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STUDIES ON MEMBRANE FUSION

III. THE ROLE OF CALCIUM-INDUCED PHASE CHANGES

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Summary

The interaction of phosphatidylserine vesicles with Ca^{2+} and Mg^{2+} has been examined by several techniques to study the mechanism of membrane fusion. Data are presented on the effects of Ca^{2+} and Mg^{2+} on vesicle permeability, thermotropic phase transitions and morphology determined by differential scanning calorimetry, X-ray diffraction, and freeze-fracture electron microscopy. These data are discussed in relation to information concerning Ca^{2+} binding, charge neutralization, molecular packing, vesicle aggregation, phase transitions, phase separations and vesicle fusion.

The results indicate that at Ca^{2+} concentrations of 1.0—2.0 mM, a highly cooperative phenomenon occurs which results in increased vesicle permeability, aggregation and fusion of the vesicles. Under these conditions the hydrocarbon chains of the lipid bilayers undergo a phase change from a fluid to a crystalline state. The aggregation of vesicles that is observed during fusion is not sufficient in itself to induce fusion without a concomitant phase change. Mg^{2+} in the range of 2.0—5.0 mM induces aggregation of phosphatidylserine vesicles but no significant fusion nor a phase change.

From the effect of variations in pH, temperature, Ca^{2+} and Mg^{2+} concentration on the fusion of vesicles, it is concluded that the key event leading to vesicle membrane fusion is the isothermic phase change induced by the bivalent metals. It is proposed that this phase change induces a transient destabilization of the bilayer membranes that become susceptible to fusion at domain boundaries.

Introduction

In previous papers of this series we have shown that Ca^{2+} (and to a limited extent Mg^{2+}) can induce fusion of vesicles prepared from various acidic phos-

pholipids [1]. Vesicle fusion was demonstrated both by differential scanning calorimetry, which monitored the rate and extent of mixing, and by electron microscopy, which revealed dramatic morphological changes accompanying vesicle fusion [1—3]. In this paper we present data on the possible mechanism by which Ca^{2+} and Mg^{2+} act to promote fusion between phospholipid membranes. The data suggest that there is a functional correlation between the effects of Ca^{2+} and Mg^{2+} in triggering fusion and the effect of these cations in altering the phase transition characteristics and the permeability properties of vesicles. Certain of the effects of Ca^{2+} are also examined in terms of their temperature dependence and cooperativity, their relation to Ca^{2+} binding on phosphatidylserine membranes, and the morphological changes observed by freeze-fracture electron microscopy. Evidence is presented which indicates that the effect of Ca^{2+} in promoting membrane fusion is related to its ability to induce phase transitions and phase separations (phase changes) in negatively charged phospholipid layers. We propose that this effect induces a transient destabilization of the bilayer, which becomes "fusion susceptible" at points of domain boundaries. Finally, it is proposed that the heat of crystallization which is evolved during the reaction of Ca^{2+} with acidic phopholipids may be an important factor in enhancing the fusion reaction.

Materials and Methods

Phosphatidylserine was purified from beef brain in this laboratory as described before [4]. The final product was re-chromatographed from silicic acid, washed with EDTA and NaCl [5] and kept in sealed ampoules under nitrogen as a solution in chloroform (10 μmol/ml) at −50° C. Other lipids and chemicals, as in the two preceding papers of this series [1,6].

The initial suspensions of multilamellar vesicles were prepared by the method of Bangham et al. [7]. These were then sonicated in a bath-type sonicator (Laboratory Supplies Co., N.Y., Model T-80-IRS) for 1 h at 20° C as before [8]. The buffer used in all experiments-contained NaCl (100 mM)/ L-histidine (2 mM)/*N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (TES) (2 mM)/EDTA (0.1 mM), adjusted to pH 7.4. The efflux rate of $^{22}Na^{+}$ and [^{14}C]sucrose from vesicle was measured as before [8]. Differential scanning calorimetry [9] and freeze-fracture electron microscopy as described elsewhere [1,2].

Ca^{2+} binding was measured by suspending phosphatidylserine (5 μmol) in 10 ml of buffer (100 mM NaCl/4 mM His/TES, pH 7.4) containing various amounts of $CaCl_2$ labeled with a $^{45}Ca^{2+}$ tracer. The suspension was shaken in a vortex mixer for 10 min at 24° C, incubated at 37° C for 30 min and then centrifuged for 0.5 h at $1 \cdot 10^5 \times g$ at 20° C. The pellet was separated carefully from the supernatant, and was resuspended in 1 ml of 1.33% sodium deoxycholate by sonicating for 10 min. The amounts of phosphate and ^{45}Ca were determined in both pellet and supernatant. In all cases, more than 90% of the phosphate added initially was found in the pellet. The concentration of Ca^{2+} was calculated from the amount present in the supernatant after subtraction of what would be bound to any phospholipid remaining in the supernatant. Ionophore A23187 (Lot No. 361-VO2-276-1) was generously provided

by Dr. R.J. Hosley of Eli Lilly Research Laboratories (Indianapolis, Ind.). ^{22}Na, ^{45}Ca and [^{14}C]sucrose were obtained from New England Nuclear, Boston, Mass. All other methodology and the source and purity of reagents have been described in the previous two papers of this series [1,6].

Results and Discussion

Correlation between increase in vesicle permeability and fusion of phospholipid vesicles

In a paper published in 1966 it was shown that the permeability of phosphatidylserine vesicles increased sharply when Ca^{2+} was added externally into the aqueous bulk phase [10]. We have re-examined this phenomenon in detail, with reference to more recent findings on the effect of Ca^{2+} on the thermotropic properties of phosphatidylserine membranes [9,11] the data on Ca^{2+} binding to vesicles [12] and the role of Ca^{2+} in producing charge neutralization, vesicle aggregation [13,14] and in triggering fusion of vesicle membranes [1—3].

One of the most interesting aspects of the Ca^{2+}-induced increase in vesicle permeability is the steep and discontinuous relationship to Ca^{2+} concentration. As shown in Fig. 1, the self-diffusion rate of Na^+ through sonicated phosphatidylserine vesicles at 24°C in 100 mM NaCl buffer is very low [15,16] and is not affected appreciably by Ca^{2+} concentrations up to 0.5 mM. However, at concentrations of Ca^{2+} between 0.5 and 2.0 mM, a sharp increase in Na^+ efflux is observed (500—1000 fold), which is accompanied by a simultaneous release of the captured Cl^- [17] and visible flocculation and precipitation of the vesicles. Fig. 1B, showing the $^{22}Na^+$ efflux rate of phosphatidylserine vesicles at the end of 2 h after the addition of Ca^{2+}, reveals that the efflux shows a steep dependence on Ca^{2+} concentration, implying a high degree of cooperativity in the action of Ca^{2+}.

The increase in vesicle permeability observed after addition of Ca^{2+} (and Mg^{2+}) externally is interpreted as being equivalent to the lowering of electrical resistance and "membrane breaking" observed with phosphatidylserine black film bilayers after the addition of Ca^{2+} on one side of the bilayer only [17,18]. When these bilayers were made in the presence of Ca^{2+} on both sides, membranes with very high electrical resistance were obtained [17,18]. To investigate a possible similar role for asymmetric Ca^{2+} distribution in altering vesicle permeability, phosphatidylserine vesicles were made in the presence of the calcium ionophore A-23187 at a molar ratio of 1 per 50 phospholipid molecules. The ability of the ionophore to transport Ca^{2+} across the bilayer should provide for a relatively rapid equilibration of the difference in Ca^{2+} concentration across the membrane of the vesicles containing the ionophore compared to vesicles composed of pure phospholipid. Vesicles were prepared as before in 100 mM NaCl buffer containing 1.0 mM sucrose and [^{14}C]sucrose as a marker for measuring changes in vesicle permeability. The ionophore-containing vesicles captured the same amount of [^{14}C]sucrose as control vesicles without ionophore made under the same conditions. Both types of vesicles were dialysed for 12 h against a large volume of buffer containing 0.5 mM $CaCl_2$ at 24°C to promote Ca^{2+} equilibration across the membranes of the test vesicles.

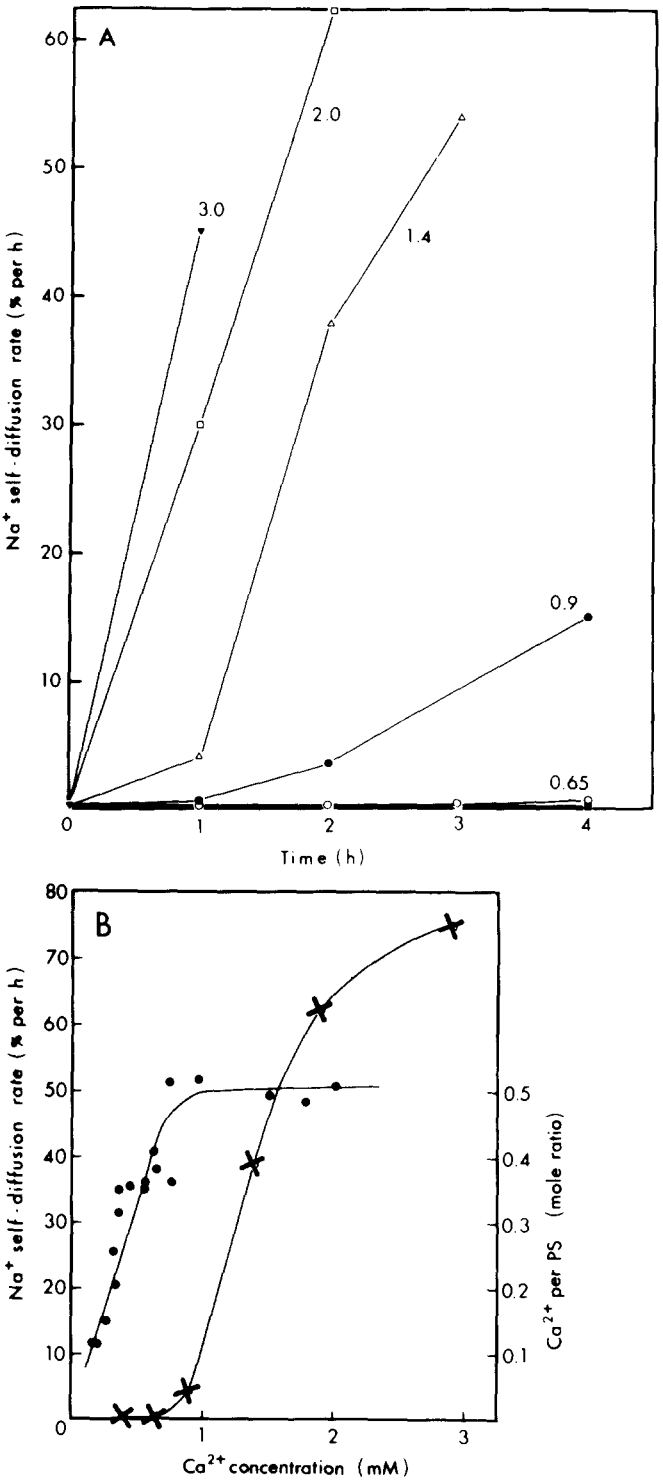

Fig. 1. The effect of Ca^{2+} at different concentrations on the permeability of phosphatidylserine vesicles to Na^+. (A) Self-diffusion rate of $^{22}Na^+$ at different time intervals after addition of Ca^{2+} to preformed sonicated vesicles: no Ca^{2+} (■); 0.6 mM (○); 0.9 mM (●); 1.4 mM (△); 2.0 mM (□); 3.0 mM (▼). The bulk phase contained NaCl (100 mM) buffered to pH 7.4. Temperature 24°C. (B) $^{22}Na^+$ efflux (from Fig. 1A) at the end of the second hour as a function of Ca^{2+} concentration (X). Mole ratio of Ca^{2+} bound to phosphatidylserine in multilamellar vesicles at different Ca^{2+} concentrations (●).

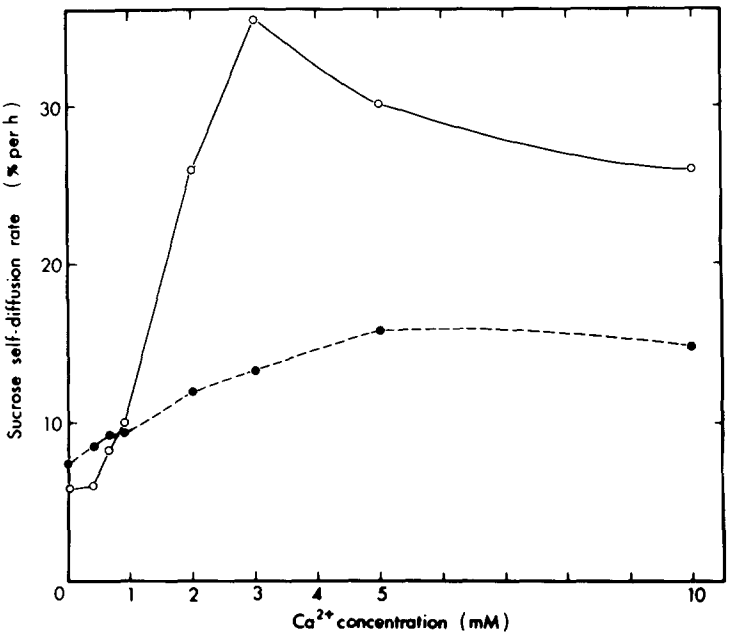

Fig. 2. The effect of Ca^{2+} at different concentrations on the permeability of sonicated phosphatidylserine vesicles to [^{14}C]sucrose. Vesicles formed with phospholipid alone (○); vesicles formed in the presence of ionophore A-23187 (●). Temperature, 24°C.

Finally, both types of vesicles were dialysed for three 1-h intervals against buffer containing different concentrations of Ca^{2+}, and the amount of [^{14}C]-sucrose present in each dialysate determined. The results, shown in Fig. 2 indicate that both types of vesicles, at low concentrations of Ca^{2+} (0.5 mM), exhibited identical permeabilities to sucrose. It is clear however, that at higher concentrations of Ca^{2+} the vesicles containing A23187 showed a significantly lower rate of release of [^{14}C]sucrose in response to the addition of external Ca^{2+}, compared to control vesicles without the ionophore. It was also noted that under these conditions the ionophore-containing vesicles required higher concentrations of Ca^{2+} (3 mM compared with 1.5 mM in control vesicles) to induce visible aggregation and the formation of cochleate cylinders in freeze-fracture electron micrographs. All the above results suggest that asymmetry of Ca^{2+} distribution across the vesicle membrane contributes significantly to the observed increase in vesicle permeability. As will be discussed in detail below, the increase in permeability induced by Ca^{2+} (at concentrations >1 mM) is accompanied by fusion of the small unilamellar vesicles into much larger structures [3].

Fig. 3A is a freeze-fracture electron micrograph of a preparation of sonicated phosphatidylserine vesicles in 100 mM NaCl buffer showing small (200—400 Å) unilamellar vesicles. No aggregation or other morphological changes were observed with these vesicles when stored at 24°C for 12 h or when dialysed for 12 h against the same buffer containing up to 0.5 mM $CaCl_2$. If, however, the suspension was dialysed for a similar time against the same buffer supplemented with 1 mM $CaCl_2$, new structures were formed which in freeze-fracture electron micrographs appear as long cochleate cylinders (Fig. 3B). Similar struc-

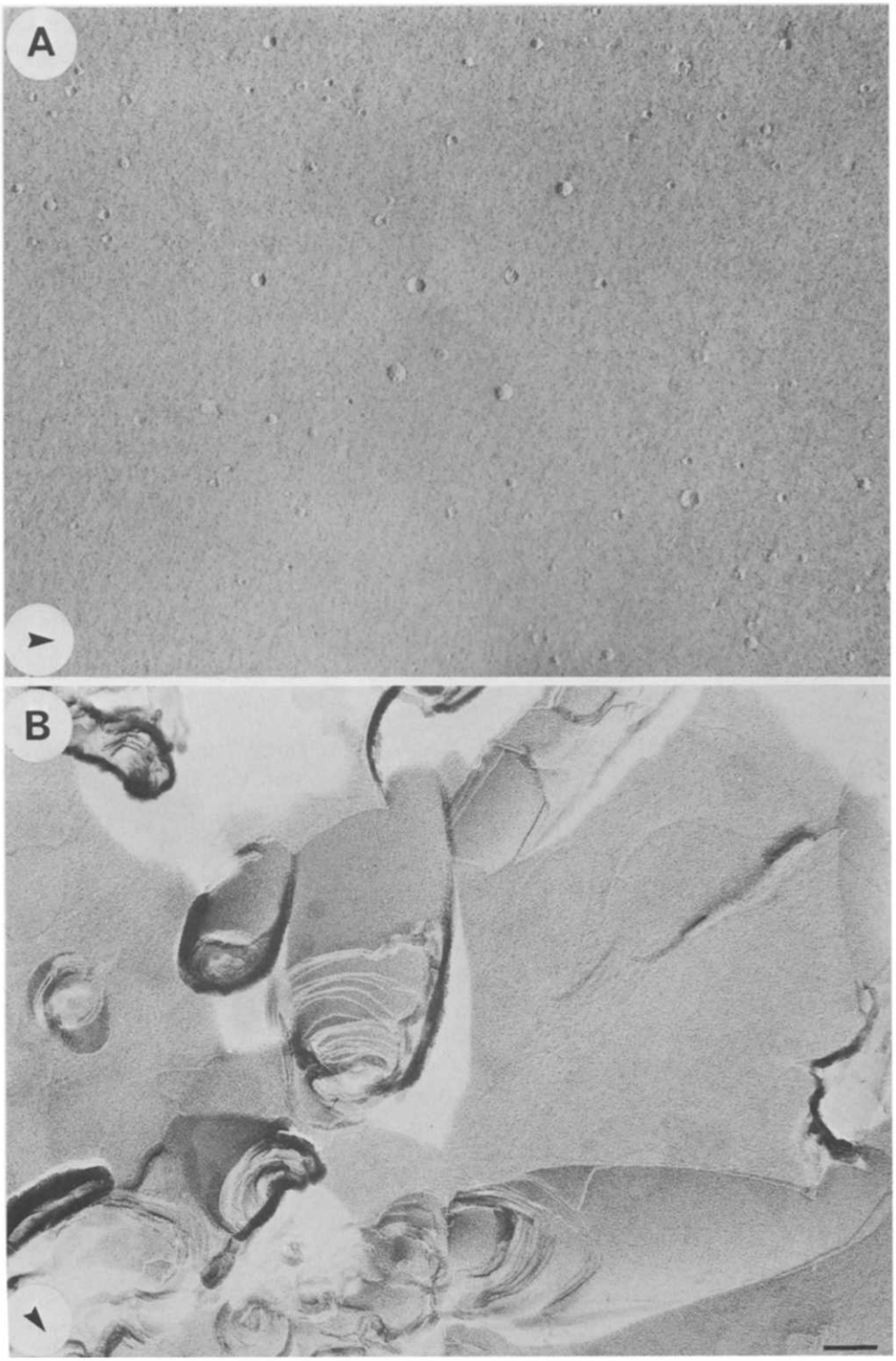

Fig. 3. Freeze-fracture electron micrographs of phosphatidylserine vesicles before and after Ca^{2+} addition. Vesicles were formed by sonication at 24°C in 100 mM NaCl buffer as indicated in methods: (A) before addition of Ca^{2+}; the vesicles were concentrated in Amicon (A-75) concentrator systems and quenched at 24°C into freon-liquid nitrogen in the presence of 30% glycerol; and (B) same preparation of vesicles as in A after dialysis against same buffer containing 1 mM $CaCl_2$ for 12 h at 24°C. The suspension was macroscopically aggregated under these conditions.

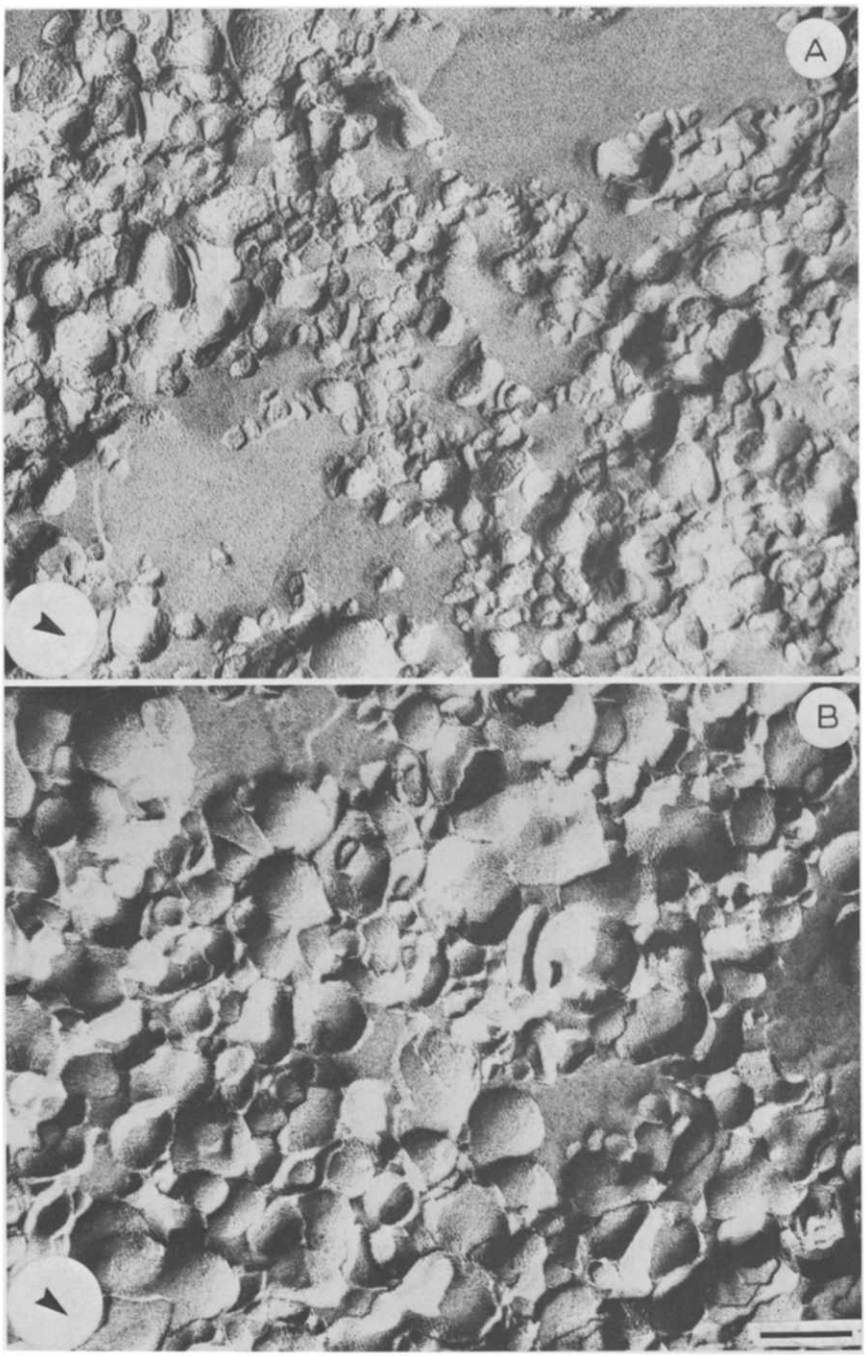

Fig. 4. Freeze-fracture electron micrograph of phosphatidylserine vesicles following addition of Mg^{2+}. Vesicles were made by sonication in NaCl buffer as in Fig. 3, and then treated as follows: (A) dialysed against NaCl buffer containing 3 mM $MgCl_2$ for 12 h at 37°C; and (B) incubated for 1 h at 37°C after addition of $MgCl_2$ to the NaCl buffer to a concentration of 10 mM.

tures were obtained following direct addition of Ca^{2+} at concentrations >1 mM at either 24 or 37°C [2], although dialysis of vesicle preparations against Ca^{2+}-containing buffer as described above produced relatively more cochleates. Samples frozen at different times after the addition of Ca^{2+} indicate that the precipitate obtained immediately after addition of Ca^{2+} contains aggreagated flat membranous structures but very few cochleate cylinders are present. Cochleate cylinders were more frequent after 1 h, however, and were the predominant structure after overnight incubation.

Freeze-fracture electron micrographs of phosphatidylserine vesicles dialysed against 3 mM Mg^{2+} at 37°C for 12 h (Fig. 4A) reveal that aggregation of intact vesicles occurs but no appreciable fusion to form larger structures is evident. The aggregation shown in Fig. 4A is very slow, and is not apparent within 3 h of dialysis at 2—3 mM Mg^{2+} at 37°C. Aggregation is evident within 2—3 h dialysis against the same buffer at 37°C containing 5 mM Mg^{2+}. Aggregation is obtained immediately following addition of higher concentrations of Mg^{2+} (10 mM) to phosphatidylserine vesicles incubated at 37°C. Under the latter conditions, following incubation at 37°C for 1 h (Fig. 4B), the aggregated vesicles are considerably larger than those without Mg^{2+} (Fig. 3) indicating that some fusion may have occurred. Earlier results [10,17] indicated that the permeability of phosphatidylserine vesicles increases sharply only when Mg^{2+} is added externally at concentrations of 5—10 mM, again indicating some relationship between an increase in vesicle permeability and vesicle fusion.

Vesicle aggregation and vesicle fusion: differentiation between simple electrostatic charge neutralization and induction of membrane fusion

To understand the possible relationship between the Ca^{2+}-triggered permeability increase and vesicle fusion, we next examined Ca^{2+} binding and its effects on the physical properties of phosphatidylserine membranes at Ca^{2+} concentrations above and below the critical concentration required to induce fusion (1 mM). It has already been shown [12] that at a Ca^{2+} concentration of 1 mM (in 100 mM NaCl at pH 7.4), the mole ratio of bound Ca^{2+} to phosphatidylserine is 0.5. More detailed data on Ca^{2+} binding to phosphatidylserine multilamellar vesicles (Fig. 1B) reveal a sharp increase in this ratio from 0.12 (at 0.2 mM Ca^{2+}) to 0.5 (at 0.75 mM Ca^{2+}), at which point a plateau is reached that is maintained up to at least 2 mM. A Hill plot of the data in the concentration range 0.2—0.7 mM gives a straight line with a slope of 2, indicating some cooperativity in Ca^{2+} binding. The plot of the ^{22}Na efflux rates through phosphatidylserine vesicles in the same range of Ca^{2+} concentration (Fig. 1B) gives a steeper dependence on Ca^{2+} concentrations compared to that obtained in the binding studies. It is evident from these results that the permeability of the vesicles is not related to Ca^{2+} binding in a simple linear fashion, and that Ca^{2+} has no appreciable effect on permeability until the molar ratio of bound Ca^{2+} to phosphatidylserine approaches the maximal value of 0.5. Since this ratio corresponds to the point of complete charge neutralization, it is pertinent to ask whether the observed fusion is the inevitable result of such electrostatic neutralization and the accompanying phenomenon of vesicle aggregation.

Data on the electrophoretic mobility of multilamellar vesicles and surface potential (ΔV) of monolayers indicate that Ca^{2+} at 0.5 mM (in the presence of

100 mM NaCl, pH 7.4) reduces the electrokinetic potential (ζ) by 13 mV and the ΔV by 37 mV. In contrast, Mg^{2+} at the same concentration reduces ΔV by only 24 mV [13]. These changes indicate that partial charge neutralization has occurred, which can be interpreted either as double layer screening or specific binding of the metal to the acidic groups of the phospholipid. Although double-layer screening has been favored by several investigators on the basis of certain experimental agreement with the Gouy-Chapman formulation [19], it appears that some degree of specific binding by Ca^{2+} must be involved since differences between Ca^{2+}, Mg^{2+} and other divalent metals cannot be rationalized on the basis of simple double layer electrostatics. The partial neutralization of charges is accompanied by a decrease in the area per molecule of phosphatidylserine in monolayers [12,13] and an increase (10—15°C) in the transition temperature (T_c) from fluid to liquid crystalline [9,11]. This latter phenomenon has been observed with phosphatidylserine membranes in the presence of both Mg^{2+} (1.5 mM) and Ca^{2+} (0.1—0.5 mM). It thus appears that at low Ca^{2+} concentrations (<0.5 mM), there is only partial neutralization of the negative charges which results in changes in surface potential, in molecular packing and in thermotropic properties but which does not affect in a major way the aggregation state of the vesicles or their permeability properties, and does not produce fusion.

As the concentration of Ca^{2+} increases from 0.5 to 1.5 mM, the ζ and ΔV potentials decrease further by 7—8 mV [13] but the permeability increases by 2—3 orders of magnitude and the thermotropic transition shifts to much higher temperatures [9]. By extrapolation from the effects of Ca^{2+} on dilaurylphosphatidylglycerol [20], which shifts the T_c by 80°C the T_c of phosphatidylserine in the presence of 1 mM Ca^{2+} could be as high as 87°C. X-ray diffraction spacings also indicate that Ca^{2+} at 1—2 mM and at a temperature of 25°C induces complete crystallization of the hydrocarbon chains [9]. Furthermore, the low angle diffractions indicate a well-packed lamellar system with a repeat distance of 54 Å [4,21]. This would involve a close apposition of the bilayers, with only a thin layer of water in between. As shown in Fig. 3, the vesicles at this point fuse into much larger membranous structures. The stoichiometry of the phosphatidylserine · Ca^{2+} complex in this concentration range is near the maximal 2 to 1 (Fig. 1B). The lack of an effect by Ca^{2+} on the kinetics of ^{22}Na efflux and vesicle fusion at concentrations lower than 1 mM can be explained if the assumption is made that all the available Ca^{2+} binding sites must be occupied in order to "trigger" a phase transition over a membrane region or domain. Concentrations of Ca^{2+} in excess of those required for complete saturation might be necessary to increase the probability that all the available Ca^{2+} sites will be occupied and that this complete occupation will persist long enough for the phase transition to be nucleated and propagated over the entire domain. The phosphatidylserine · Ca^{2+} complex, at Ca^{2+} concentrations above 1 mM, could therefore involve a polymeric arrangement similar to that proposed earlier [13], while at lower concentrations it could involve only dimers.

It is clear therefore that in the region of 0.1—1.5 mM Ca^{2+}, a highly cooperative event takes place which results in aggregation and fusion of phosphatidylserine vesicles and that this is also accompanied by a release of their

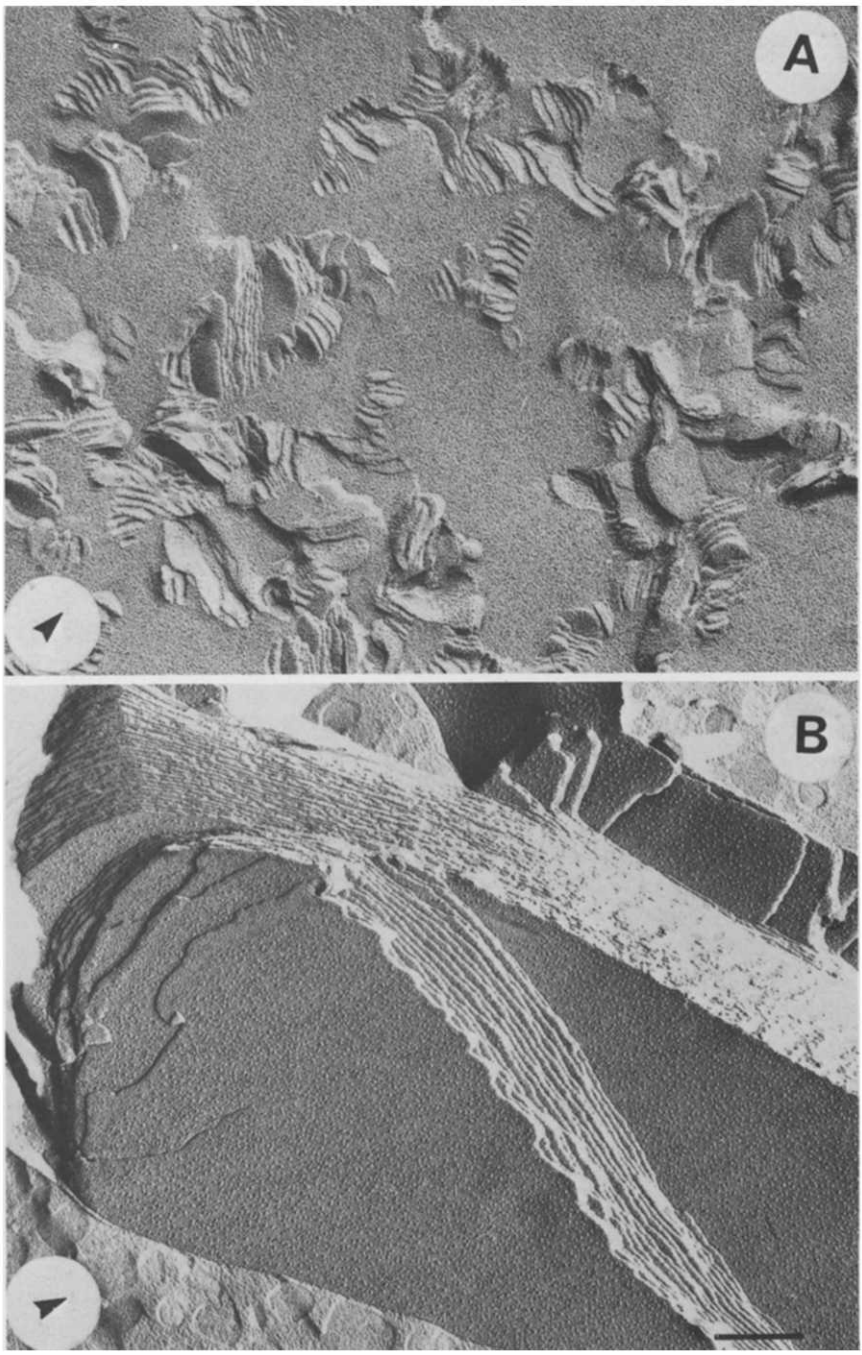

Fig. 5. Freeze-fracture electron micrograph of phosphatidylserine vesicles prepared in NaCl buffer as in Fig. 3 and then treated as follows: (A) equilibrated at 0°C, after which Ca^{2+} (2 mM) was added and further incubated for 1 h. The sample was quenched from 0°C; and (B) the vesicle suspension was titrated to pH 3.5 with 0.1 HCl at 20°C and then incubated further for 1 h before quenching from 20°C. In both cases macroscopic aggregation was evident.

contents, loss of the normal phase transition and crystallization of the hydrocarbon chains. Mg^{2+} at similar or slightly higher concentrations (2—5 mM) produces only a slow aggregation of the same vesicles (Fig. 4), presumably due to at least partial charge neutralization. Importantly, relatively little fusion and no increase in vesicle permeability occur under these conditions. It is evident therefore that simple charge neutralization and vesicle aggregation are not sufficient to induce fusion.

Correlation between phase transition, phase separation and fusion

As mentioned above, Ca^{2+}-induced fusion of phosphatidylserine vesicles is accompanied by a phase change which results in crystallization of the acyl chains of the phospholipid bilayer. The experiments described below were designed to investigate the role of phase changes as a possible factor of major importance in the induction of membrane fusion.

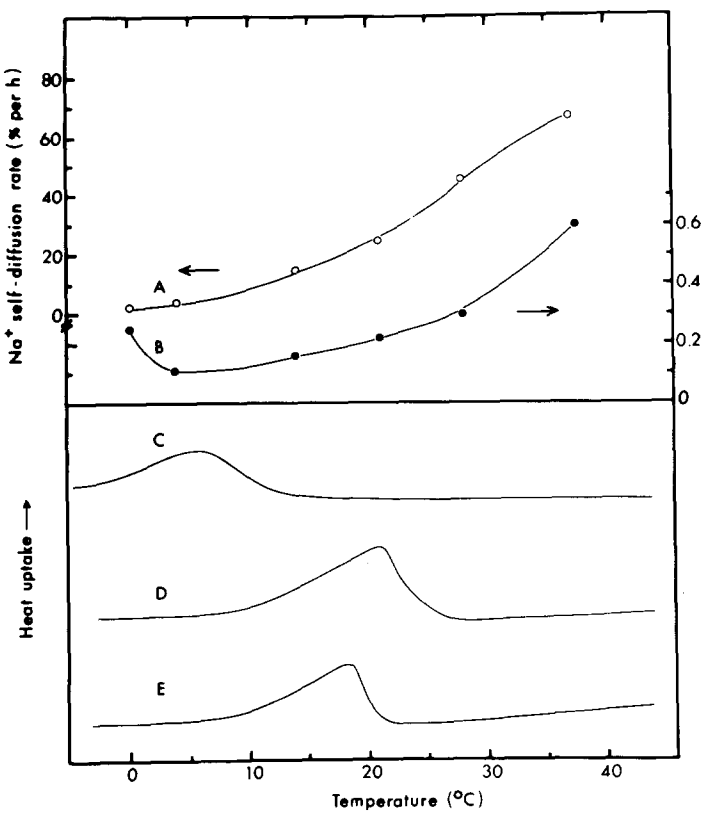

Fig. 6. Permeability and calorimetric changes of phosphatidylserine membranes in different ionic environments: A and B, efflux rates of $^{22}Na^{+}$ through sonicated vesicles at different temperatures. (A) in the presence of 1.2 mM $CaCl_2$ and 100 mM NaCl buffer, pH 7.4, and (B) in the presence of 100 mM NaCl buffer alone; C, D, and E, differential scanning calorimeter tracing of non-sonicated phosphatidylserine multilamellar vesicles, showing the endothermic transition from solid to liquid crystalline: (C) in 100 mM NaCl buffer alone, pH 7.4; (D) in 100 mM NaCl, pH 3.5; and (E) in 100 mM NaCl, pH 7.4, containing 5 mM $MgCl_2$.

If Ca^{2+} is added (2 mM) to phosphatidylserine vesicles which have been pre-equilibrated at 0°C, a precipitate forms but the morphology of the aggregates (Fig. 5A) is entirely different from that described in Fig. 3B. As shown in Fig. 5A the structures revealed by freeze-fracture electron microscopy appear as flattened spheroids (discs) aggregated along their flat surfaces. The small average size of these disks indicates that only very limited vesicle fusion could have occurred. In contrast, much larger aggregated flat membranous structures (Fig. 5B) were obtained when the same phosphatidylserine vesicles were suspended in NaCl buffer (no Ca^{2+}) after which the pH of the suspension medium was reduced to 3.5 and incubated for 1 h at 20°C. Under these conditions, not only was macroscopic aggregation of the vesicles observed, but large membranous structures were found in freeze-fracture electron micrographs (Fig. 5B) indicating that massive fusion of the original vesicles has taken place.

The striking differences in fusion behavior observed in Fig. 3, 4 and 5 can be accounted for by considering the thermotropic properties of phosphatidylserine membranes (Fig. 6). Since the mid-point of the endothermic transition of phosphatidylserine vesicles is at 6°C (Fig. 6, curve C), these membranes would be mostly frozen at 0°C and completely fluid at temperatures above 12°C. When phosphatidylserine vesicles are dialysed against 1.2 mM Ca^{2+} at different temperatures, the increase in Na^+ efflux is very large at temperatures above 10°C, but considerably smaller at temperatures 0—5°C when the membranes would be below their T_c (Fig. 6, curve A). Curve B in Fig. 6 shows that the $^{22}Na^+$ efflux from the same phosphatidylserine vesicles in NaCl buffer (no Ca^{2+}) remains very low in this temperature range *. It thus appears that although Ca^{2+} induces aggregation of phosphatidylserine vesicles at all temperatures, its effect in increasing the Na^+ efflux and producing fusion is evident only at temperatures higher than the T_c, when the acyl chains of the phospholipid would be in a fluid state. Since Ca^{2+} induces crystallization of these chains, it is reasonable to conclude that Ca^{2+}-induced vesicle fusion is related to a phase change (fluid-to-solid) rather than to the aggregation induced by charge neutralization seen in Fig. 5A at low temperatures.

The vesicle fusion event shown in Fig. 5B induced by lowering the pH of the medium in which the vesicles were suspended, could also be related to a phase change. As shown in Fig. 6 (curve D) the T_c of phosphatidylserine membranes is increased considerably by decreasing the pH to 3.5. At 20°C the titration of the vesicles to that pH would have brought them beyond the onset of crystallization.

The effect of Mg^{2+} on the thermotropic properties of phosphatidylserine membranes is also shown in Fig. 6 (curve E). As described earlier [9,11,22], Mg^{2+} induces a 10—12°C shift of the T_c of acidic phospholipids to higher temperatures. It is clear, however, from the position of the endothermic peak that when phosphatidylserine vesicles are incubated at 24 or 37°C in the presence of Mg^{2+}, they would still be fluid (above the onset of crystallization).

* The efflux of $^{22}Na^+$ through phosphatidylserine vesicles increases considerably as the temperature decreases below 5°C. This result is consistent with the anomalous increase in permeability within the temperature region of the phase transition of phosphatidylglycerol vesicles observed earlier [5]. The sonicated phosphatidylserine suspension at 0°C appears to contain small vesicles similar in size to those observed at higher temperature (Fig. 3A).

Fig. 7. Freeze-fracture electron micrograph of phosphatidylserine membranes following addition of Mg^{2+}. Experimental conditions are as in Fig. 4B, except that vesicles were pre-equilibrated at 12°C, then $MgCl_2$ (10 mM) added and the preparation incubated for 1 h at 12°C.

If a phase change is a prerequisite to vesicle fusion, the addition of Mg^{2+} at 37°C should not induce fusion of phosphatidylserine vesicles, which is in agreement with the observations presented in Fig. 4, indicating mainly aggregation of vesicles.

The relationship between the effect of Mg^{2+} on membrane phase transitions and membrane fusion can also be seen by comparison of the structure shown in Fig. 4 with those shown in Fig. 7. In the latter case, phosphatidylserine vesicles were first equilibrated at 12°C after which Mg^{2+} was added at a concentration of 10 mM, followed by incubation for 1 h at 12°C. Freeze-fracture electron microscopy reveals that the particles formed under these conditions are multilamellar and considerably larger, indicating that a higher degree of fusion is occurring at 12°C than at higher temperatures. Similar large multilamellar vesicles were obtained by dialysis of phosphatidylserine vesicles against 5 mM Mg^{2+} at 12°C for a period of 3 h, while dialysis at 37°C under these conditions produced mostly aggregated small vesicles with only few larger vesicles, indicating a relatively small degree of fusion. Therefore, it appears that fusion

can be enhanced at concentrations of 5—10 mM Mg^{2+} when the temperature is decreased to 12°C. An explanation for this enhancement of membrane fusion by a decrease in temperature can be obtained by examination of the calorimetric data for phosphatidylserine vesicles before and after addition of Mg^{2+} shown in Fig. 6. At 12°C, in the presence of only NaCl (no Mg^{2+}), phosphatidylserine membranes are fluid (above their T_c = 6°C). When Mg^{2+} is added, the transition is shifted to higher temperatures so the same membranes will be solid-like in the final state (below their T_c = 18°C). The higher degree of fusion obtained under these conditions (compared to 37°C where Mg^{2+} does not induce phase change) indicates that the phase change induced by the divalent metal ion could be related intimately to the mechanism of membrane fusion. Indeed as discussed below, it is possible that the phase change is actually providing the driving force for the fusion reaction.

The increased degree of fusion observed at 10 mM Mg^{2+} (Fig. 4B) compared to 2 mM Mg^{2+} (Fig. 4A) does not appear to be related to a further shift of the transition to higher temperatures. The endothermic transition of phosphatidylserine at 10 mM $MgCl_2$ (mid-point T_c : 19.5°C) is only 1.0°C higher than that obtained at 2.5 mM $MgCl_2$ (results not shown) under the same conditions used for curve E, Fig. 6. These results indicate that the phosphatidylserine vesicles would be "fluid" at 37°C, even in 10 mM $MgCl_2$. Therefore, the relatively small but appreciable degree of fusion (Fig. 4B) and also the increase in permeability of phosphatidylserine vesicles [10,17] observed at 10 mM Mg^{2+} and 37°C could not be due to a phase transition. This phenomenon is presently under further investigation.

Comparison of the effects of Ca^{2+} (and Mg^{2+}) on different phospholipid vesicles indicates that the phenomena described above are not specific to phosphatidylserine. Although the concentration of the bivalent metal necessary to induce changes in vesicle permeability and fusion can differ widely, it appears that there is a good correlation between the three main events: (1) a phase transition or separation; (2) a drastic increase in membrane permeability; and (3) fusion of bilayers from different vesicles. The increase in permeability was shown to occur at 0.1—0.2 mM Ca^{2+} for phosphatidic acid and 10—20 mM Ca^{2+} for phosphatidylglycerol [17]. We have also shown [1] that fusion of these vesicles occurs at exactly the same concentrations of Ca^{2+}. However, the bivalent metal specificity differs between phospholipids; both Ca^{2+} and Mg^{2+} can fuse phosphatidic acid vesicles, but phosphatidylglycerol vesicles have been shown to fuse only in the presence of Ca^{2+} [1]. Although fusion has not been established, the formation of cochleate cylinders was observed when dilauryl-phosphatidylglycerol was dispersed in aqueous solutions containing equimolar amounts of $MgCl_2$ when heated above 20°C [44]. We have also shown that the Ca^{2+}-induced fusion can be observed with both sonicated unilamellar vesicles, and with hand-shaken multilamellar vesicles [1].

A good correlation between increased membrane permeability, phase separation and membrane fusion is also observed in vesicles containing phosphatidylserine mixed with either phosphatidylcholine or cholesterol. Vesicles composed of phosphatidylserine and phosphatidylcholine at equimolar ratios do not show an increase in permeability to Ca^{2+} even at 10 mM, at which point they aggregate [17]. Calorimetric observations on these vesicles have failed to detect

either phase separation or vesicle fusion [3]. However, when incubated with 10 mM Ca^{2+}, the same phospholipids at a 2 to 1 ratio show both a large increase in permeability [17], undergo a phase separation [3,9] and fusion [3]. Unilamellar (sonicated) vesicles composed of an equimolar mixture of phosphatidylserine and cholesterol show an increase in permeability at 2 mM Ca^{2+} but not at 1 mM [17], and recent freeze-fracture data obtained in this laboratory reveals the formation of large vesicles at 2 mM Ca^{2+}, while at 1 mM Ca^{2+} the vesicles simply aggregate but do not fuse into larger structures. It is not clear at present how the Ca^{2+}-induced fusion of phosphatidylserine/cholesterol vesicles is related to phase transitions because the endothermic transition of phosphatidylserine is not observable in the presence of equimolar amounts of cholesterol. Finally, no evidence for fusion, phase separation or increase in permeability has been observed with pure phosphatidylcholine vesicles or phosphatidylserine/phosphatidylcholine vesicles containing up to 40% phosphatidylserine incubated with Ca^{2+} at concentrations up to 10 mM [17,3].

Discussion

Phase changes induced by Ca^{2+} as the driving force for membrane fusion

It is evident from the experiments discussed above that there is a strong correlation between the aggregation of vesicles, an increase in vesicle permeability, and the onset of phase changes and vesicle fusion. All these events are initiated in a highly cooperative fashion by increasing Ca^{2+} (and in certain instances Mg^{2+}) concentrations. The concentrations of Ca^{2+} needed vary widely with different phospholipids or mixtures, but the general characteristics of all these systems are very similar. As reported here, and in a previous study [1] vesicle aggregation is not always followed by fusion and the other events mentioned above. Recent calculations based on superposition of the electrostatic repulsive interactions and Van der Waals attractive interactions [23] indicate that for vesicles with a charge density exceeding 1/10 of electronic charge per phospholipid molecule, there is only small probability for the vesicles to approach within 25 Å of each other, at an ionic strength (0.1 M) corresponding to $\kappa = 1.25 \cdot 10^7\ cm^{-1}$, where $1/\kappa$ is the Debye length. When Ca^{2+} or Mg^{2+} are added they neutralize the charges, thus reducing the barrier to close approach of the vesicles, which are then observed to aggregate as mentioned above.

Charge neutralization which leads to aggregation can also be followed by fusion, but in itself is not sufficient to induce fusion. The additional "key" event required for fusion seems to be the phase change from fluid to crystalline acyl chain packing that is induced by Ca^{2+} at concentrations above 1 mM. However, since the final product of the interaction of acidic phospholipids with Ca^{2+} is stable bilayer membranes of very low electrical conductance [18] or completely crystalline multilamellar systems [9] which are not susceptible to further fusion [1], it is reasonable to consider that it is transient events associated with the initial addition of Ca^{2+} which are responsible for creating the intermediate unstable state that leads to fusion.

Membrane fusion involves mixing of the components of two different membranes with subsequent formation of one membrane. Since the initial mem-

branes are stable structures, mixing and subsequent fusion clearly cannot occur until the membranes are destabilized. A few experimental facts can be used to indicate the high initial stability of phospholipid membranes. For instance, phospholipid vesicles are quite impermeable to cations [15,16]. Moreover, the movement of molecules from the inside to the outside monolayer of a phosphatidylcholine vesicle is a very slow process [24,25], as is also the exchange and mixing of molecules between pure phospholipid vesicles at temperatures above the T_c without specific exchange proteins [3,6,26,27]. Finally, the activation energy for the dissolution of phosphatidylserine molecules from a monolayer into the aqueous phase is approximately 30 kcal/mol [28]. All the above facts suggest that transfer and mixing of molecules between two adjacent bilayers would be extremely unfavorable energetically. The Ca^{2+}-induced phase changes in acidic phospholipid membranes discussed earlier could be responsible for altering the energetics of the system by providing an unstable transition state of relatively low free energy of activation, thus increasing the probability for the process.

It is proposed here that addition of Ca^{2+} induces a transient unstable state during which the bilayer is highly susceptible to fusion but which becomes stable again after Ca^{2+} equilibration or removal. The molecular events responsible for creating this unstable state will be discussed below as three separate but intimately related phenomena: (1) Ca^{2+} asymmetry across the phospholipid bilayer; (2) the boundaries between two different phospholipid domains (solid and fluid) within the same membrane; and (3) the transient local release of heat liberated by the exothermic crystallization of the phospholipid acyl chains.

Membrane instability induced by calcium asymmetry

Calcium asymmetry has been proposed earlier [18], as responsible for the low electrical resistance and instability of phosphatidylserine bilayers following addition of Ca^{2+} on one side only, while phosphatidylserine bilayers made with Ca^{2+} on both sides were stable and had high electrical resistance. Differences in surface energy between the opposing sides of the bilayer were suggested at that time as the reason for the instability of such asymmetric membranes. Surface energy differences were thought to result [18] from an asymmetric charge distribution (one monolayer is neutralized by Ca^{2+}) and also from an asymmetric membrane structure (one monolayer is condensed by Ca^{2+}).

Further experimental support for the role of Ca^{2+}-asymmetry in producing membrane instability is provided by the data shown in Fig. 2. These data indicate that when the difference in Ca^{2+} concentration on either side of the vesicle membrane is reduced by the presence of the divalent-cation ionophore A23187 within the vesicle membrane, the ability of external Ca^{2+} to increase membrane permeability and to induce vesicle fusion is significantly reduced.

We can now expand the above considerations to take into account the phase changes induced by Ca^{2+} which were observed more recently [9], and relate the observed membrane instability to the membrane fusion reaction. As Ca^{2+} is added (concentration lower than 1 mM) to the bulk phase in which phosphatidylserine vesicles are suspended, Ca^{2+} is able to bind only to the outside monolayer, because of the extremely low ionic permeability of these vesicles.

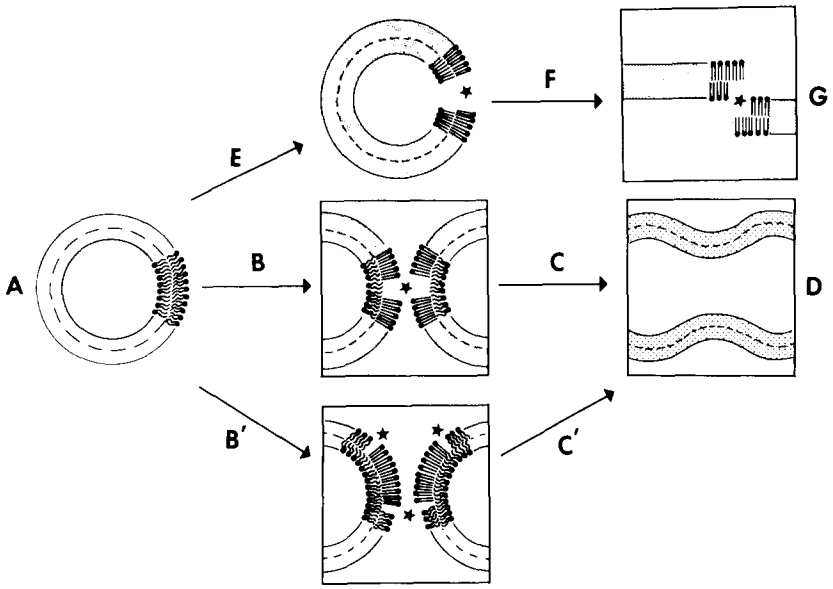

Fig. 8. Schematic representation of proposed events in fusion between phospholipid vesicles resulting from Ca^{2+}-induced phase changes. The stippled areas of the vesicle membranes indicate crystallized domains, the broken lines indicate the position of the bilayer midplanes and the asterisks indicate structural defects, domain boundaries, or regions of transient hydrocarbon-water contact. As indicated in the text, these transient energy states may be generated by the mismatch of monolayer regions which have undergone a Ca^{2+}-induced phase transition (separation) with the remaining fluid regions of the vesicle. It is proposed that fusion is initiated at such points. Phospholipid molecules in fluid domains are represented with "wavy" acyl chains and in solid domains with straight line acyl chains.

This produces certain charge neutralization, with concomitant condensation of the area per phosphatidylserine molecule and some aggregation of the vesicles. These effects can be induced by both Ca^{2+} and Mg^{2+} but, as mentioned, are not enough to trigger instability (increase in permeability) or fusion.

At higher Ca^{2+} concentrations (>1 mM), further condensation of the phospholipids will occur, resulting in complete crystallization of the outer monolayer of the vesicle membrane. This condensation of the outer monolayer may well create structural defects with resulting exposure of hydrocarbon regions to water (Fig. 8, Step B). The inner phospholipid monolayer will still be in a fluid state (Fig. 8, Step B), and the overall effect of this partial crystallization of the membrane would be expected to create a very unstable state. We suggest that it is because of this unstable state that the vesicle membrane becomes highly permeable and also susceptible to fusion. This interaction is illustrated in Fig. 8 (Steps A—D) where fusion is proposed as occurring at high energy points of hydrocarbon contact with water (Fig. 8, Step C). This "half-frozen" destabilized membrane state is a transient event, however, since as Ca^{2+} gains access to the inner monolayer (due to the altered membrane permeability) the differences in molecular packing and surface charge between the inner and outer monolayers will disappear and thus create a Ca^{2+}-condensed, completely frozen, highly stable membrane (Fig. 8, Step D). As discussed earlier [2], it is not unreasonable to suggest that as the bilayer of the vesicles freezes, the phospholipid molecules can no longer accomodate the high curvature of the original

fluid vesicles and will rupture (Fig. 8, Step E) to form flattened bilayer disks in which the hydrocarbon edges are exposed to contacts with water (Fig. 8, Step F). Since contact between the hydrocarbon core and water is energetically unfavorable these structures would tend to fuse laterally at points of exposed hydrocarbon/water interface producing large membranous sheets (Fig. 8, Steps F and G). This phenomenon is equivalent to the coalescence of oil droplets in water, except in this case the colaescence (fusion) occurs only in two dimensions.

Domain boundaries as regions susceptible to fusion

In considering the points at which fusion between vesicles will occur, the above discussion has introduced the concept of two-dimensional coalescence at the exposed hydrocarbon regions of transient bilayer defects. We can further extend this concept by considering domain boundaries within phospholipid membranes as focal points for fusion following the initial collision and aggregation. These domain boundaries could be regions of structural discontinuity between fluid and solid domains in the same membrane. One can envisage such transient domain boundaries in phosphatidylserine membrane being produced as Ca^{2+} initiates crystallization in certain areas before the whole membrane became solid. Such domain boundaries would, of course, also exist in mixed phosphatidylserine/phosphatidylcholine membranes, where Ca^{2+} induces a phase separation of the two phospholipids. We therefore propose that in areas where two membranes are closely apposed (<10 Å separation) these domain boundaries will serve as focal points for mixing of molecules from one membrane to the other, with consequent fusion (Fig. 8, Steps A—D). The structural discontinuities present in domain boundaries had been proposed earlier as responsible for the higher permeability of lipid bilayers at the phase transition temperature [5]. The concept of maximal lateral compressibility was also introduced to account for a similar observation on valinomycin induced transport [29]. Subsequently, other studies of membranes undergoing a phase transition have shown increased binding of proteins [30] and fluorescent probes [31] as well as enhanced membrane transport of dyes [32] and enzymatic activities [33]. Thus it is reasonable to consider that domain boundaries could also serve as focal points for fusion between apposed membranes, since they would involve a lowering of the activation energy for mixing of molecules from two different membranes. Whether fluctuations in structure induced in the region of the phase transition temperature can induce fusion in pure phospholipid membranes without Ca^{2+} is still unresolved [6,26,34,35]. However, the presence of fatty acids [36], proteins [6,37] and peptides [38] has been shown recently to enhance mixing of vesicle components with resulting growth in vesicle size compatible with the process of fusion.

Liberation of heat associated with Ca^{2+}-induced phase changes

Finally, another potentially important point to be considered in relation to the energetics and kinetics of membrane fusion is the local heat of crystallization that can be expected to be released by the interaction of Ca^{2+} with phosphatidylserine. This parameter has not been considered before, but it could be a crucial factor for the fusion reaction. It is well known that the melting of the

acyl chains of various phospholipids is an endothermic reaction with a heat content of 6—9 kcal/mol, which is primarily determined by the length of the hydrocarbon chain [39]. Since Ca^{2+} induces crystallization of the chains of phosphatidylserine at the same concentration as it induces fusion, the fusion reaction may be expected to be exothermic. Preliminary experiments with a microcalorimeter indicate that addition of Ca^{2+} (2 mM) to phosphatidylserine vesicles is an overall exothermic reaction with a heat content of at least 2 kcal/mol (Papahadjopoulos, D. and Epand, R., unpublished observations).

It is proposed that the heat which is evolved during the crystallization of phosphatidylserine by Ca^{2+} could produce local heating in certain regions of two apposed membranes, which would increase the rate of mixing between the membranes with subsequent fusion, especially at domain boundaries. The evolved heat would produce an increase in entropy in the neighborhood of the regions undergoing the transition. Thus, the initiation of the fusion-crystallization reaction would increase the degree of disorder in neighboring hydrocarbon chains, and also in the phospholipid headgroups and associated water.

Since the initial effect of Ca^{2+} as seen by the increase in vesicle permeability is a highly cooperative event, the heat evolved during the local crystallization is expected to be concentrated in large local quanta. It should also be noted that when the process of heating starts, the vesicles may already be aggregated so that the heat is dissipated initially through only a thin layer of water between adjacent bilayers, or laterally within each bilayer. The final outcome would of course be absorption of heat by the whole environment and formation of more ordered crystalline structures of Ca^{2+}-phospholipid.

In view of the ubiquity of membrane fusion phenomena in cellular activities [40] and the documented role of Ca^{2+} in regulating these processes [40—43], the possible involvement of Ca^{2+}-induced phase changes in phospholipid bilayer regions as an important part of the mechanism of fusion in natural membranes is worthy of serious considerations.

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