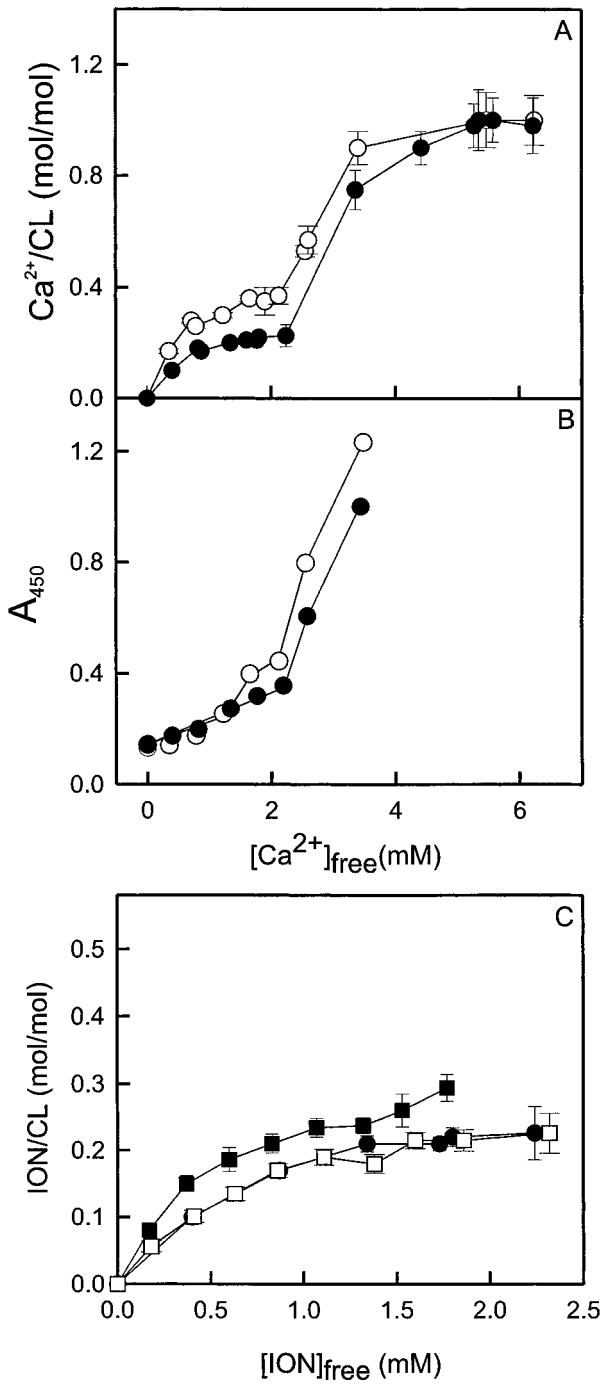


FIGURE 8 (*A*) Ca^{2+} binding and (*B*) turbidity measurements (A_{450}) to CL LUVs in the absence (●) or presence (○) of the ionophore A23187, as a function of the free Ca^{2+} concentration. The lipid concentration was 1.5 mM, and dialysis was performed for 4 h at 20–25°C. (*C*) Binding of Ca^{2+} (●), Sr^{2+} (□), and Ba^{2+} (■) to CL LUVs in the lamellar phase, under similar conditions. All measurements were made in triplicate; average values (± SEM) are presented.

same. In summary, these binding studies indicate that Ca^{2+} binding to CL in the lamellar phase saturates at a binding ratio of ~0.35, whereas in the H_{II} phase a stoichiometric 1:1 complex is formed.

Binding experiments with radioactive Sr^{2+} or Ba^{2+} revealed binding characteristics very similar to those of Ca^{2+}. As shown in Fig. 8 *C*, binding saturated at approximately the same cation/CL binding ratio in the lamellar phase. Within this concentration range, there was only a small increase in turbidity, indicating that the vesicles remained in the lamellar phase (not shown). The saturation of cation binding to lamellar CL at a ratio of 0.35 implies that in the kinetic fusion studies, even though these are performed at a relatively high cation concentration, the initial binding of cation to the outer monolayer lipid of dispersed vesicles would remain relatively low, corresponding to a ratio of 0.35.

DISCUSSION

Within the context of the discussion about the molecular mechanisms of membrane fusion processes, pure lipid systems, particularly those that can convert from a lamellar (L_α) to a hexagonal (H_{II}) configuration, have received a great deal of attention. One example is DOPE-Me, where vesicle fusion occurs preferentially at temperatures just below the L_α-H_{II} transition temperature T_H (Ellens et al., 1984, 1986, 1989; Bentz and Ellens, 1988; Siegel et al., 1989). While it had been proposed originally that the mechanism of the L_α-H_{II} transition and vesicle fusion in the DOPE-ME system would involve IMIs (Siegel, 1986a,b,c, 1987; Bentz and Ellens, 1988), recent cryo-transmission electron microscopy studies of DOPE-Me (Siegel et al., 1994) and dipalmitoleoyl-PE (Siegel and Epand, 1997) suggest that the transition and fusion are mediated by stalks evolving into TMCs and, subsequently, ILAs (Siegel et al., 1994). Accordingly, theoretical work of Siegel (1993) shows that the formation of stalks is energetically more favorable than the formation of IMIs. A stalk mechanism for membrane fusion processes had been proposed originally by Markin et al. (1984) and Chernomordik et al. (1985, 1987), the stalk representing in fact a hemifusion configuration between two interacting lipid bilayer membranes. There is accumulating evidence to indicate that stalks are also involved as dynamic intermediates in protein-mediated biological membrane fusion (Chernomordik and Zimmerberg, 1995; Chernomordik et al., 1995b,c, 1997; Vogel et al., 1993; Yeagle et al., 1994).

CL, examined in the present study, represents another phospholipid that can undergo an L_α-H_{II} transition. The ^{31}P-NMR data in Fig. 1 show that, in the Sr^{2+}-CL complex at equilibrium with 10 mM of the cation in the medium, the transition occurs at ~20–25°C. We also observed an L_α-H_{II} transition in the presence of Ba^{2+}, the transition occurring in the same temperature range of 20–25°C, in agreement with the T_H reported previously for the Ba^{2+}-CL complex (Vasilenko et al., 1982). In the presence of Ca^{2+} or Mg^{2+}, the T_H of CL is below 0°C (Fig. 1). Because CL without divalent cations is in the lamellar phase, the addition of Sr^{2+} or Ba^{2+} to CL vesicles at temperatures above 20–25°C or the addition of Ca^{2+} or Mg^{2+} at any temperature above 0°C

will induce an L_α-H_{II} transition in the system, the final equilibrium structure in each case being H_{II}. Our present results demonstrate, however, that the fusion behavior of CL LUVs in the presence of Sr^{2+} or Ba^{2+} is very different from that in the presence of Ca^{2+} or Mg^{2+}, despite the similarity of the final cation-CL complexes. Clearly, the nature of the final cation-CL complex is not the primary determinant of whether a nonleaky fusion event will occur during the initial cation-induced interaction between CL vesicles.

Below 25°C, Sr^{2+} and Ba^{2+} induce a limited but sustained extent of CL vesicle fusion, as evidenced by mixing of aqueous vesicle contents, along with a comparatively slow release of vesicle contents to the external medium (Fig. 3). On the other hand, above 25°C, the rates of release of vesicle contents in the presence of Sr^{2+} or Ba^{2+} increase abruptly (Figs. 3 and 5). At the same time, mixing of vesicle contents, although increasing in terms of initial rate, becomes a highly transient process (Fig. 3). These results are indicative of a correlation between the T_H of the cation-CL complex at equilibrium and the occurrence of membrane destabilization upon exposure of CL vesicles to the cation. In other words, the leakage of vesicle contents above the T_H appears to be due to rapid collapse of the vesicles into H_{II} phase precursors (Siegel et al., 1994; Siegel and Epand, 1997). This is also evident from the experiment in which *N*-NBD-PE was incorporated into the CL vesicle bilayer to probe the kinetics of the transition (Fig. 6). Furthermore, the second-order kinetics of the release process imply that the formation of H_{II} phase precursors requires vesicle-vesicle interaction. In summary, the behavior of CL vesicles in the presence of Sr^{2+} or Ba^{2+} above the T_H is consistent with a cation-driven L_α-H_{II} transition, leading to extensive leakage, mixing of aqueous vesicle contents being limited and of a very transient nature. However, it should be pointed out that the stopped-flow time-resolved fluorescence data presented in Fig. 3 B suggest that, even at temperatures above the T_H, fusion and mixing of aqueous vesicle contents do precede leakage.

A different picture emerges for CL LUVs in the presence of Ca^{2+} or Mg^{2+}, where a correlation between the T_H of the final cation-CL complex and the behavior of the vesicles is not at all apparent. Specifically, with Ca^{2+}, nonleaky fusion of the vesicles appeared to occur under conditions where the final Ca^{2+}-CL complex is H_{II} (Fig. 4). Furthermore, with increasing temperature the rate of leakage decreased (Figs. 4 and 5). This remarkable behavior of the Ca^{2+}-CL system is not due to a peculiarity of the assay used. It has been suggested that the Tb/DPA fusion assay might report false-positive "fusion" in systems of aggregated, leaky vesicles due to trapping of the fluorescent Tb/DPA complex within the vesicle aggregates (Kendall and McDonald, 1982). However, from the Tb/DPA signal in the Sr^{2+}-CL system above the T_H (Fig. 3), it can be concluded that whenever leakage of aqueous contents from aggregated vesicles occurs, it results in a rapid quenching of the Tb fluorescence. Thus the sustained high levels of fluorescence, seen in the presence of Ca^{2+}, must represent nonleaky fusion of the vesicles. Apparently, upon exposure of CL LUVs to Ca^{2+}, even at temperatures much higher than the T_H, the L_α-H_{II} transition is retarded. This delay of the transition is also evident from the fluorescence determination of the kinetics of the transition (Fig. 6) and from the morphological characterization of the system (Fig. 7).

It is not clear at this point why in the Ca^{2+}-CL system the L_α-H_{II} transition is retarded in favor of sustained nonleaky fusion of the vesicles, whereas in the Sr^{2+}-CL system the transition is more rapid, resulting in fast and extensive leakage. It is likely that in either case, the initial ion-induced interaction between the vesicles proceeds in a similar manner. In terms of the modified stalk model (Siegel, 1993; Siegel et al., 1994; Siegel and Epand, 1997) this would involve rapid formation of stalks and TMCs, representing hemifusion intermediates, evolving subsequently into ILAs, corresponding to complete fusion. It is not likely that, in the Sr^{2+}-CL system, initial TMC aggregates evolve directly to H_{II} phase precursors, because this would not be expected to result in mixing of aqueous vesicle contents, while we clearly did observe a contents mixing signal (Fig. 3, *A* and *B*). Therefore, we suggest that it is a quantitative rather than a qualitative difference between the Sr^{2+}-CL and the Ca^{2+}-CL systems that is responsible for their diverging fusion and leakage behavior. The difference does not seem to be related to the cation-CL binding ratio, because the binding of Ca^{2+} to CL in the lamellar phase is not appreciably different from that of Sr^{2+} (Fig. 8). One option is that the nature of the complexes formed with the different cations is different, where one type of complex would permit predominant fusion and the other would result in rapid H_{II} phase formation, depending on such factors as the size of the cations and the specific interaction of the cation with the CL headgroup. Different *"trans"* and *"cis"* cation-lipid complexes have been described for the phosphatidylserine (PS) system in the presence of Ca^{2+} or Mg^{2+} (Wilschut et al., 1981), resulting in dramatically different fusion behavior. Another—and in our view, plausible—option is that in the CL system the asymmetrical distribution of the ions across the vesicle bilayer is involved. The initial presence of the ions at just the external surface of the vesicles may limit stalk and TMC formation and, thus, favor TMC-to-ILA conversion. Depending on the leakiness of the initial fusion events involved in this TMC-to-ILA conversion, the ions would access the vesicle interior. This, in turn, would promote more extensive stalk and TMC formation, which is likely to produce rapid lateral TMC aggregation and formation of H_{II} phase precurors. In other words, the fast, relatively nonleaky, fusion of CL in the presence Ca^{2+} and the sustained asymmetrical distribution of Ca^{2+} across the bilayer would kinetically prevent the system from efficiently assembling into H_{II} tubes. On the other hand, with Sr^{2+} the initially more leaky fusion would allow the ions to access the vesicle interior, resulting in a more rapid completion of the L_a-H_{II} transition. Indeed, the initial rate of leakage of vesicle contents in the presence of Sr^{2+} is comparatively

high, whereas with Ca^{2+} the initial rate of leakage is comparatively very low (Fig. 5). It is important to emphasize that, in the fusion and leakage studies, the Ca^{2+}-CL system is kinetically inhibited from undergoing the L_a-H_{II} transition in favor of nonleaky fusion of the vesicles. On the other hand, in the equilibrium dialysis studies of Fig. 8, a much more concentrated suspension of CL vesicles is exposed to the cations for a prolonged period of time. The jump in Ca^{2+} binding at 2.5 mM free cation suggests that, under these conditions, even in the absence of ionophore, the Ca^{2+} ions eventually reach the vesicle interior, establishing a final equilibrium.

Although the present results are consistent with the stalk mechanism of fusion, it is important to note that our observations do not prove that fusion in CL vesicle systems induced by divalent cations does indeed proceed via this mechanism. One could even argue that, because the vesicles respond so differently to different divalent cations (under conditions where in all cases the final cation-CL complex is hexagonal), the initial interaction between CL vesicles in the presence of Ca^{2+} does not involve the formation of stalks or ILAs at all, but rather proceeds via an entirely different mechanism. The stalk and modified stalk theories have been derived for zwitterionic (PE) systems without consideration of electrostatic effects (Siegel, 1993; Siegel and Epand, 1997). Furthermore, it is well established that PS vesicles fuse very efficiently in the presence of Ca^{2+} (Wilschut et al., 1980, 1981, 1983). This fusion process is very unlikely to proceed via a mechanism involving the formation of H_{II}-like structures, the final Ca^{2+}-PS complex being lamellar (Cullis et al., 1985; Hope and Cullis, 1980). It is possible that Ca^{2+} is inducing fusion of CL vesicles by a mechanism similar to that of PS vesicles.

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