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Modulation of Membrane Fusion by Membrane Fluidity: Temperature Dependence of Divalent Cation Induced Fusion of Phosphatidylserine Vesicles[†]

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ABSTRACT: We have investigated the temperature dependence of the fusion of phospholipid vesicles composed of pure bovine brain phosphatidylserine (PS) induced by Ca^{2+} or Mg^{2+}. Aggregation of the vesicles was monitored by 90° light-scattering measurements, fusion by the terbium/dipicolinic acid assay for mixing of internal aqueous volumes, and release of vesicle contents by carboxyfluorescein fluorescence. Membrane fluidity was determined by diphenylhexatriene fluorescence polarization measurements. Small unilamellar vesicles (SUV, diameter 250 Å) or large unilamellar vesicles (LUV, diameter 1000 Å) were used, and the measurements were done in 0.1 M NaCl at pH 7.4. The following results were obtained: (1) At temperatures (0–5 °C) below the phase transition temperature (T_c) of the lipid, LUV (PS) show very little fusion in the presence of Ca^{2+}, although vesicle aggregation is rapid and extensive. With increasing temperature, the initial rate of fusion increases dramatically. Leakage of contents at the higher temperatures remains limited initially, but subsequently complete release occurs as a result of collapse of the internal aqueous space of the fusion products. (2) SUV (PS) are still in the fluid state down to 0 °C, due to the effect of bilayer curvature, and fuse rapidly in the entire temperature range from 0 to 35 °C in the presence of Ca^{2+}. The initial rate of leakage is low relative to the rate of fusion. At higher temperatures (15 °C and above), subsequent collapse of the vesicles' internal space causes complete release. At 0–5 °C, collapse does not occur, and the extent of fusion is limited; as the vesicles grow in size and start to resemble LUV, the fusion process slows down. (3) With Mg^{2+}, LUV (PS) aggregate massively in the entire temperature range from 0 to 35 °C, but fusion and release of contents do not occur. (4) SUV (PS) do fuse in the presence of Mg^{2+}. The initial rate of leakage relative to fusion is severalfold higher than during Ca^{2+}-induced fusion of SUV. At low temperatures, this results in an early and complete release of vesicle contents. At 20 °C and above, complete release does not occur. Fusion stops spontaneously as the vesicles grow in size, and part of the aqueous contents is retained in the vesicles for a prolonged period of time. The results point out that a cation-induced isothermal phase transition of the bilayer lipids is not a prerequisite for the fusion process. Prerequisite for fusion appears to be that the vesicle is in an overall fluid state when the cation has bound to its outer surface.

The development of the fluorescent Tb/dipicolinic acid (Tb/DPA)[1] method registering the mixing of the aqueous contents of phospholipid vesicles (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980, 1981) has considerably facilitated the study of membrane fusion in model systems. Particularly, the divalent cation induced interaction between vesicles containing acidic phospholipids such as phosphatidylserine (PS), cardiolipin, phosphatidic acid or phosphatidylinositol has been investigated extensively (Wilschut et al., 1980, 1981, 1982, 1983; Düzgüneş et al., 1981a,b, 1984a; Bentz

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[1] Abbreviations: CF, carboxyfluorescein; DPA, dipicolinic acid; DPH, diphenylhexatriene; PS, phosphatidylserine; PC, phosphatidylcholine; SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

et al., 1983a; Bearer et al., 1982; Sundler & Papahadjopoulos, 1981; Sundler et al., 1981) and reviewed recently (Nir et al., 1983; Düzgüneş et al., 1984b; Düzgüneş, 1985).

It is well established that at 25 °C, upon interaction with Ca^{2+}, PS vesicles undergo massive aggregation and fusion (Papahadjopoulos et al., 1977; Düzgüneş & Ohki, 1977; Portis et al., 1979) with eventual formation of cochleate structures, consisting of dehydrated, tightly packed Ca^{2+}/PS bilayers (Papahadjopoulos et al., 1975). Obviously, such a structural reorganization must finally result in a complete release of aqueous vesicle contents. However, using the Tb/DPA assay, we have demonstrated for both small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) that mixing of aqueous vesicle contents within the vesicle interior clearly precedes the release of contents (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980, 1983; Nir et al., 1980), indicating that during the initial stages Ca^{2+}-induced fusion of PS vesicles is essentially nonleaky. In contrast with Ca^{2+}, Mg^{2+} does not induce any mixing or release of contents with LUV (PS), although massive vesicle aggregation occurs (Wilschut et al., 1981). With SUV, a limited degree of fusion occurs, which appears to be related to the highly curved bilayer of this type of vesicle (Wilschut et al., 1981; Hoekstra, 1982a), but there is no transformation to cochleate structures (Papahadjopoulos et al., 1975, 1977; Portis et al., 1979).

The effect of temperature on the above processes has been investigated previously by freeze–fracture electron microscopy and by determination of the release of vesicle contents (Papahadjopoulos et al., 1977). At 0 °C, Ca^{2+} does not induce a transformation of PS vesicles to cochleate structures, while the rate of release of vesicle contents is considerably lower than at 25 °C. This temperature dependence has been related to the fluidity of the vesicle membranes prior to fusion, the gel to liquid-crystalline phase transition temperature (T_c) of PS bilayers being approximately 8 °C (Newton et al., 1978; Papahadjopoulos et al., 1978). Since the T_c of the final Ca^{2+}/PS complex is very high (>100 °C; Portis et al., 1979; Newton et al., 1978), at 25 °C Ca^{2+} can induce an isothermal phase transition, whereas at 0 °C, the bilayer already being in the gel state, such an isothermal transition would not occur. This and other observations have led to the suggestion that the key event in vesicle fusion is the isothermal phase transition induced by the metal ion (Papahadjopoulos et al., 1977, 1978).

With respect to the above early studies, the following should be noted. First, the work has been carried out with SUV. It is now recognized that the phase transition of small vesicles is broadened and shifted to lower temperatures (Suurkuusk et al., 1976; Papahadjopoulos et al., 1976). Therefore, at 0 °C SUV (PS) would be expected to be fluid or at least within the temperature range of the phase transition. Second, the experimental approaches followed in the earlier studies neither met a rigorous criterion for fusion, i.e., mixing of aqueous vesicle contents, nor allowed the investigation of the initial kinetics of the process. The development of methodologies for preparation of large unilamellar vesicles (Szoka & Papahadjopoulos, 1978; Szoka et al., 1980) with phase transition characteristics similar to those of multilamellar vesicles (Düzgüneş et al., 1983) and the availability of an assay for mixing of aqueous vesicle contents that allows kinetic measurements (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980, 1983) have made possible the present study, in which the effect of the bilayer fluidity on the Ca^{2+}- or Mg^{2+}-induced fusion of PS vesicles is examined.

MATERIALS AND METHODS

Phosphatidylserine (PS) was purified from bovine brain as described (Papahadjopoulos & Miller, 1967), chromatographed on silicic acid, and finally converted to the sodium salt by washing with EDTA and NaCl (Papahadjopoulos et al., 1973). Alternatively, PS was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Diphenylhexatriene (DPH) was obtained from Aldrich (Beerse, Belgium). The source and purity of other reagents have been described before (Wilschut et al., 1980).

Preparation of vesicles and fluorescence measurements were carried out essentially as described before (Wilschut et al., 1980, 1981). Briefly, SUV were prepared by sonication in either (a) 15 mM $TbCl_3$/150 mM sodium citrate, (b) 150 mM dipicolinic acid (DPA, sodium salt), or (c) 100 mM carboxyfluorescein (CF, sodium salt). The aqueous media were buffered with 2.0 mM L-histidine and 2.0 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) at a final pH of 7.4. LUV were prepared by reverse-phase evaporation (Szoka & Papahadjopoulos, 1978) and subsequent extrusion (Olson et al., 1979) through a 0.1-μm pore-size Unipore membrane (Bio-Rad, Richmond, CA). LUV were made in either (a) 2.5 mM $TbCl_3$/50 mM sodium citrate, (b) 50 mM DPA (sodium salt)/20 mM NaCl, or (c) 50 mM CF (sodium salt), all buffered as described above. Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-75. The elution buffer contained 100 mM NaCl, 2.0 mM L-histidine, 2.0 mM TES, and 1.0 mM EDTA (pH 7.4). Lipid phosphorus was determined according to Bartlett (1959). Typical values for the encapsulation of Tb, DPA, and CF in the vesicles, determined as described (Wilschut et al., 1980), were (in nanomoles per mole of lipid) 11.1, 210, and 221, respectively, for LUV and 1.7, 16.3, and 9.1, respectively, for SUV.

Fluorescence measurements were done in an SLM-4000 fluorometer, equipped with two 90° emission channels. Concentrated solutions of $CaCl_2$ or $MgCl_2$ were introduced with a Hamilton syringe into a magnetically stirred dispersion containing either a 1:1 mixture of Tb and DPA vesicles or CF-containing vesicles. The temperature was controlled by circulating water through a jacket aroung the cell and measured with a thermocouple in the cuvette. To prevent condensation on the surface of the cuvette, dry air was passed through the sample compartment at lower temperatures. Measurements were done in a final volume of 1.0 mL containing 100 mM NaCl, 2.0 mM L-histidine, 2.0 mM TES, and 0.1 mM EDTA. The pH of the medium at the experimental temperature was adjusted to 7.4. Final lipid concentration was 0.05 mM. In experiments where $MgCl_2$ was used to induce vesicle interaction, 0.1 mM EGTA was also included in the medium (Wilschut et al., 1981). Excitation of the Tb/DPA complex was at 276 nm; fluorescence was measured through the monochromator set at 545 nm with a Corning 3-68 cutoff filter (transmitting wavelengths above 530 nm) between the sample and the monochromator. The Tb fluorescence scale was calibrated such that the 100% value corresponded to the maximal fluorescence intensity that could be obtained with the amount of Tb present (Wilschut et al., 1980). Therefore, the fluorescence intensity measured at any time during the fusion assay represents the percentage of the total amount of Tb that has become associated with DPA. CF was excited at 430 nm in LUV experiments and at 493 nm with SUV. Emission was measured through a Corning 3-68 cutoff filter. The residual fluorescence intensity of CF inside the vesicles (5% and 10% of the maximal value for SUV and LUV, respectively) was taken as 0% release. Maximal fluorescence intensity, obtained after complete disruption of the vesicles

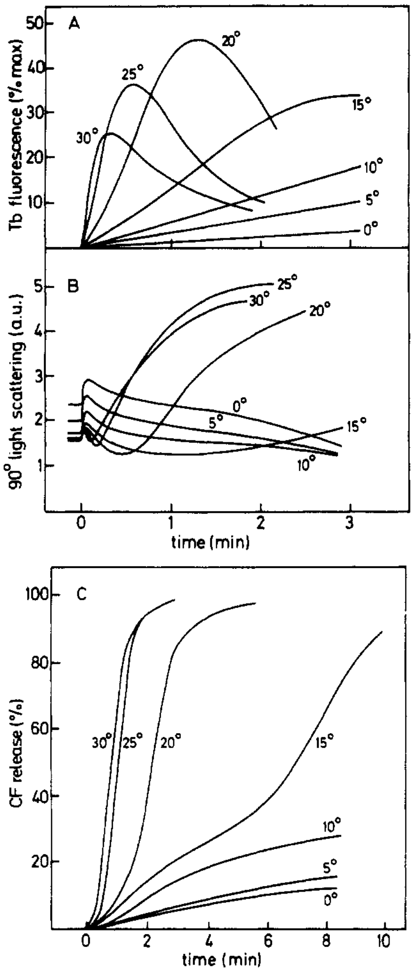

FIGURE 1: Ca^{2+}-induced fusion of LUV (PS) at different temperatures. $CaCl_2$ (0.1 M) was injected into a cuvette containing a 1:1 mixture of Tb and DPA vesicles or CF vesicles equilibrated at the temperatures indicated. Final Ca^{2+} concentration, 5.0 mM; lipid concentration, 0.05 mM. Tb fluorescence (A) and 90° light scattering at 276 nm (B) were recorded simultaneously; CF release (C) was measured in parallel experiments.

by addition of Triton X-100 (0.1% v/v), was taken as the value for 100% release.

Light scattering at 276 nm was measured, simultaneously with Tb fluorescence, in the second emission channel through a Corning 7-54 band-pass filter.

Fluorescence polarization measurements were performed in a Perkin-Elmer MPF 43 fluorometer with a polarization accessory. A small aliquot of a 2.0 mM solution of DPH in tetrahydrofuran was added to the vesicle suspension (0.5 μmol/mL) to give a DPH:PS molar ratio of approximately 1:750 and allowed to equilibrate for 2 h at 25 °C. Readings were taken at fixed temperatures, controlled and measured as described above. The excitation wavelength was 360 nm and the emission wavelength 430 nm. Polarization was calculated as described before (Shinitzky & Barenholz, 1974) with a correction factor for $I_\perp$ to account for the intrinsic polarization of the instrument (Chen & Bowman, 1965).

RESULTS AND DISCUSSION

Ca^{2+}-Induced Fusion of LUV (PS). Figure 1 shows the fusion characteristics of LUV (PS) in the presence of 5 mM Ca^{2+} at different temperatures. Rapid aggregation of the vesicles occurred in the entire temperature range (0–30 °C). At the lower temperatures, aggregation was accompanied by only very little mixing of aqueous vesicle contents. The refractoriness of LUV (PS) to fusion at 0 °C was confirmed by freeze–fracture electron microscopic examination of a vesicle population produced by incubation of LUV (PS) with Ca^{2+} at 0 °C and subsequent dispersal of the vesicle aggregates with EDTA (Wilschut et al., 1981). No significant increase of the vesicle diameter was observed (results not shown). With increasing temperature, the rate of mixing of vesicle contents increased dramatically (Figure 1A). At temperatures above 15 °C, a subsequent decrease of the fluorescence intensity became evident due to release of vesicle contents.

In separate experiments, we determined the kinetics of release of vesicle contents directly, employing the dequenching of CF fluorescence upon dilution (Blumenthal et al., 1977; Weinstein et al., 1977; Portis et al., 1979; Wilschut et al., 1980, 1981). Relatively little release occurred between 0 and 10 °C (Figure 1C). At 15 °C and at higher temperatures, release clearly exhibited a biphasic character, the rapid secondary release phase coinciding with the decrease of Tb fluorescence in the corresponding fusion measurements. These results confirm our earlier conclusion (Wilschut et al., 1980, 1983) that little leakage of contents occurs during the initial rounds of fusion between the vesicles. Extensive release seems to depend on multiple fusion events, and we have proposed (Wilschut et al., 1980) that it is due to collapse of the internal aqueous space of the vesicles, following their fusion, as an intermediate stage during the formation of cochleate cylinders (Papahadjopoulos et al., 1975).

The process of collapse is clearly reflected in the 90° light scattering at 276 nm presented in Figure 1B. Note the relatively high scatter intensity of the vesicles at 0 and 5 °C, before Ca^{2+} addition. This is a reflection of the fact that the vesicles are partly in the gel state in this temperature region (see also Figure 3A). Ca^{2+}-induced vesicle aggregation was observed as an initial increase and subsequent decrease of the scatter intensity. This pattern, seen between 0 and 10 °C, clearly resembled the light-scattering curve of Mg^{2+}-induced aggregation of LUV (PS) at 25 °C, a condition which does not lead to fusion or collapse of the vesicles (Wilschut et al., 1981). At higher temperatures, a large subsequent increase of the scatter intensity appeared (Figure 1B). This increase probably represents the formation of tightly packed Ca^{2+}/PS complexes during collapse of the vesicles, since at all temperatures where it was observed, it coincided with extensive release of vesicle contents (Figure 1C).

Ca^{2+}-Induced Fusion of SUV (PS). Fusion of SUV (PS) in the presence of Ca^{2+} is characterized by a lower threshold cation concentration (1.0 mM) than fusion of LUV, which requires a minimal Ca^{2+} concentration of approximately 2.5 mM (Wilschut et al., 1980). The temperature dependence of SUV (PS) fusion at 2.0 mM Ca^{2+} is presented in Figure 2. The initial rate of SUV fusion exhibited a much less pronounced temperature dependence than that of LUV fusion. Even at 0 °C, fusion of SUV proceeded at a considerable rate, which was only 5-fold lower than the rate at 30 °C. By contrast, LUV fusion was over 100-fold slower at 0 °C than at 30 °C.

With SUV in the temperature range from 0 to 10 °C, an only limited release of CF was observed (Figure 2C), the initial rate of which was considerably slower than the rate of Tb fluorescence increase. Tb fluorescence intensity levelled off after about 15 s at approximately 45% maximum fluorescence and remained constant for a relatively long period of time. This indicates that in this temperature region the fusion process slowed down considerably after a limited number of fusion events.

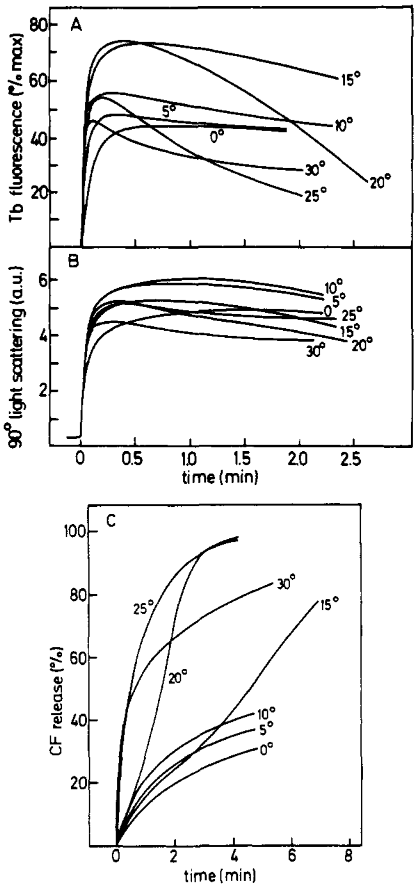

FIGURE 2: Ca^{2+}-induced fusion of SUV (PS) at different temperatures. $CaCl_2$ (0.1 M) was injected into a cuvette containing a 1:1 mixture of Tb and DPA vesicles or CF vesicles equilibrated at the temperatures indicated. Final Ca^{2+} concentration, 2.0 mM; lipid concentration, 0.05 mM. Tb fluorescence (A) and 90° light scattering at 276 nm (B) were recorded simultaneously; CF release (C) was measured in parallel experiments.

At 15 and 20 °C, Tb fluorescence intensity reached very high values (Figure 2A), indicating extensive fusion in combination with relatively little initial release. While at 15 °C Tb fluorescence remained rather high for at least 2 min, at 20 °C a subsequent decline was observed. Consistent with this observation, in the corresponding CF release experiments (Figure 2C), a distinct subsequent release process, presumably due to collapse of the vesicles, was seen. This release phase started after 3–4 min at 15 °C, but earlier at 20 °C. At the higher temperatures, initial and subsequent stages of release became indistinguishable. At 30 and 35 °C, release did go to completion eventually, but for reasons as yet not understood this required a longer period of time than that at 20 or 25 °C. This effect could also be recognized in the corresponding Tb fluorescence curves (Figure 2A). Fluorescence was maintained at a higher level at 30 °C than at 20 or 25 °C.

Collapse of the vesicles is not distinguishable as such in the light-scattering pattern of SUV (Figure 2B). The initial scatter intensity of SUV is low due to the small size of the vesicles. Therefore, aggregation and fusion produce a more pronounced relative increase in scattering than with LUV, which masks the subsequent increase caused by collapse of the vesicles. That collapse of the vesicles does contribute to the scatter signal can be seen by comparison of Figure 2B with the light-scattering curve observed for Mg^{2+}-induced fusion of SUV at 25 °C (Figure 4B), a condition not leading to collapse of the fusion product.

The following picture emerges from the above results. In the temperature range from 0 to 10 °C, the extent of SUV fusion remains limited, as the rate of the process slows down substantially after an initial rapid phase. This decrease can be understood in terms of a gradual reduction of the degree of bilayer curvature as the vesicles grow in size and begin to resemble LUV, which have very little tendency to fuse at these low temperatures (Figure 1A). Above 15 °C, SUV fusion becomes very extensive. In addition, at these temperatures, the subsequent release process appears. In this respect, it should be noted that the release curves at 15 and 20 °C are very similar to the corresponding curves obtained with the LUV. It would thus appear that collapse and associated massive release of contents occur only after the vesicles have reached a sufficiently large size. Collapse is probably the result of entry of Ca^{2+} into the vesicle interior during the fusion process. Subsequent binding of the ion to the lipid of the interior monolayer would then allow the interior surfaces at opposite ends of the vesicle to interact, thus forcing the internal volume out. It is quite conceivable that such a mechanism requires a certain minimal size of the fusion product.

Relative Kinetics of Aggregation and Fusion Induced by Ca^{2+}. Fusion of phospholipid vesicles consists of two distinct but kinetically coupled stages: first, aggregation of the vesicles and second, fusion of the bilayers. At 25 °C, Ca^{2+}-induced fusion of SUV (PS) occurs without any appreciable delay after vesicle aggregation (Wilschut et al., 1980; Nir et al., 1980, 1982, 1983; Bentz et al., 1983b). Thus, aggregation is the rate-limiting step in the overall process. With LUV, on the other hand, the fusion step per se is a substantially slower process than with SUV, and at 25 °C, the aggregation step is not exclusively rate limiting to the overall process (Nir et al., 1982, 1983; Bentz et al., 1983b). Our present results with LUV demonstrate a reduction of the overall rate of fusion with decreasing temperature and, ultimately, at 0 °C an essentially negligible rate of fusion. Yet, under those conditions rapid and extensive aggregation of the vesicles occurs, thus indicating that fusion per se has become the exclusive rate-limiting step in the overall process.

With SUV, the rate of aggregation can be estimated from the initial rate of increase of light scattering. Values of this parameter and of the corresponding rate of Tb fluorescence increase (calculated from the curves in Figure 2A,B) show that the rate of aggregation changed only marginally in the temperature range studied, whereas the rate of fusion at 0 °C was 5-fold lower than at 30 °C. This indicates that with SUV at lower temperatures the fusion step per se is also starting to become rate limiting to the overall process of aggregation and fusion.

Bilayer Fluidity and the Rate of Fusion in the Presence of Ca^{2+}. In order to correlate the fusion capacity of PS vesicles with the physical state of the bilayer lipids, we determined the fluidity of LUV and SUV at different temperatures, employing the fluorescence polarization of DPH. The results are shown in Figure 3. LUV (PS) showed a broad but well-defined transition centered around 8 °C. This transition temperature is very similar to the value observed previously for multilamellar vesicles (Newton et al., 1978; Portis et al., 1979). By contrast, SUV (PS) did not show a well-defined phase transition in the temperature region down to 0 °C, which is in line with earlier results showing that in small vesicles the phase transition is broadened and shifted to lower temperatures due to the high curvature of the vesicle bilayer (Suurkuusk et al., 1976; Papahadjopoulos et al., 1976).

In the presence of 0.5 mM Ca^{2+}, a concentration not inducing vesicle aggregation, the phase transition of LUV (PS) was only marginally shifted to higher temperatures (Figure

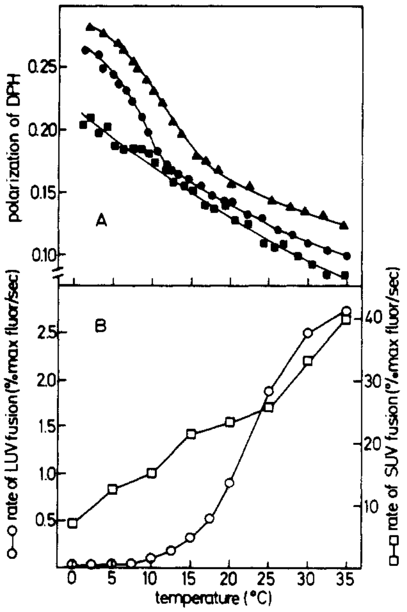

FIGURE 3: Fluidity of LUV and SUV (PS) (A) and the initial rates of Ca^{2+}-induced fusion (B) at different temperatures. Fluorescence polarization of DPH was determined at fixed temperatures as described under Materials and Methods. (■) SUV without Ca^{2+}; (●) LUV without Ca^{2+}; (▲) LUV in the presence of 0.5 mM Ca^{2+}. Initial rates of fusion were determined from the tangents to Tb fluorescence curves, recorded at sufficiently high chart speeds. LUV fusion was at 5.0 mM Ca^{2+} (○), SUV fusion at 2.0 mM Ca^{2+} (□).

3A). This minor change is not due to limited binding of Ca^{2+} to the PS bilayer: Binding under these conditions is considerable and results in approximately 70% neutralization of the surface charge (Portis et al., 1979; Ekerdt & Papahadjopoulos, 1982). It thus would appear that the presence of Ca^{2+} at one side of the bilayer only and the lack of intermembrane contact prevent the separate vesicles from undergoing a major phase change at 0.5 mM Ca^{2+}. What remains unknown at this stage is the T_c of separate vesciles in the presence of 5 mM Ca^{2+}, before intermembrane contact is established. This quantity could not be measured because the vesicles aggregated and fused rapidly under these conditions. However, just before vesicle aggregation, Ca^{2+} will bind to the outer vesicle surface only, thus creating a transient condition, possibly similar to the stable condition at 0.5 mM Ca^{2+}, in which the T_c of the vesicles is shifted only slightly to higher values.

The temperature dependence of the initial rate of Ca^{2+}-induced fusion of LUV and SUV is shown in Figure 3B. The rate of fusion of LUV was negligible below 5 °C, while very high rates were observed above 15–20 °C (see also Figure 1A). These temperatures coincide with the lower and upper limits of the phase transition of LUV in the presence of Ca^{2+}, but at the stage before aggregation (Figure 3A). Therefore, it appears that, after initial binding of Ca^{2+}, the membrane has to be in the fluid state to undergo fusion.

Sun et al. (1978, 1979) have suggested that fusion is optimal *at* the phase transition temperature of the vesicles after Ca^{2+} binding, but before aggregation and fusion. In contrast, our results show that the rate of fusion increases steadily with temperature. It should be noted, however, that Sun et al. (1978, 1979) examined the increase in size of the vesicles after EDTA treatment following a long incubation with Ca^{2+} and did not determine the initial kinetics of fusion.

Temperature Dependence of the Effects of Mg^{2+} on LUV and SUV (PS). Mg^{2+} induced aggregation of LUV (PS), but no fusion or release of vesicle contents, in the entire temperature range from 0 to 35 °C (results not shown). The absence of fusion under these conditions was confirmed by freeze–fracture electron microscopy. Importantly, no fusion occurred between 8 and 18 °C, where Mg^{2+} could, in principle, be expected to induce an isothermal phase transition in PS bilayers, the T_c of the final Mg^{2+}/PS complex being centered around 18 °C (Portis et al., 1979). If an isothermal phase transition would occur in isolated vesicles and were critically involved in the fusion process, one would expect fusion to occur in this temperature region.

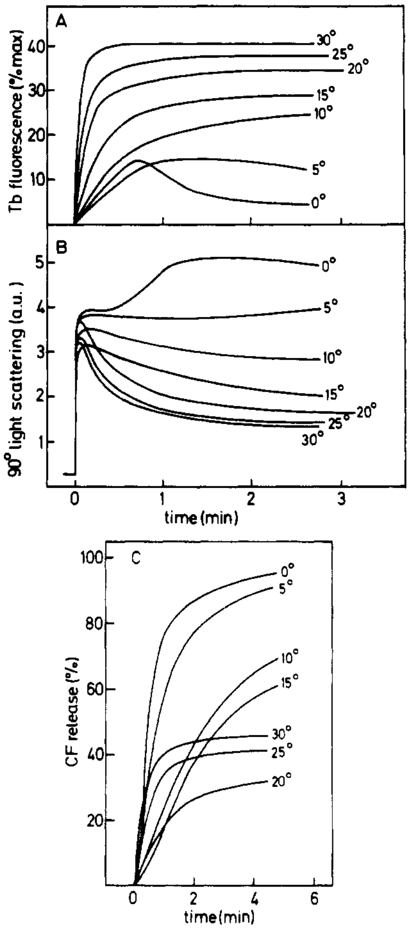

FIGURE 4: Mg^{2+}-induced fusion of SUV (PS) at different temperatures. $MgCl_2$ (0.2 M) was injected into a cuvette containing a 1:1 mixture of Tb and DPA vesicles or CF vesicles equilibrated at the temperatures indicated. Final Mg^{2+} concentration, 8.0 mM; lipid concentration, 0.05 mM. Tb fluorescence (A) and 90 °C light scattering at 276 nm (B) were recorded simultaneously. CF release (C) was measured in parallel experiments.

In contrast to LUV, SUV (PS) do fuse in the presence of Mg^{2+} at 25 °C. The extent of fusion is limited: The process ceases spontaneously after a few rounds of fusion. Despite the relatively high initial leakiness of Mg^{2+}-induced fusion of SUV, the fused vesicles do not collapse, which is reflected by a steady level of fluorescence intensity with no further release of contents (Wilschut et al., 1981).

The temperature dependence of Mg^{2+}-induced fusion of SUV (PS) is presented in Figure 4. In the entire temperature range studied, an initial increase of Tb fluorescence intensity was observed. Clearly at 0 °C, but also at 5 °C, this initial increase was followed by a subsequent decrease. Accordingly, essentially complete release of vesicle contents was observed at these temperatures (Figure 4C). At higher temperatures, Tb fluorescence developed with progressively higher initial rates (Figure 4A), and no subsequent decrease was seen within a period of 3 min after the induction of fusion. At 10 and 15 °C, the subsequent decline did appear at later times (not shown) consistent with the results of the corresponding leakage measurements, where extensive and eventually complete release was observed (Figure 4C). At 20 °C and above, the Tb fluorescence remained constant for at least 30 min, indicating, in agreement with the corresponding release measurements, that part of the vesicle contents remained sequestered in the fusion product.

The extensive release at the lower temperatures can be explained by an early collapse of the vesicles. The relatively high leakiness of the initial fusion events per se allows Mg^{2+} to gain access to the vesicle interior, where it can bind to the inner half of the vesicle bilayer. Binding of the ion to both sides of the bilayer and the vesicles being aggregated may provide the necessary and sufficient condition for an isothermal phase transition to occur, provided that the ambient temperature is below the T_c of the ion/PS complex. This, in turn, may induce a destabilization and an early collapse of the vesicles. The T_c of the Mg^{2+}/PS complex being 18 °C (Portis et al., 1979), an isothermal phase transition can be expected to occur at all temperatures below this value. Extensive release of contents was observed in this temperature range (Figure 4C). Early collapse of the vesicles at low temperature was further indicated by the change in light scattering during the fusion process (Figure 4B). Clearly at 0 °C, but also at 5 °C, a subsequent increase of the scatter intensity was seen, indicating the formation of collapsed gel-phase structures. At temperatures above the T_c of the Mg^{2+}/PS complex (>18 °C), despite the initial leakiness of fusion and the expected influx of Mg^{2+}, the bilayers remain in the fluid state. Since the vesicles stop fusing after a limited increase in size, collapse is not observed, and part of the initial contents is retained in the fusion products.

CONCLUSIONS

In Figure 5, we schematically summarize our findings on the effects of Ca^{2+} and Mg^{2+} on large and small PS vesicles at 0 and 25 °C. The dramatic differences in fusion behavior are instructive for understanding the role of membrane fluidity and vesicle size in defining the rate and extent of fusion. The use of LUV with a large enough diameter to have an unstrained bilayer and phase transition characteristics similar to those of multilamellar vesicles (Düzgüneş et al., 1983) has made it possible to demonstrate that binding of Ca^{2+} in a "cis" complex (Portis et al., 1979) to the outer monolayer lipids of isolated PS vesicles does not produce a major shift of the phase transition temperature (Figure 3A). Efficient fusion of the vesicles is observed only in the temperature region above this marginally shifted T_c (Figures 1 and 3B), suggesting that a prerequisite for fusion is that the isolated vesicles, after binding of Ca^{2+}, be in a fluid state. The well-documented, isothermal phase transition that Ca^{2+} induces in PS vesicle systems (Jacobson & Papahadjopoulos, 1975; Papahadjopoulos et al., 1977; Portis et al., 1979) would appear to be a result of, rather than a requirement for, the fusion process. This conclusion is supported by the following observations: (i) Using an assay that monitors the kinetics of lateral lipid phase separations (Hoekstra, 1982b), Hoekstra (1982a) has demonstrated that the Ca^{2+}-induced formation of a condensed lipid phase in PS vesicle systems and also the lateral phase separations in mixed PS/PC vesicles are much slower processes than the fusion of the vesicles. (ii) Ba^{2+} and Sr^{2+} induce rapid fusion of LUV (PS) at temperatures above the transition temperatures of the respective final cation/PS complexes (Düzgüneş et al., 1984a), indicating that a cation-induced isothermal phase transition of the vesicle lipids is not a requirement for fusion.

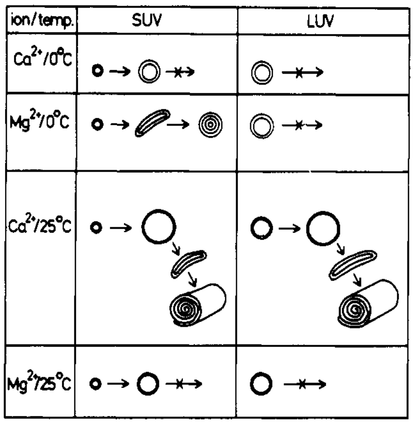

FIGURE 5: Schematic survey of the effects of Ca^{2+} and Mg^{2+} on LUV and SUV (PS) at 0 and 25 °C. The filled membranes represent bilayers in the liquid-crystalline state; the "open" (double line) membranes are in the gel state. At 0 °C, in the presence of Ca^{2+} or Mg^{2+}, LUV (PS) show very little, if any, fusion, although vesicle aggregation in either case is rapid and extensive. SUV, on the other hand, do fuse at 0 °C. Fusion is rapid with Ca^{2+}, but the extent is limited: as the vesicles grow in size and start to resemble LUV, which are in the gel state at this temperature, fusion slows down considerably. Release of contents remains limited under these conditions. This implies that Ca^{2+} does not gain easy access to the vesicle interior and collapse is prevented. With Mg^{2+} at 0 °C, SUV fusion is slow but quite leaky. The metal ion thus gains an early access to the vesicle interior as a result of which the internal space of the vesicles collapses and the vesicles probably continue to fuse at areas of structural defects ("leaky points") to finally produce multilamellar vesicles. At 25 °C, LUV (PS) fuse extensively in the presence of Ca^{2+}. Leakage is limited initially, but eventually the fused vesicles collapse and their contents are released massively. With Mg^{2+} at 25 °C, LUV aggregate but do not fuse or release contents. SUV at 25 °C fuse quite rapidly in the presence of Ca^{2+}. Initial release of contents is slower than fusion, indicating a limited leakiness of the fusion per se, but eventually Ca^{2+} gains access to the vesicle interior causing collapse and complete release of contents. With Mg^{2+} at 25 °C, SUV fuse to a limited extent: the process stops as the vesicles grow in size and start to resemble LUV. The fusion products do not collapse, and even though the initial leakiness is higher than with Ca^{2+}-induced SUV fusion, a high percentage of the aqueous contents is retained.

Although binding of Ca^{2+} in a cis complex to the outer surface of isolated PS vesicles is not a sufficient condition to produce a major shift in the T_c of the lipids, our results do not exclude the formation of microdomains consisting of partly condensed lipid in the outer vesicle monolayer at a temperature *above* the actual phase transition. The fluorescence polarization data in Figure 3A show that at a given temperature above the T_c (for example, 25 °C), the fluidity of the membrane is slightly lower in the presence of Ca^{2+} than in its absence. This could, indeed, be taken to indicate that a fraction of the outer monolayer lipid is motionally restricted due to formation of microdomains consisting of partly condensed lipid. The formation of such microdomains would imply that laterally fluctuating phase boundaries exist on the surface of the vesicles, which could be nucleation points for fusion. Recently, Hui et al. (1983) have observed Ca^{2+}-induced formation of microdomains in mixtures of PS and PC under conditions where no macroscopic, morphologically distinguishable segregation of the two lipid species occurs.

As proposed earlier (Portis et al., 1979; Wilschut et al., 1981; Hoekstra, 1982a), the ability of Ca^{2+} to induce fusion of PS vesicles is likely to be related to its capacity to form a dehydrated "trans" complex between PS molecules on apposing bilayers, thus bringing the membranes in close proximity. This suggestion is supported by observations demonstrating the formation of a Ca^{2+}/PS complex with a special binding mode, related to aggregation *and* fusion of the vesicles (Ekerdt & Papahadjopoulos, 1982). These results point to a critical role of intermembrane contact in triggering the physical changes in the vesicle bilayer that are crucial to the fusion process. Our present observations, suggesting that Ca^{2+} does not induce drastic structural changes in PS vesicle systems at the level of isolated vesicles (Figure 3A), are in line with this notion.

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