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Calcium/Magnesium Specificity in Membrane Fusion: Kinetics of Aggregation and Fusion of Phosphatidylserine Vesicles and the Role of Bilayer Curvature[†]

Jan Wilschut,[‡] Nejat Düzgüneş, and Demetrios Papahadjopoulos*

ABSTRACT: We have investigated the relative abilities of Ca^{2+} and Mg^{2+} to induce the fusion of phospholipid vesicles composed of pure bovine brain phosphatidylserine (PS). Vesicle aggregation was monitored by light-scattering measurements, fusion by the terbium/dipicolinic acid assay for mixing of internal vesicle volumes, release of vesicle contents by carboxyfluorescein fluorescence, and changes in vesicle size by freeze–fracture electron microscopy. Either small unilamellar vesicles (SUV, diameter 250 Å), prepared by sonication, or large unilamellar vesicles (LUV, diameter 1000 Å), prepared by reverse-phase evaporation and extrusion through a polycarbonate membrane, were used. Measurements were done in a 0.1 M NaCl medium at 25 °C and pH 7.4. In the presence of either Mg^{2+} or Ca^{2+} at above-threshold concentrations, both types of vesicles massively aggregate. Mg^{2+} does not induce the fusion of LUV or any release of vesicle contents. It does induce the fusion of SUV, which is accompanied by a transient release of vesicle contents, but the extent of fusion is limited to a small increase in vesicle size, beyond which there is no further mixing or release of vesicle contents. By contrast, Ca^{2+} induces rapid and extensive fusion of LUV or SUV, while eventually all internal contents are lost due to collapse of the vesicles. The Mg^{2+}-induced fusion of SUV proceeds less rapidly and is more leaky than the initial Ca^{2+}-induced fusion of SUV. Mixtures of Ca^{2+} and Mg^{2+} behave either competitively or cooperatively, depending on the relative cation concentrations and on the type of vesicle used. The initial rate of Ca^{2+}-induced fusion of LUV is inhibited by Mg^{2+} at any Ca^{2+} concentration. At low, near-threshold Ca^{2+} concentrations, Mg^{2+} cooperates in inducing the fusion of SUV, whereas at higher Ca^{2+} concentrations, Mg^{2+} is inhibitory. The results are discussed in terms of the degree of dehydration and the change in lipid fluidity induced by interaction of the divalent cations with the vesicles. Crucial to the specificity of Ca^{2+} in inducing the fusion of PS vesicles seems to be its ability to form an anhydrous complex between apposed bilayers.

The well-documented involvement of Ca^{2+} in various biological membrane fusion phenomena (Poste & Allison, 1973; Rubin, 1974; Douglas, 1975) has prompted extensive studies on the interactions of divalent cations with phospholipid vesicles composed of phosphatidylserine (PS)[1] or other acidic phospholipids. Several of our recent studies have emphasized the contrasting effects of Ca^{2+} and Mg^{2+} on the physical properties of acidic phospholipid bilayers (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978), and we have proposed correlations of these discrepancies with the diverse abilities of the two cations to induce vesicle fusion (Papahadjopoulos et al., 1977,

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[†] From the Cancer Research Institute and the Department of Pharmacology, School of Medicine, University of California, San Francisco, California 94143. *Received October 10, 1980.* This investigation was supported by Research Grant GM-26259 from the National Institutes of Health. J.W. is a recipient of a fellowship grant from the Netherlands Organization for the Advancement of Pure Research (ZWO). N.D. is a recipient of National Institutes of Health Fellowship CA-06190 from the National Cancer Institute.

[‡] Present address: Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands.

[1] Abbreviations used: PS, phosphatidylserine; DPA, dipicolinic acid; CF, carboxyfluorescein; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

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1978; Newton et al., 1978; Portis et al., 1979). Thus, Ca^{2+}, in contrast to Mg^{2+}, can induce an isothermal phase transition in PS bilayers at 25 °C, which is accompanied by the release of the heat of crystallization of the acyl chains (Portis et al., 1979). In addition, X-ray diffraction studies have revealed that Ca^{2+} can also induce essentially complete removal of water from the interbilayer space. It has been proposed that this effect of Ca^{2+} is due to the formation of an anhydrous trans complex involving the head groups of PS molecules in apposed bilayers (Portis et al., 1979). The evidence for the Mg^{2+}/PS complex, on the other hand, indicates that an appreciable amount of water, corresponding to a layer approximately 10 Å thick, remains between the apposed bilayers. This would not allow the formation of a trans complex, and we have suggested, therefore, that a Mg^{2+} ion binds to only one bilayer surface in a so-called cis complex (Portis et al., 1979).

We have proposed that the formation of an anhydrous (possibly "trans" membrane) Ca/PS complex between two colliding vesicles is responsible for the initiation of the observed fusion events (Portis et al., 1979). Mg^{2+}, on the other hand, forming a hydrated cis complex with PS bilayers, would be expected to be ineffective, apart from causing vesicle aggregation. However, in terms of vesicle fusion, Mg^{2+} is not completely ineffective toward small sonicated PS vesicles; limited increases in vesicle size have been observed (Papahadjopoulos et al., 1977). In addition, Mg^{2+} induces a transient but significant release of internal contents from sonicated PS vesicles (Portis et al., 1979). These observations prompted us to investigate the Mg^{2+}-induced interaction of PS vesicles by using the Tb/DPA assay for mixing of aqueous vesicle contents (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980). In addition to the small sonicated vesicles composed of bovine brain PS studied in previous work, we also used larger unilamellar PS vesicles (approximately 1000 Å in diameter). Their response to Mg^{2+} is strikingly different from that of the small sonicated vesicles. In this paper, we report the contrasting effects of Mg^{2+} on large and small vesicles and compare them to the effects of Ca^{2+}.

Materials and Methods

Phosphatidylserine (PS) was purified from bovine brain as described previously (Papahadjopoulos & Miller, 1967), chromatographed on silicic acid, and finally washed with EDTA and NaCl (Papahadjopoulos et al., 1973). The source and purity of other reagents have been described before (Wilschut et al., 1980).

Vesicles, either small unilamellar vesicles (SUV; diameter ~250 Å) or large unilamellar vesicles (LUV; diameter ~1000 Å), were prepared by sonication or reverse-phase evaporation (Szoka & Papahadjopoulos, 1978) and subsequent extrusion through a 0.1-μm pore-size Unipore membrane (Olson et al., 1979), respectively, as described (Wilschut et al., 1980). SUV were made in either (a) 15 mM $TbCl_3$ and 150 mM sodium citrate, (b) 150 mM dipicolinic acid (DPA, sodium salt), or (c) 100 mM carboxyfluorescein (CF, sodium salt). LUV were made in either (a) 2.5 mM $TbCl_3$ and 50 mM sodium citrate, (b) 50 mM DPA (sodium salt) and 20 mM NaCl, or (c) 50 mM CF (sodium salt). In addition, all aqueous media contained 2.0 mM L-histidine and 2.0 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) adjusted to a final pH of 7.4. Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-75 (elution buffer, 100 mM NaCl, 2.0 mM L-histidine, 2.0 mM Tes, and 1.0 M 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 were (in nmol/μmol of lipid) 11.1, 210, and 221, respectively, for LUV and 1.7, 16.3, and 9.1, respectively, for SUV.

In the Tb/DPA assay for mixing of aqueous contents during vesicle fusion (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980), $TbCl_3$ is encapsulated as the $Tb(citrate)_3{}^{6-}$ complex in one population of vesicles and the sodium salt of dipicolinic acid (DPA) in another. Vesicle fusion allows the formation of the $Tb(DPA)_3{}^{3-}$ complex (hereafter referred to as the Tb/DPA complex), which is accompanied by a 10^4-fold enhancement of the Tb fluorescence (Barela & Sherry, 1976). Thus, fusion can be followed by continuous monitoring of the Tb fluorescence intensity. EDTA (0.1 mM) and Ca^{2+} (>1 mM) prevent the formation and/or cause the dissociation of the Tb/DPA complex in the external medium. We found a combination of EDTA (0.1 mM) and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) (0.1 mM) to be more effective than EDTA alone in quenching the Tb fluorescence in the presence of Mg^{2+}. In addition to mixing of vesicle contents, release to the external medium was measured by employing the increase in fluorescence intensity that occurs upon dilution of carboxyfluorescein (CF), initially encapsulated at a high, self-quenching concentration in the vesicles (Weinstein et al., 1977; Blumenthal et al., 1977; Portis et al., 1979; Wilschut et al., 1980).

Measurements were done in an SLM-4000 fluorometer (SLM Instruments) equipped with two 90° emission channels, allowing fluorescence and light scattering to be monitored simultaneously. Concentrated solutions of $CaCl_2$ or $MgCl_2$ were introduced, with a Hamilton syringe, into a magnetically stirred dispersion containing either a mixture of Tb and DPA vesicles or CF-containing vesicles. The temperature was maintained at 25 °C, and the final volume was 1.0 mL containing 100 mM NaCl, 2.0 mM L-histidine, 2.0 mM Tes, 0.1 mM EDTA, and 0.1 mM EGTA, pH 7.4. 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 (>530 nm) placed between the sample and the monochromator. Light scattering was measured, simultaneously with Tb fluorescence, in the second emission channel by using a Corning 7-54 band-pass filter. The Tb fluorescence scale was calibrated such that the 100% value corresponded to the fluorescence intensity that could maximally 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 (>530 nm). 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 by addition of Triton X-100 (0.1% v/v), was taken as the value for 100% release. Freeze–fracture electron microscopy was carried out essentially as described before (Vail et al., 1974).

Results

Mg^{2+}-Induced Vesicle Aggregation, Fusion, and Release of Vesicle Contents. We have investigated the mixing of internal aqueous volumes during Mg^{2+}-induced interaction of PS vesicles by using the Tb/DPA assay for vesicle fusion. Vesicle aggregation was monitored simultaneously by light scattering. Release of vesicle contents was measured in separate experiments under conditions otherwise identical with those in the fusion assay. The results for LUV and SUV are

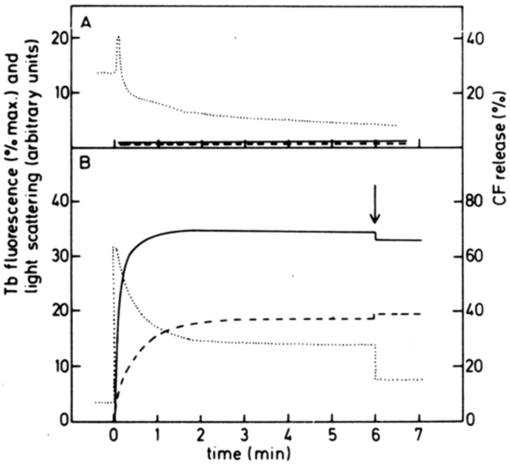

FIGURE 1: Mg^{2+}-induced aggregation and fusion of phosphatidylserine vesicles: (A) LUV; (B) SUV. At time zero, $MgCl_2$ was injected as a 0.2 M solution into the vesicle dispersion at a total lipid concentration of 0.05 mM (ratio of Tb to DPA vesicles in the fusion assay was 1:1, 0.025 mM each). Final Mg^{2+} concentration, 20 mM (LUV) and 8 mM (SUV). Solid lines, Tb fluorescence; dotted lines, light scattering at 276 nm; dashed lines, CF release. In the SUV experiments, EDTA was added (as a 0.2 M solution) after 6 min to a final concentration of 16 mM (arrow); the EDTA solution was pretitrated with NaOH such that the final pH of the medium was 7.4. The final fluorescence and scattering intensities are corrected for the dilution due to the addition of EDTA.

shown in parts A and B of Figure 1, respectively.

Mg^{2+} in concentrations as high as 20 mM did not induce any measurable mixing or release of the contents of LUV. Yet, above 5 mM Mg^{2+}, massive aggregation occurred, which was very obvious, particularly in the case of the CF-containing vesicles where relatively few, large, orange-colored aggregates were present in a colorless, nonfluorescent medium. Aggregation was reflected in the light-scattering curve (Figure 1A) as an initial increase and a subsequent decrease. This profile is similar to that seen during Ca^{2+}-induced aggregation of LUV (Wilschut et al., 1980) except for the large secondary increase in scatter intensity in the presence of Ca^{2+}, which was not observed with Mg^{2+}. This secondary increase has been attributed to a change in the refractive index of the material due to the formation of a tightly packed, collapsed, anhydrous structure (Wilschut et al., 1980). Therefore, in accordance with previous observations (Papahadjopoulos et al., 1977), Mg^{2+} did not induce the formation of such a collapsed structure, which is consistent with the complete retention of vesicle contents (Figure 1A).

With SUV, a different picture emerged (Figure 1B). After introduction of Mg^{2+} (8 mM) into the mixture of Tb and DPA vesicles (1:1 ratio), the Tb fluorescence started to develop immediately, but leveled off at approximately 35% and remained constant subsequently. Addition of excess EDTA at this stage resulted in disaggregation and almost complete fixation of the Tb fluorescence intensity (Figure 1B), indicating the physical separation of the Tb/DPA complex from the external medium. The release of vesicle contents was of a transient nature and leveled off at approximately 40%. Apparently, part of the vesicle contents is retained within the structure, which is reflected in both the Tb fluorescence and the CF fluorescence curves. This is in contrast to the situation with Ca^{2+}, where an eventual complete release of contents is observed (Wilschut et al., 1980). Due to the small initial size of the SUV, their aggregation initially caused a more pronounced relative increase in light scattering than that seen with LUV (Figure 1). Subsequently, as with LUV, a considerable decrease in scatter intensity was observed. By contrast, Ca^{2+} induces a sustained, very high scatter intensity, presumably due to the formation of tightly packed Ca/PS complexes (Wilschut et al., 1980).

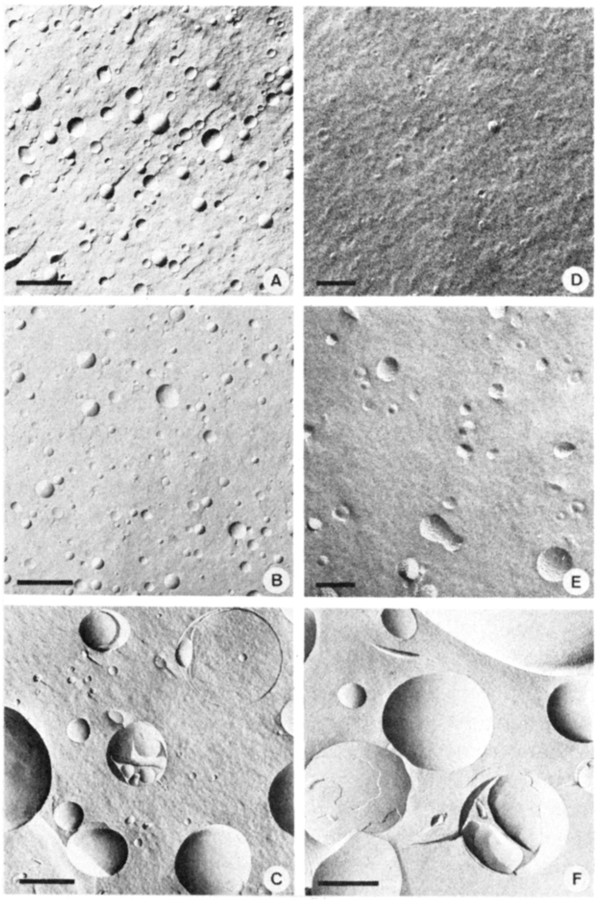

FIGURE 2: Freeze–fracture electron micrographs of phosphatidylserine vesicles before and after interaction with Ca^{2+} or Mg^{2+}. Vesicles, prepared in the 0.1 M NaCl medium in which the fusion assay is done, were incubated for 10 min at 25 °C with $CaCl_2$ or $MgCl_2$ at a lipid concentration of ~1.5 mM. The aggregates were spun down at 25 °C in an Eppendorf microcentrifuge, and, to disaggregate the vesicles, the pellets were treated with 0.1 M EDTA (pretitrated with NaOH to compensate for the release of H^+ ions upon binding of metal ion to EDTA at pH 7.4). The EDTA was added to at least a 2-fold molar excess over the amount of metal ion bound to the lipid. (A) LUV, control; (B) LUV, 10 mM Mg^{2+}; (C) LUV, 5 mM Ca^{2+}; (D) SUV, control; (E) SUV, 8 mM Mg^{2+}; (F) SUV, 3 mM Ca^{2+}. Bars represent 0.5 μm in (A–C) and (F) and 0.1 μm in (D) and (E).

Extent of Mg^{2+}- (and Ca^{2+}-) Induced Vesicle Fusion. The above differences between the effects of Mg^{2+} on LUV and SUV suggest that the fusion observed with the smaller vesicles is related to their high degree of bilayer curvature. This in turn would imply that the extent of Mg^{2+}-induced SUV fusion is likely to be limited: After the "strain" in the highly curved bilayer has been relieved as a result of limited increase in vesicle size, fusion would be expected to stop spontaneously. We, therefore, examined the extent of Mg^{2+}- (and Ca^{2+}-) induced fusion of PS vesicles by freeze–fracture electron microscopy. It is clear from the results in Figure 2 that Mg^{2+} caused no detectable change in the diameter of LUV and only a limited increase in the size of SUV (the average diameter increased from approximately 200 to approximately 400 Å, with the largest vesicle approximately 1000 Å), whereas Ca^{2+} induced a dramatic increase in vesicle size with both LUV and SUV (up to approximately 0.8-μm average diameter). These observations provide morphological evidence which supports our previous conclusion that Mg^{2+} is not inducing any fusion at all between LUV and only a limited number of fusion events between SUV. A more quantitative analysis of the increase in vesicle size during fusion is beyond the scope of this study and would require a more detailed morphological approach.

The limited extent of SUV fusion as induced by Mg^{2+} was further examined by changing the ratio of Tb to DPA vesicles in the fusion assay. This allows the estimation of the relative multiplicity of fusion events. For example, one round of binary fusion in a 1:1 mixture of Tb and DPA vesicles results in maximally 50% Tb fluorescence (the Tb–Tb and DPA–DPA fusions are nonproductive in terms of fluorescence), whereas in a 1:9 mixture of Tb and DPA vesicles 90% of the Tb present will become fluorescent, since the probability of a Tb–Tb fusion is 0.1. By contrast, if there were many rounds of fusion (multiple fusion events), the difference between the 1:1 and the 1:9 mixture would become negligible as all the Tb in the fused vesicles would then be able to complex with DPA. In experiments with the 1:9 mixture (data not shown), the maximal extent of Tb fluorescence was considerably higher (55%) than that in the 1:1 mixture (34%). These values, adjusted appropriately (Nir et al., 1980a) to take into account the extent of vesicle contents released during fusion (40%, Figure 1B), become 91% and 54%, respectively. These values for the calculated extent of fusion are very close to the theoretically expected values for binary fusion discussed above and indicate that according to this criterion the vesicles are undergoing approximately one round of fusion on the average. It should be noted that more detailed observations are needed in order to evaluate accurately the number of fusion events per vesicle under these conditions. Although both electron microscopy and the Tb assay indicate that Mg^{2+} induces only a limited number of fusion events between SUV, it is not possible to compare directly the increase in size (microscopy) with the extent of fusion (Tb fluorescence) because of the following factors: (i) the vesicle concentrations in these experiments differ by a factor of 30; (ii) the exact determination of vesicle size from freeze–fracture replicas is difficult because the fracture plane does not necessarily go through the largest diameter of the vesicle; (iii) for electron microscopy the sample was centrifuged in the presence of Mg^{2+} (see legend to Figure 2) which could have produced some larger vesicles.

Relative Kinetics of Aggregation and Fusion. Ca^{2+}-induced fusion of PS vesicles has been shown to occur without any appreciable delay relative to aggregation (Wilschut et al., 1980; Nir et al., 1980a). The evidence was based mainly on a comparison of the rate of vesicle fusion at a saturating Ca^{2+} concentration with the potential rate of aggregation under these conditions as calculated by Nir & Bentz (1978) and Nir et al. (1980b) and as determined by Lansman & Haynes (1975). It appeared that the rate constant for Ca^{2+}-induced Tb fluorescence development was very similar to the rate constant for aggregation. In addition, Tb fluorescence development was second order with respect to the vesicle concentration in a wide concentration range, indicating that the rate-limiting step was vesicle–vesicle aggregation rather than fusion per se.

We have now performed similar analyses for Mg^{2+}-induced SUV (PS) fusion. First, we examined the saturation with Mg^{2+}. The initial rate of fusion increased with Mg^{2+} concentration in a range up to ~15 mM, above which a saturation was observed (Figure 3). The actual rate of fusion observed at saturation is approximately 10-fold lower than the corresponding rate in the presence of saturating Ca^{2+} concentrations [Figure 3B; see also Wilschut et al. (1980)]. If we assume that in the presence of saturating concentrations of either Ca^{2+} or Mg^{2+} the rate of vesicle aggregation has reached the same maximal value, it would follow that the Mg^{2+}-induced fusion, in contrast to Ca^{2+}-induced fusion, is delayed with respect to aggregation. However, the above assumption that the rate of aggregation is the same in the presence of saturating Ca^{2+} or Mg^{2+} levels may not be entirely correct. Nir et al. (1980b) have shown that even under these extreme conditions (very high cation concentrations) a residual electrostatic energy barrier would be expected to cause a reduction of the rate of vesicle aggregation, and, according to these calculations, this reduction would be larger for Mg^{2+} than for Ca^{2+}, owing to the lower affinity of Mg^{2+} for PS than that of Ca^{2+}. Therefore, we approached the question of kinetics of aggregation vs. fusion from another direction and determined the kinetic order of the initial rate of Tb fluorescence development.

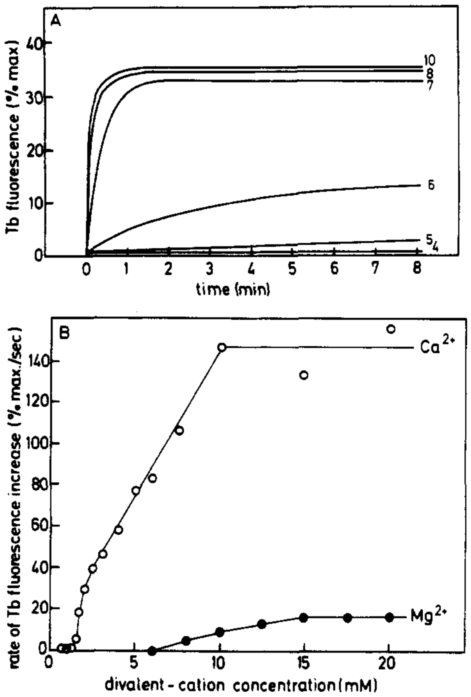

FIGURE 3: Dependence of SUV fusion on the Mg^{2+} concentration. $MgCl_2$ was injected as a 0.2 M solution into a 1:1 mixture of Tb and DPA vesicles (total lipid concentration, 0.05 mM) to the final millimolar concentrations indicated. (A) Tb fluorescence curves; (B) initial rates of the Tb fluorescence increase determined from the tangents to the fluorescence curves. (At the higher rates of fusion, fluorescence was recorded at very high recorder chart speeds.) For comparison, the corresponding results obtained with Ca^{2+} are also shown (Wilschut et al., 1980).

The initial rate of vesicle aggregation can be expected to be second order with respect to vesicle concentration. If the mixing of vesicle contents were also second order, this can be taken as an indication that fusion occurs without any appreciable delay. The dependence of the increase in Tb fluorescence on vesicle concentration in the presence of 8 mM Mg^{2+} is shown in Figure 4. An order of 1.6 was observed in the vesicle concentration range, where with Ca^{2+} the order was exactly 2.0. This indicates that the dependence of the rate of fusion on the frequency of vesicle–vesicle collisions is significantly less pronounced with Mg^{2+} than with Ca^{2+}, suggesting that, indeed, Mg^{2+}-induced fusion is delayed with respect to aggregation. At very high vesicle concentrations with either Mg^{2+} or Ca^{2+}, the order of the fusion reaction decreased (Figure 4). This phenomenon, which has been discussed before (Wilschut et al., 1980), shows that under those conditions the rate of fusion is almost completely independent of the rate of vesicle aggregation.

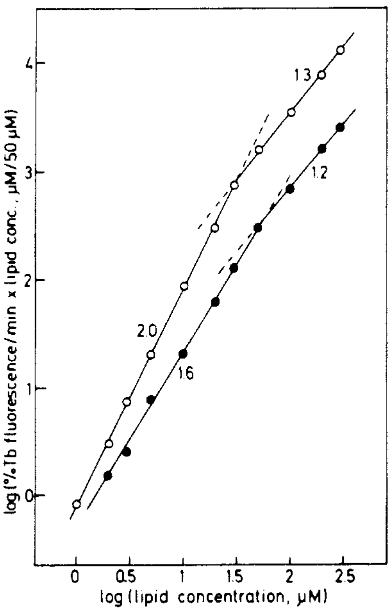

FIGURE 4: Dependence of SUV fusion on the vesicle concentration. Fusion was initiated by addition of $MgCl_2$ (as a 0.2 M solution) to a 1:1 mixture of Tb and DPA vesicles at various lipid concentrations. In all cases, the fluorescence scale was calibrated to the amount of Tb present. Initial rates of Tb fluorescence increase (percent of maximal fluorescence per minute) were then corrected for the actual lipid concentration, relative to 50 μM, and plotted in a double-logarithmic manner against the lipid concentration: (●) 8 mM Mg^{2+}; (○) 2 mM Ca^{2+} [results shown for reasons of comparison; see also Wilschut et al. (1980)].

Table I: Relative Kinetics of Ca^{2+}- or Mg^{2+}-Induced SUV Aggregation, Fusion, and Release of Vesicle Contents[a]

divalent cation concn (mM)	initial rate of increase: light scattering (unit increase/min)	Tb fluorescence (% max/min)	CF release (% max/min)
Ca^{2+}: 1.0	2.8 (0.70)	4.0	1.4 (0.35)
1.25	30 (0.60)	50	12 (0.24)
1.5	217 (0.62)	350	45 (0.13)
2.0	1025 (0.57)	1800	126 (0.07)
3.0	2010 (0.72)	2780	222 (0.08)
Mg^{2+}: 4.0	5.0 (250)	0.02	0.3 (15)
5.0	62 (155)	0.4	2.4 (6.0)
6.0	119 (34)	3.5	25 (7.1)
7.0	469 (6.6)	71	107 (1.0)
8.0	1110 (3.7)	300	291 (0.10)

[a] Fusion was initiated by addition of $CaCl_2$ (0.1 M) or $MgCl_2$ (0.2 M) either to a 1:1 mixture of Tb and DPA vesicles (SUV) or to CF-containing SUV. Total lipid concentration was 0.05 mM. Initial rates of increase of light scattering (at 276 nm), Tb fluorescence, and CF release were determined. The arbitrary units in which the light scattering is expressed are the same for the measurements with Ca^{2+} and Mg^{2+}. Values in parentheses indicate the ratio of initial rates of aggregation over fusion (column two) or the ratio of initial rates of release of contents over fusion (column four).

Further evidence to corroborate the above conclusion that Mg^{2+}-induced fusion is delayed with respect to aggregation was obtained from a direct comparison of the rates of fusion with the rates of aggregation as determined from changes in light scattering. Table I presents values for the rate of aggregation and the corresponding rates of Tb fluorescence increase at various Mg^{2+} and Ca^{2+} concentrations. The ratio of the two values appeared to be fairly constant through a wide range of Ca^{2+} concentrations: Under any condition where there was vesicle aggregation, fusion could be observed to occur, at the same relative rate. With Mg^{2+}, on the other hand, aggregation (when measured under the same conditions as Ca^{2+}-induced aggregation) was consistently faster than Tb fluorescence increase. Particularly at relatively low Mg^{2+} concentrations, such as 5 mM, there was appreciable aggregation but negligible mixing of vesicle contents. At higher Mg^{2+} concentrations, the ratio of the rate of aggregation to that of fusion dropped, indicating that these phenomena have distinct dependencies on the Mg^{2+} concentration.

Relative Kinetics of Fusion and Release of Contents. Ca^{2+}-induced fusion of PS vesicles is relative nonleaky. It has been estimated that SUV fusion is accompanied by the release of only 10% of the entrapped volume per fusion event, while LUV fusion is essentially nonleaky (Wilschut et al., 1980). Massive release of contents does occur, but as a secondary phenomenon related to the collapse of the vesicle structure during the formation of cochleate cylinders (Papahadjopoulos et al., 1975). In view of the very limited extent of Mg^{2+}-induced fusion of SUV (only a few rounds of fusion, see above) and the absence of the collapse of the structure, the eventual level of CF released (40%, Figure 1B) would appear to be relatively high. This is suggestive of a greater leakiness of the Mg^{2+}-induced fusion event as compared to Ca^{2+}-induced SUV fusion. We therefore analyzed the initial rates of Tb and CF fluorescence increase as measures of fusion and release of vesicle contents, respectively. The results are presented in columns 3 and 4 of Table I. With Ca^{2+}, the ratio of the rate of CF release to that of Tb fluorescence increase was low and fairly constant throughout the Ca^{2+} concentration range. With Mg^{2+}, this ratio was consistently several-fold higher than that with Ca^{2+}, indicating that the Mg^{2+}-induced fusion events are more leaky than the initial Ca^{2+}-induced fusion. In addition, the ratio of the rate of release to that of fusion was very high even at the lower Mg^{2+} concentration, suggesting that leakage of vesicle contents is occurring during initial aggregation when fusion is still quite limited.

Effects of Mg^{2+} on Ca^{2+}-Induced Vesicle Fusion: Synergism and Inhibition. In previous studies (Düzgüneş, 1978; Portis et al., 1979) we have proposed that Mg^{2+} could have a synergistic effect on Ca^{2+}-induced PS vesicle aggregation and fusion. Considerable aggregation and CF release could be induced with a mixture of Ca^{2+} and Mg^{2+} at concentrations of both cations which, when present separately, were ineffective. It was proposed that this apparent synergism is related to a Mg^{2+}-induced reduction of the surface charge density and thus of the electrostatic repulsion between the vesicles, which would allow the formation of a trans Ca/PS complex even at low (in the absence of Mg^{2+}, subthreshold) Ca^{2+} concentrations (Portis et al., 1979). In this view, Mg^{2+} is displaced from the PS vesicle surface during vesicle aggregation due to the formation of the trans complex which is specific for Ca^{2+}. This displacement could be substantiated by binding experiments, while freeze–fracture electron microscopy revealed the formation of cochleate cylinders from PS vesicles in the presence of 5 mM Mg^{2+} and only 0.05 mM Ca^{2+} (Portis et al., 1979). However, the drawback of any comparison with fast fusion events is that the binding and the electron microscopy experiments were done under equilibrium conditions, both procedures involving dialysis. These results, therefore, do not necessarily prove that a similar phenomenon also occurs during the very first contact between the vesicles. Therefore, it was of considerable interest to study the effect of Mg^{2+} on Ca^{2+}-induced fusion as monitored with the Tb/DPA assay.

Ca^{2+} and Mg^{2+} were introduced simultaneously in various ratios into a mixture of Tb and DPA vesicles, and the initial rate of the Tb fluorescence increase was determined. Figure

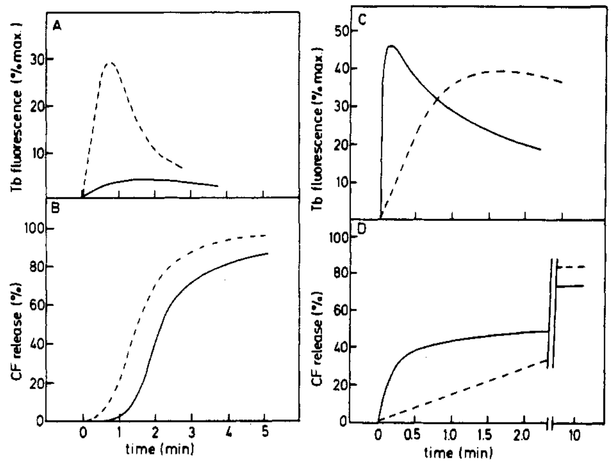

FIGURE 5: Effects of Mg^{2+} on the Ca^{2+}-induced fusion of phosphatidylserine vesicles. $CaCl_2$ (0.1 M) and $MgCl_2$ (0.2 M) were premixed in the desired ratios and added either to a 1:1 mixture of Tb and DPA vesicles (A and C) or to CF-containing vesicles (B and D). In all cases, the total lipid concentration was 0.05 mM. The solid lines are results obtained with both Mg^{2+} and Ca^{2+} present; the dashed lines represent the corresponding controls in the absence of Mg^{2+}. (A and B) LUV, 4 mM Ca^{2+}/10 mM Mg^{2+}; (C and D) SUV, 1.25 mM Ca^{2+}/5 mM Mg^{2+}.

5A shows that 10 mM Mg^{2+} strongly inhibited the fusion of LUV induced by 4 mM Ca^{2+}. Also, at other concentrations of Ca^{2+} or Mg^{2+}, inhibition was observed which became more pronounced at higher Mg^{2+} concentrations (Figure 6A). With SUV (Figure 5C), a synergistic effect of Mg^{2+} was observed at Ca^{2+} concentrations that are (in the absence of Mg^{2+}) just below or near the threshold [1.2 mM; see Wilschut et al. (1980)]. At higher Ca^{2+} concentrations, however, Mg^{2+} inhibited the rate of fusion of SUV (Figure 6B). Both effects, synergism and inhibtion, again became more pronounced at higher Mg^{2+} concentrations.

The above results indicate that Mg^{2+} can exert a synergistic effect on Ca^{2+}-induced fusion only in the case of SUV, where it will, at sufficiently high concentrations, induce fusion even in the absence of Ca^{2+}. Thus, it would appear that the observed synergism at near-threshold Ca^{2+} concentrations (Figure 6B) is the result of the cooperation of the two cations not only in reducing the electrostatic repulsion between the vesicles but also in fusing the vesicle bilayers. In accordance, the Ca^{2+}-induced fusion of LUV is inhibited by Mg^{2+} at all concentrations, since in this case Mg^{2+} cannot cooperate with Ca^{2+}, because of its intrinsic inability to fuse these large vesicles. These results then suggest that Ca^{2+} does not displace Mg^{2+} from the PS bilayer at the first instance of vesicle contact. However, as mentioned previously, eventually Ca^{2+} binding does become predominant, and cochleates are formed, probably due to extensive formation of trans Ca/PS complexes. Since this must be accompanied by extensive release of vesicle contents, we examined the leakage of CF from PS vesicles in the presence of mixtures of Ca^{2+} and Mg^{2+}. It appeared that indeed considerable release of contents occurs. With SUV in the presence of 5 mM Mg^{2+} and 1.25 mM Ca^{2+} (Figure 5D), initially a "Mg^{2+}-type" release was observed. However, the CF fluorescence did not reach a saturation level as with Mg^{2+} alone. Rather, it continued to increase slowly, indicating an eventual release of most of the internal contents due to collapse of the vesicles, as observed with Ca^{2+} alone. With LUV in the presence of 10 mM Mg^{2+} and 4 mM Ca^{2+} (Figure 5B), leakage of vesicle contents occurred after a pronounced lag period, but almost complete release was observed subsequently. This indicates that after aggregation the vesicle contents are released in a delayed reorganization process, which, like in the case of SUV, presumably represents the extensive formation of collapsed Ca/PS complexes. Although membrane fusion may occur during this reorganization process, it is not accompanied by mixing of aqueous vesicle contents separated from the external medium and therefore presumably involves a different mechanism than the initial fusion of PS vesicles as induced by Ca^{2+} alone.

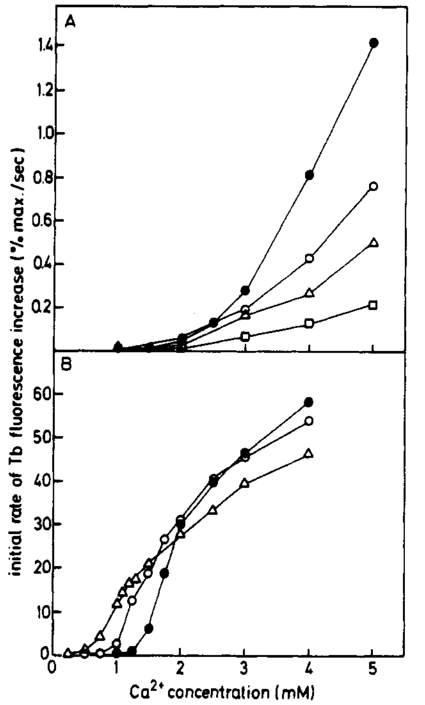

FIGURE 6: Effects of Mg^{2+} on the initial rates of Ca^{2+}-induced fusion of phosphatidylserine vesicles. $CaCl_2$ (0.1 M) and $MgCl_2$ (0.2 M) were premixed in the desired ratios and added to a 1:1 mixture of Tb and DPA vesicles at a total lipid concentration of 0.05 mM. Initial rates of Tb fluorescence increase were determined from the tangents to the fluorescence curves. (A) LUV; (B) SUV. (●) No Mg^{2+}; (○) 3 mM Mg^{2+}; (△) 5 mM Mg^{2+}; (□) 10 mM Mg^{2+}.

Discussion

The present study demonstrates the intrinsic inability of Mg^{2+} to induce the fusion of membranes composed of pure bovine brain phosphatidylserine at 25 °C, except for the small vesicles produced by sonication. This incompetency of Mg^{2+} is in marked contrast to the effect of Ca^{2+}, which induces fast and extensive fusion of PS vesicles almost irrespective of the initial degree of bilayer curvature (Wilschut et al., 1980). Although previous investigations (Papahadjopoulos et al., 1977; Portis et al., 1979) had provided evidence that Mg^{2+} is much less potent than Ca^{2+} in PS vesicle fusion, the absolute specificity of Ca^{2+} over Mg^{2+} in this system could be established only with the use of large unilamellar vesicles (Figure 1A). The diversity between the effects of the two cations does not extend to vesicle aggregation. Either ion induces massive aggregation, although with Mg^{2+}, due to its lower affinity for PS (Newton et al., 1978; Nir et al., 1978), higher concentrations are required than with Ca^{2+} [Table I and Figure 3; see also Papahadjopoulos et al. (1977) and Portis et al. (1979)].

The fusion of SUV (PS) as induced by Mg^{2+} is clearly related to the strong curvature of the bilayer in these vesicles (Figure 1). Yet, despite this critical, stimulatory effect of bilayer curvature, both the rate and extent of the Mg^{2+}-induced fusion of SUV are considerably lower than with Ca^{2+}-induced fusion. First, fusion with Mg^{2+} is delayed with respect to vesicle aggregation (Figures 3 and 4; Table I), indicating that the fusion per se is rate limiting. By contrast, with Ca^{2+}, the overall rate of fusion is limited by the frequency of vesicle–

vesicle collisons (Wilschut et al., 1980; Nir et al., 1980a). Second, the extent of Mg^{2+}-induced fusion of SUV is restricted to a doubling or tripling of the vesicle diameter (Figure 2), which underlines the crucial role of strong bilayer curvature: it is quite reasonable to suggest that fusion stops after a limited increase in vesicle size, as the "strain" in the original small vesicles is relieved. Furthermore, although leakage of vesicle contents accompanies the initial Mg^{2+}-induced fusion events, after the fusion has ceased there is no further release (Figure 1B). With Ca^{2+}, eventually the entire internal volume of the vesicles is lost as a result of collapse of the vesicles (Portis et al., 1979; Wilschut et al., 1980) and formation of cochleate cylinders (Papahadjopoulos et al., 1975) consisting of crystalline, anhydrous Ca/PS complexes (Newton et al., 1978). Such complexes are not formed with Mg^{2+}, which is presumably the reason for the absence of collapse of the vesicles and the partial retention of internal aqueous space. Finally, from the ratio of the initial rates of CF release and Tb fluorescence increase (Table I), it follows that Mg^{2+}-induced fusion of SUV is several-fold more leaky than Ca^{2+}-induced fusion. Moreover, the variation of this ratio with the Mg^{2+} concentration suggests that following vesicle aggregation at least part of the leakage may be occurring without concomitant fusion. For example, 6 mM Mg^{2+} induces relatively fast release of CF, but only very slow mixing of vesicle contents (Table I).

The differences between Ca^{2+} and Mg^{2+} in their ability to induce PS vesicle fusion must be related to the differences in the physicochemical properties of their respective complexes with PS (Newton et al., 1978; Papahadjopoulos et al., 1978; Portis et al., 1979). It has been proposed that in addition to any specific effects of an isothermal phase transition, the degree of bilayer dehydration may in itself be crucial to the initiation of fusion induced by Ca^{2+} (Portis et al., 1979). In agreement with this supposition, the model for membrane fusion proposed by McIver (1979) and recent observations by Ohki & Düzgüneş (1979) emphasize the role of interfacial water and the nature of the membrane surface. As ions are bound to a lipid bilayer, they will become at least partly dehydrated, and in addition they will destroy, more or less extensively, the stabilizing hydration layer at the lipid–water interface (Hauser et al., 1975, 1977). There is ample evidence now that this hydration layer accounts for strong repulsive forces between lipid bilayers at short distances of separation (Cowley et al., 1978). Ca^{2+} is more easily dehydrated than Mg^{2+} (Gresh, 1980); it binds more avidly to PS (Newton et al., 1978) and has been shown to form more anhydrous complexes than Mg^{2+} (Portis et al., 1979).

Inasmuch as dehydration plays a critical role in membrane fusion, the specificity of Ca^{2+} over Mg^{2+} is likely to arise during vesicle–vesicle contact. Thus, Ca^{2+} by forming a trans complex between the apposed bilayers will conceivably dehydrate the interbilayer space completely (Papahadjopoulos et al., 1978; Hauser et al., 1977), whereas Mg^{2+} would not be able to remove the water that keeps the membranes separated. The trans Ca/PS complex formed during initial vesicle aggregation may or may not be entirely crystalline, although a certain degree of condensation in the lipid packing would be expected to occur.

In conclusion, the difference in the abilities of Ca^{2+} and Mg^{2+} to induce fusion appears to be related to differences in factors such as the energy of dehydration of the ions and the lipid–water interface and the specific chemistry of the complex, which will in turn be related to the preferred coordination of the ions (Williams, 1976), the conformation of the polar group of the lipid (Gresh, 1980), and the average distance between the lipid molecules in the bilayer. Since the regular packing of lipid molecules is severely affected in a highly curved bilayer (Huang & Mason, 1978), the geometry of the Mg^{2+} binding to the lipid polar groups in SUV would be different than in the case of the LUV. This difference could explain the relative propensity of SUV to fuse with Mg^{2+}. At sufficiently high concentrations, the binding of Mg^{2+} to the outer surface of the (PS) SUV may cause transient molecular packing defects (evidenced by the high initial rate of release; Table I and Figure 1). These may serve as nucleation points for fusion as proposed earlier (Papahadjopoulos et al., 1977), the regular hydration layer being also affected by packing defects in the lipid. Fusion would then proceed essentially through hydrophobic association within these dicontinuities. The delay of fusion relative to aggregation in the presence of Mg^{2+} could arise from the necessity to align the nucleation points in apposed bilayers, this process being limited by their lateral diffusion along the surface of the bilayer.

The results obtained with mixtures of Ca^{2+} and Mg^{2+} indicate that the initial metal/PS complex may be different from the final complex. Previous equilibrium dialysis experiments (Newton et al., 1978; Portis et al., 1979) have demonstrated that in the presence of Mg^{2+} considerably more Ca^{2+} is bound to PS than in its absence. This was attributed to the formation of an intermembrane Ca^{2+}/PS complex after vesicle aggregation as facilitated by Mg^{2+}. Thus, because of the high binding constant of the trans Ca/PS complex, under these aggregating conditions Ca^{2+} would displace Mg^{2+} from the bilayer surface. Our present results (Figures 5 and 6), however, disfavor an early displacement of Mg^{2+} by Ca^{2+}. Fusion appears to be governed by the initial relative binding of the ions to dispersed vesicles, since under those conditions Ca^{2+} and Mg^{2+} are expected to compete with each other, and in the fusion measurements the effects of the ions are indeed essentially competitive. With SUV, either cation has the intrinsic capability of inducing fusion. In that case (Figure 6B), an apparent synergism can be observed, but only when both cations are present at near-threshold concentrations, such that they, when present separately, would be relatively ineffective due to electrostatic repulsion between the vesicles. With both ions present simultaneously, a substantial reduction of this repulsion is achieved, causing an increase in the rate of vesicle aggregation. Under these conditions, Ca^{2+} and Mg^{2+} cooperate in inducing fusion. At higher Ca^{2+} concentrations, Mg^{2+} is not required to achieve rapid vesicle aggregation. Then the competitive effect of Mg^{2+} becomes apparent as a relative inhibition of the rate of fusion, since the Mg^{2+}-induced fusion is a slower process than the Ca^{2+}-induced fusion (Figure 3). The competitive effect of Mg^{2+} is even more clearly demonstrated by the results obtained with LUV (Figure 6A). Mg^{2+} is expected to increase the rate of vesicle aggregation, particularly at the lower Ca^{2+} concentrations, but the initial rate of fusion is inhibited under any condition, because Mg^{2+} is intrinsically incapable of inducing LUV fusion (Figure 1A).

It is well established that Ca^{2+}, in contrast to Mg^{2+}, plays a vital role in such natural processes involving membrane fusion as exocytosis, secretion, and synaptic vesicle fusion (Poste & Allison, 1973; Rubin, 1974; Douglas, 1975). In view of the same specificity observed in the present study, we would like to postulate that the specific interaction of Ca^{2+} with certain acidic phospholipids is a key event in several biological fusion phenomena (Papahadjopoulos, 1978; Papahadjopoulos et al., 1977, 1978). As to a role for Mg^{2+}, it has been speculated that this ion might exert a synergistic effect on Ca^{2+}-induced fusion by promoting membrane apposition

(Portis et al., 1979; Papahadjopoulos et al., 1978). Although our present results may raise serious doubt in this respect, recent experimental data indicate that in mixed phosphatidylserine–phosphatidylethanolamine vesicle systems Mg^{2+} does enhance fusion induced by Ca^{2+} (Düzgüneş et al., 1981). However, it appears that Mg^{2+} alone also induces considerable fusion of these vesicles. This observation as well as the significantly less pronounced discrepancy between the effects of Ca^{2+} and Mg^{2+} in vesicle systems containing other acidic phospholipids than PS (Van Dijck et al., 1975; Papahadjopoulos et al., 1976) points to a rather complex relationship between the specificity of Ca^{2+} over Mg^{2+} and lipid composition.

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