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Studies on the Mechanism of Membrane Fusion: Role of Phosphate in Promoting Calcium Ion Induced Fusion of Phospholipid Vesicles†

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ABSTRACT: The role of phosphate in enhancing the calcium-induced fusion of phosphatidylserine (PS) vesicles has been examined by using the new fluorescent (terbium/dipicolinic acid) assay described by Wilschut et al. (1980) [Wilschut, J., Düzgüneş, N., Fraley, R., & Papahajopoulos, D. (1980) *Biochemistry* (preceding paper in this issue)]. In the presence of physiological levels of phosphate, the calcium concentration required for fusion of PS vesicles was lowered significantly (3–4-fold), and the rate of vesicle fusion was increased dramatically (up to 1000-fold). The fusion of PS vesicles by calcium and phosphate is shown to be specific and critically dependent on temperature, pH, ion concentrations, and the composition of the calcium phosphate crystalline phase present during the incubation. The results indicate that a significant enhancement in vesicle fusion occurs only when calcium phosphate precipitation is initiated in the presence of PS vesicles, suggesting that crystal nucleation on the vesicle surface is a prerequisite for fusion. Calcium and phosphate were shown to promote phospholipid phase separations and vesicle fusion under conditions (e.g., mixtures of PS and phosphatidylcholine) in which calcium alone is ineffective, indicating that formation of PS calcium phosphate complexes may facilitate the molecular segregation of PS into distinct domains. These experiments underline the important role that phosphate may play in calcium-mediated fusion phenomena in biological membranes.

The fusion of biological membranes is of fundamental importance in diverse cellular processes such as fertilization (Gwatkin, 1976), viral infection (Murayama & Okada, 1965), myoblast differentiation (Buckingham, 1977), trichocyst discharge in paramecia (Matt et al., 1978), and neurotransmitter release from presynaptic vesicles (Kelly et al., 1979). The involvement of Ca^{2+} and its interaction with acidic phospholipids have been implicated in a critical regulatory role in these membrane fusion phenomena (Poste & Allison, 1973; Douglas, 1975). In this respect, investigations of the interaction of Ca^{2+} with acidic phospholipids in well-defined model membrane systems (Papahadjopoulos et al., 1976; Hauser et al., 1977; Ohnishi & Ito, 1974) are particularly useful in extending our understanding of the mechanism(s) of membrane fusion.

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While the Ca^{2+}-induced fusion of phosphatidylserine (PS)[1] vesicles has been studied extensively and the interaction of Ca^{2+} with PS vesicles is well understood (Portis et al., 1979; Wilschut & Papahadjopoulos, 1979), the involvement of additional factors in Ca^{2+}-mediated fusion of biological membranes is not precluded. Rather, the greater sensitivity of biological membranes to Ca^{2+}-induced fusion necessarily implicates the presence of other factors favoring fusion. Gratzl & Dahl (1978) have reported that the fusion of secretory vesicles isolated from rat liver is half-maximal at Ca^{2+} concentrations near 10^{-6} M, whereas fusion of PS vesicles requires millimolar Ca^{2+} concentrations (Portis et al., 1979). It has been suggested that glycoproteins may facilitate fusion since treatment of the vesicles with proteases or neuraminidase decreases the percentage of fused vesicles (Gratzl & Dahl, 1978). Similarly, the aggregation and subsequent fusion of chromaffin granules, which occurs at Ca^{2+} concentrations greater than 6 μM, are thought to be promoted by the Ca^{2+}-binding protein synexin (Creutz et al., 1979). In addition, we have shown that the Ca^{2+} levels required for aggregation and fusion of sonicated PS vesicles can be substantially lowered in the presence of Mg^{2+} (Portis et al., 1979).

It has been reported recently that the fusion of human erythrocytes (Baker & Kalra, 1979) and erythrocyte ghosts (Zakai et al., 1976, 1977) can be promoted by the combined action of Ca^{2+} and phosphate ions. The addition of Ca^{2+} to erythrocyte ghosts incubated in phosphate buffer caused fusion of 60–80% of the ghosts in 15 min, whereas incubation with Ca^{2+} alone was ineffective. It was suggested that a calcium phosphate complex formed between adjacent erythrocytes or ghosts, causing agglutination and eventual cell fusion.

Since PS is known to form specific complexes with Ca^{2+} and phosphate (Cotmore et al., 1971), the possible role of these ions in promoting membrane fusion has been examined. In this paper, we present experiments which indicate that the presence of phosphate enhances dramatically the Ca^{2+}-induced fusion of PS vesicles. The results are discussed with respect to membrane fusion and the role of phospholipids in biological calcification.

Materials and Methods

Lipids and Other Materials. Phosphatidylcholine (PC) was purified from egg yolk as described by Papahadjopoulos & Miller (1967). Phosphatidylethanolamine (PE), prepared from egg PC by transesterification, and phosphatidylserine (PS), obtained from bovine brain by DEAE chromatography and EDTA wash, were purchased from Avanti (Birmingham, AL). All the above phospholipids were shown to yield one spot (50 μg spotted) by thin-layer chromatography on silica gel plates, employing the two-dimensional system of Rouser et al. (1970). Cholesterol, obtained from A. B. Fluka (Buch, Switzerland), was recrystallized three times from methanol, and its purity was confirmed chromatographically on silicic acid plates by using the solvent system described by Mangold (1969). Lipid concentrations were determined as described by Bartlett (1959). All other chemicals were of the highest purity available.

Vesicle Preparation. Large unilamellar vesciles (LUV) were prepared by the reverse-phase evaporation method of Szoka & Papahadjopoulos (1978) as modified by Wilschut et al. (1980). A typical preparation contained 10 μmol of phospholipid (and 10 μmol of cholesterol where indicated) in 0.33 mL of aqueous buffer and 1.0 mL of diethyl or diisopropyl ether. The buffer contained 2 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (Tes), 2mM L-histidine (pH 7.4), and either (1) 75 mM carboxyfluorescein (CF), (2) 4 mM terbium (Tb) chloride and 40 mM sodium citrate, or (3) 40 mM dipicolinic acid (DPA) and 20 mM sodium chloride. The LUV were sized to 0.1 μm by successive extrusions through 0.2- and 0.1-μm Unipore filters (Olson et al., 1979), and unencapsulated material was removed by gel filtration on a Sephadex G-75 column (employing 2 mM Tes, 2 mM L-histidine (pH 7.4), 100 mM sodium chloride, and 1.0 mM EDTA as the elution buffer). LUV preparations used for the determination of Tb and DPA encapsulation were freed of EDTA (as described by Wilschut et al., 1980) by rechromatographing the sample on a Sephadex G-75 column equilibrated in the above buffer, minus EDTA.

Fluorescence Measurements. Fluorescence and light-scattering measurements were performed simultaneously with an SLM-4000 fluorometer. The sample chamber was equipped with a magnetic stirrer, and temperature was controlled with a thermostated circulating water bath. The sample temperature was determined with a thermistor immersed in the cuvette. All fluorescence measurements were performed at an absorbance <0.2 O.D. units at the excitation wavelength, in 1.0-cm path-length cuvettes. The Tb/DPA complex was excited at 276 nm, fluorescence was measured at 491 nm, and light scattering was measured with a Corning 7-54 band-pass filter. CF excitation was at 430 nm, emission was detected at >530 nm by using a Corning 3-68 filter, and light scattering was measured at 430 nm. Tb encapsulation was determined by the addition of 20 μM DPA to vesicles which had been disrupted by sonication (20 min) in the presence of 2% deoxycholate. DPA encapsulation was similarly measured following the addition of 5 μM $TbCl_3$ to disrupted vesicles. $CaCl_2$ and phosphate (sodium salt) were added directly to the sample cuvette with a Hamilton syringe from 100 mM and 1 M stock solutions, respectively.

Ca^{2+} Measurements. Ca^{2+} concentrations were monitored continuously during the vesicle fusion reaction with a Ca^{2+}-selective electrode, operating with a neutral carrier incorporated in a poly(vinyl chloride) membrane. The potential was measured with a digital pH meter (Fisher), and millivolts were recorded. The electrode was calibrated in Ca^{2+} solutions of known concentrations.

Differential Scanning Calorimetry. An aliquot of the centrifuged vesicle preparation (5 μmol in 20–50 μL) was placed in hermetically sealed aluminum calorimeter pans at 25 °C and transferred to a differential scanning calorimeter (DSC-2, Perkin-Elmer). The sample was initially cooled to −10 °C and then heated at a rate of 5 °C/min (sensitivity = 1 mcal/s).

Electron Microscopy. Samples corresponding to various time points in the calcium phosphate induced fusion of PS/Chol vesicles (Figure 2b, curve 2) were examined by electron microscopy (EM) by using both negative staining and freeze–fracture techniques. Reaction conditions identical with those described in the text were employed, and reactions were stopped by chilling the samples on ice. The samples were then concentrated by brief centrifugation at 4 °C and the pellets resuspended in $^1/_{50}$ volume of the same buffer at 0 °C. All EM procedures were also performed at 4 °C to ensure reaction termination. For freeze–fracture, an aliquot of the concentrated sample (~10 μmol of phospholipid/mL) was made 30% in glycerol, frozen in liquid nitrogen cooled Freon 22, and

[1] Abbreviations used: PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; DPA, dipicolinic acid; CF, carboxyfluorescein; HAP, hydroxylapatite; ACP, amorphous calcium phosphate; OCP, octacalcium phosphate.

fractured, and a carbon/platinum replica was prepared as described (Fisher & Branton, 1974). For negative staining, the concentrated sample was diluted with the original buffer to 1 μmol/mL, absorbed for 1 min to a glow-discharged, carbon-stabilized, and Formvar-covered grid, and stained with cold 1% sodium phosphotungstate for 30 s. Specimens were examined with a Philips 300 electron microscope operating at 80 KV.

Results

Previous investigations of the interactions of acidic lipids with calcium and phosphate ions have emphasized the role of these lipids in bone calcification. The basis for these studies was the early observation (Anderson, 1967; Bonucci, 1967) that membrane vesicles, termed matrix vesicles, might serve as the initial site for calcium deposition during mineralization. Biochemical analysis of these vesicles revealed a high concentration of acidic phospholipids, with PS and PI comprising the bulk of this lipid class (Vogel & Boyan-Salyers, 1976). It was shown subsequently that a Ca^{2+}/phospholipid phosphate complex, with a constant 1:1 Ca^{2+} to phosphate stoichiometry, could be isolated from bone (Boskey & Posner, 1976) and that this complex could initiate hydroxylapatite (HAP) formation from metastable calcium phosphate solutions (Boskey & Posner, 1977). Purified acidic phospholipids were shown to be equally effective in inducing HAP formation. Although it is apparent from the above studies that acidic phospholipids interact with Ca^{2+} and phosphate and that they may be involved in the calcification process, the exact nature of their involvement and its effects on membrane structure are poorly understood. The possible role of calcium and phosphate ions in promoting membrane contact and fusion has not been investigated in any detail.

The fusion of PS vesicles can be followed conveniently by using the fluorescent assay described by Wilschut & Papahadjopoulos (1979) and Wilschut et al. (1980). This assay monitors the mixing of aqueous vesicle contents between two populations of phospholipid vesicles containing $TbCl_3$ and DPA. The formation of the Tb/DPA complex produces a 10^4-fold enhancement in Tb fluorescence. Formation of the fluorescent Tb/DPA complex in the external medium is prevented by the presence of EDTA or Ca^{2+}, which rapidly dissociates the complex; consequently, only the mixing of vesicular contents, separated from the outside medium, is registered as fusion. Leakage of the complex from the vesicles or entry of Ca^{2+} into the intravesicular space causes a rapid decrease in the fluorescence signal. The actual extent of vesicle content leakage can be determined in parallel vesicle preparations by the enhancement of fluorescence intensity that occurs upon release and dilution of carboxyfluorescein (CF), entrapped in vesicles at self-quenching concentrations (Blumenthal et al., 1977). The formation of crystalline calcium phosphate phases can be conveniently followed by light-scattering measurements (Robertson, 1973), simultaneous with the fluorescence measurements. Together, these assays provide a sensitive description of the interaction of PS vesicles with calcium and phosphate.

The addition of 2 mM Ca^{2+} to PS vesicles results in no release of CF (Figure 1a, curve 1) and in only slight (10%) enhancement of Tb fluorescence (Figure 1b, curve 1). However, when 2 mM Ca^{2+} is added to PS vesicles in buffer containing 10 mM phosphate, an extremely rapid but transient increase in Tb fluorescence is observed (Figure 1b, curve 2), and the decay in Tb fluorescence occurs concomitant with extensive leakage of CF (>90% in 3 min; Figure 1a, curve 2) from a similar vesicle preparation.

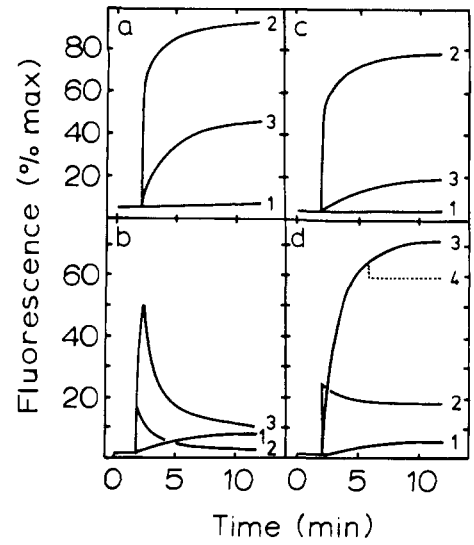

FIGURE 1: Calcium phosphate induced fusion of PS and PS/Chol vesicles and release of vesicle contents. Maximal CF fluorescence was determined in the presence of 0.1% (v/v) Triton X-100. The value of 100% Tb fluorescence was determined as described under Materials and Methods. Fluorescence intensity is expressed as a percentage of the maximal values. All incubations were performed at 30 °C at a phospholipid concentration of 100 μM. Tb- and DPA-containing vesicles were mixed in a 1:1 ratio (50 μM each). (a) CF release from PS vesicles; (b) fusion of PS vesicles; (c) CF release from PS/Chol vesicles; (d) fusion of PS/Chol vesicles. (Curve 1) 2 mM $CaCl_2$ alone; (curve 2) preincubation (2 min) with 10 mM phosphate (pH 7.7) followed by addition of 2 mM $CaCl_2$; (curve 3) preincubation (2 min) with 2 mM $CaCl_2$ followed by addition of 10 mM phosphate (pH 7.7); (curve 4) incubation as in curve 3 except that EDTA (4 mM) was added 5 min after the addition of 10 mM phosphate.

Since the order of addition of Ca^{2+} and phosphate was shown to be an important factor in the ability of these ions to promote fusion of human erythrocytes (Baker & Kalra, 1979), the effect of reversing the order of Ca^{2+} and phosphate addition was examined. Under these conditions, the leakage of CF is only 50% after 10 min (Figure 1a, curve 3); the Tb fluorescence reaches a maximal value of 50% and declines rapidly (Figure 1b, curve 3). Similar levels of vesicle fusion, induced by Ca^{2+} alone, can be achieved only at much higher (5–7 mM) Ca^{2+} concentrations (Wilschut et al., 1980). The difference observed in the levels of Tb fluorescence and in CF release when Ca^{2+} is added prior to phosphate may result from the enhanced binding of Ca^{2+} to PS bilayers in the absence of competitive binding of Ca^{2+} to phosphate. Several reports have indicated that the binding of phosphate to acidic lipids is dependent on prior binding of Ca^{2+} (Boskey & Posner, 1977; Bader, 1964); the possibility will also be discussed below that the effect on vesicle fusion may be a function of whether the initial calcium phosphate nucleation and crystallization occur in bulk solution or on the vesicle surface.

The transient nature of Tb fluorescence (Figure 1b) in the presence of phosphate probably reflects both the rapid leakage of vesicular contents and the entry of Ca^{2+} into the vesicles, two events which would result in dissociation of the fluorescent Tb/DPA complex. As it is well-known that cholesterol is effective in reducing the permeability of membranes which are above their transition temperature (Papahadjopoulos et al., 1972; Oldfield & Chapman, 1972; Petersen & Chan, 1977), PS vesicles containing 50 mol % cholesterol were examined for leakage and fusion in the presence of phosphate (Figure 1c,d). When Ca^{2+} is added to PS/Chol vesicles preincubated with 10 mM phosphate, the leakage of CF (Figure 1c, curve 2) is not as extensive as that observed for pure PS vesicles (Figure 1a), and the level of Tb fluorescence

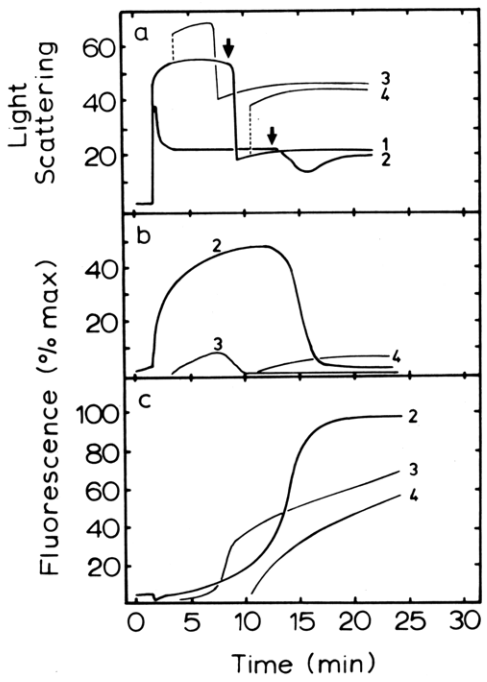

FIGURE 2: Calcium phosphate crystallization, PS/Chol vesicle fusion, and CF release at pH 7.7. Incubations were at 25 °C at a phospholipid concentration of 100 μM. Light scattering is expressed in arbitrary units. (a) Light scattering at 430 nm; (b) fusion of PS/Chol vesicles; (c) CF release from PS/Chol vesicles. In all the above experiments, 2 mM $CaCl_2$ was added to the sample 2 min prior to the addition of 50 mM phosphate (pH 7.7). (Curve 1) No vesicles present; (curve 2) vesicles present at $T = 0$ min; (curve 3) vesicles added at $T = 2$ min; (curve 4) vesicles added at $T = 11$ min. The arrows denote the onset of the amorphous to crystalline calcium phosphate transformation detected by monitoring changes in the free Ca^{2+} concentration in parallel experiments performed under identical conditions.

is maintained at 20% (Figure 1d, curve 2). A striking difference is observed, however, when phosphate is added to PS/Chol vesicles preincubated with Ca^{2+}; the leakage of CF under these conditions is only 20% (Figure 1c, curve 3), and the Tb fluorescence is maintained at very high levels (~80%) for the entire 10-min period recorded. Importantly, the high level of Tb fluorescence is maintained after the addition of excess EDTA, indicating a physical separation of the fluorescent complex from the external EDTA-containing media. The attainment of such high values for total Tb fluorescence indicates that multiple fusion events occur, since a maximum of only 50% of the Tb would be expected to complex with DPA if vesicles fuse in binary fashion.

Shown in Table I is a summary of the encapsulation data for Tb, DPA, and CF in PS and PS/Chol vesicles. All the encapsulation values are in relatively close agreement to the expected entrapped volume of 3.0 μL/μmol of phospholipid calculated for 0.1-μm diameter vesicles.[2] The inclusion of 50 mol % cholesterol does not significantly alter the efficiency of encapsulation of the various aqueous solutes,[3] indicating that the dramatic differences observed between PS (Figure 1) and PS/Chol (Figure 2) vesicles with respect to fusion and to leakage cannot be attributed to gross alterations in vesicle size or structure. Instead, the sustained Tb fluorescence observed with the PS/Chol vesicles most likely reflects a reduction in membrane permeability by cholesterol.

Table I: Summary of the Encapsulation Efficiencies of Different Liposome Preparations Used in This Study[a]

lipid composition	encapsulation efficiency (nmol/μmol of phospholipid)		
	Tb	DPA	CF
PS	14.8 (3.7)	120 (3.0)	140 (2.8)
PS/Chol (1:1)	14.8 (3.7)	112 (2.8)	145 (2.9)
PS/PE/Chol (1:4:5)	15.2 (3.8)	128 (3.2)	155 (3.1)

[a] Vesicle preparation, phospholipid determinations, and quantitation of $TbCl_3$ and DPA encapsulation were as described under Materials and Methods. The concentrations of the encapsulated Tb, DPA, and CF solutions were 4, 40, and 75 mM, respectively. CF concentrations were determined by absorbance measurements ($\epsilon_{493} = 72\,000$) following the addition of Triton X-100 (0.1%) to disrupt the vesicles. All preparations contained 10 μM phospholipid and the material to be encapsulated in 0.33 mL of buffer. The values for encapsulation efficiency are expressed as nmol of encapsulated material per μmol of phospholipid. Shown in parentheses is the ratio of encapsulated volume (μL) to the amount of phospholipid (μmol).

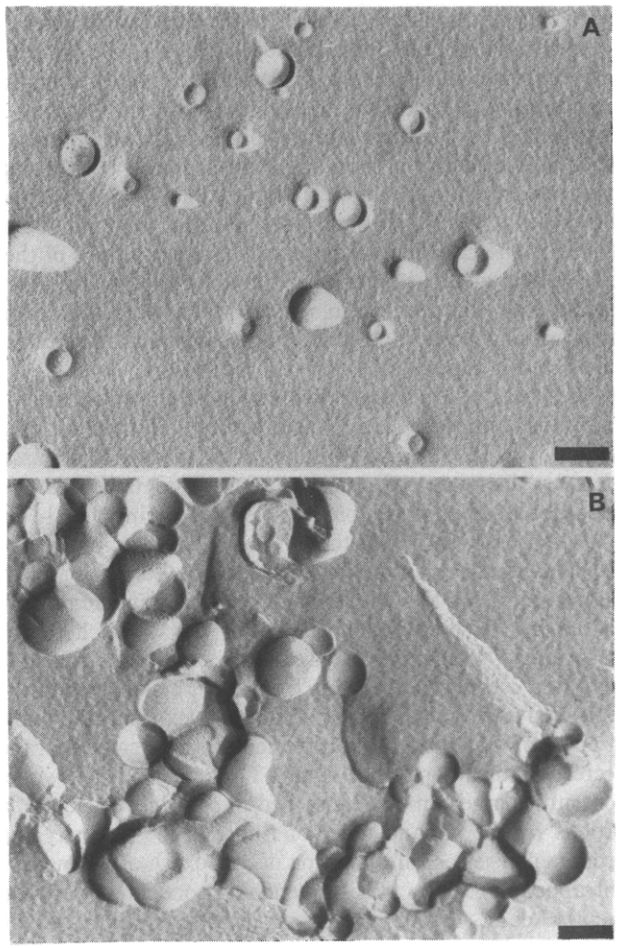

FIGURE 3: Freeze–fracture electron micrographs of PS/Chol vesicles exposed to $CaCl_2$ only, or to $CaCl_2$ and subsequently to 50 mM phosphate. Sample preparations were as described under Materials and Methods. (a) PS/Chol vesicles pretreated with 2 mM $CaCl_2$ for 2 min; (b) PS/Chol vesicles pretreated with 2 mM $CaCl_2$ for 2 min, followed by addition of 50 mM phosphate (pH 7.7). The reaction was terminated after 1 min. Bar: 0.2 μm. Both magnifications are 66 000×.

The interaction of calcium phosphate with PS/Chol vesicles and the ability of different calcium phosphate phases to induce vesicle fusion and CF release were examined in detail (Figure 2 and Figure 4). At pH 7.7 (Figure 2), the addition of 50

[2] This calculation is based on the following (Szoka & Papahadjopoulos, 1978): a phospholipid surface area of 72 $Å^2$ per molecule, condensed to 58 $Å^2$ in the presence of cholesterol; a cholesterol surface area of 38 $Å^2$; and a uniform population of spherical unilamellar vesicles of diameter $D = 12V/A$, where V is the encapsulated volume and A is the total surface area of a lipid monolayer.

[3] With the values given above, it is calculated that PS/Chol vesicles should entrap slightly larger (33%) volumes than PS vesicles.

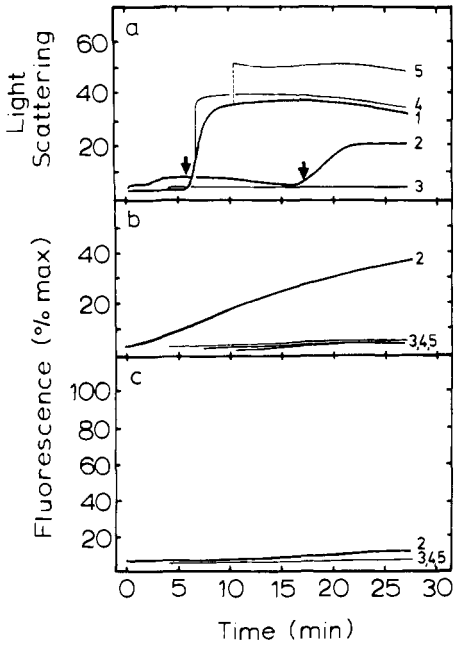

FIGURE 4: Calcium phosphate crystallization, PS/Chol vesicle fusion, and CF release at pH 6.7. The experimental conditions were identical with those described in the legend to Figure 2, except all incubations were performed at pH 6.7. (a) Light scattering at 430 nm; (b) fusion of PS/Chol vesicles; (c) CF release from PS/Chol vesicles. (Curve 1) No vesicles present; (curve 2) vesicles present at $T = 0$ min; (curve 3) vesicles added at $T = 2$ min; (curve 4) vesicles added at $T = 7$ min; (curve 5) vesicles added at $T = 11$ min. The arrows denote the onset of the amorphous to crystalline calcium phosphate transformation detected by monitoring changes in the free Ca^{2+} concentration in parallel experiments performed under identical conditions.

mM phosphate to a buffer solution containing 2 mM Ca^{2+} results in the spontaneous formation of a crystalline calcium phosphate phase as indicated by the observed increase in light scattering. Robertson (1973) has shown that the first crystalline phase detectable by light-scattering measurements has a Ca^{2+}/phosphate ratio close to that of octacalcium phosphate (OCP). This phase transforms rapidly (~8 min) to a more basic apatic (HAP) phase, indicated by a sharp decrease in light scattering (Figure 2, curve 1). When the experiment is repeated with PS/Chol vesicles present (Figure 2, curve 2), the extent of OCP formation is reduced, and the secondary transition is delayed several minutes.[4] Wuthier & Eanes (1975) have observed that the addition of PS vesicles to calcium phosphate solutions retards the amorphous calcium phosphate (ACP) to HAP transition. In contrast, the addition of PS/Chol vesicles to a preexisting OCP phase results in a more rapid initiation of the crystalline transition (Figure 2a, curve 3), an effect consistent with results (Boskey & Posner, 1977), which indicates that acidic phospholipids can initiate HAP formation. The latter results indicate that the interaction of PS vesicles with calcium phosphates and their ability to retard or promote crystal nucleation are strictly dependent on the composition of the initial crystalline phase.

The fusion of PS/Chol vesicles added during various phases of crystal growth is shown in Figure 2b,c. A large enhancement in Tb fluorescence is observed only when PS/Chol vesicles are present prior to the formation of OCP (Figure 2b, curve 2). Figure 2c shows that a large increase in CF release accompanies the OCP to apatite transition. The addition of vesicles following the transition also results in substantial CF leakage. The observation that vesicle addition to the preformed apatite phase does not promote fusion is consistent with the results of Baker & Kalra (1979) which show that preformed precipitates of calcium phosphate are ineffective in inducing the fusion of human erythrocytes.

Electron microscopy confirms that fusion of Ca^{2+}-treated vesicles occurs upon exposure to phosphate (Figure 2b, curve 2). Figure 3a shows a freeze–fracture micrograph of PS/Chol vesicles after treatment with 2 mM $CaCl_2$ for 2 min. A well-dispersed population of vesicles is seen with a mean diameter of approximately 1000 Å, indicating that no aggregation or fusion has occurred. However, the addition of 50 mM phosphate (pH 7.7) to these vesicles for 1 min produces aggregates of vesicles which are larger in size and more irregular in shape (Figure 3b). Similar results were obtained with negative staining.

At pH 6.7 (Figure 4a), the formation of OCP does not occur spontaneously but occurs only after several minutes of incubation (Figure 4a, curve 1). The secondary transition is delayed for several hours under these conditions (data not shown). When PS/Chol vesicles are added to the incubation mixture (Figure 4a, curve 2), the delay in the formation of OCP is even more pronounced, in agreement with a study indicating that acidic lipid vesicles stabilize ACP (Wuthier & Eanes, 1975). Again, vesicle fusion, measured by increased Tb fluorescence, occurs only when PS/Chol vesicles are present initially in the incubation mixture; subsequent addition of vesicles to preformed ACP or OCP or during the transition does not result in enhanced Tb fluorescence (Figure 4b). As suggested from the results in Figure 2c, little CF leakage is observed at pH 6.7 in the absence of apatite formation (Figure 4c).

The results in Figures 2 and 4 indicate that both vesicle leakage and fusion are critically dependent on calcium phosphate crystallization. Since the nucleation and growth of calcium phosphate crystals are known to be extremely sensitive to ion concentrations, pH, and temperature (Walton, 1965; Meyer & Eanes, 1978), these parameters were investigated and optimized systematically with respect to enhancement of Tb fluorescence (Figure 5). The dependence of vesicle fusion on calcium and phosphate concentrations is shown in Figure 5a,b. In the presence of 10 mM phosphate (Figure 4a), the threshold for Ca^{2+}-induced fusion is 1.25 mM, and the optimal concentration for fusion is between 1.5 and 2.0 mM Ca^{2+}. Phosphate concentrations as low as 2.5 mM (Figure 5b) enhance vesicle fusion in the presence of 2 mM Ca^{2+}. The rate and extent of fusion increase with phosphate levels, but the rapid formation of an apatic phase at higher concentrations (50 mM) causes a rapid decline in Tb fluorescence.

The pH dependence of calcium phosphate induced fusion of PS/Chol vesicles is shown in Figure 5c. Little increase in Tb fluorescence occurs at neutral pH, and maximum levels are attained between pH 7.4 and 7.7. In contrast, the fusion of vesicles by Ca^{2+} alone is independent of pH in this range (data not shown). Zakai et al. (1976) similarly observed that fusion of erythrocyte ghosts by Ca^{2+} and phosphate occurs at or above pH 7.0 and is maximal at pH 7.5. These results agree with those of Cotmore et al. (1971), indicating that formation of the calcium/PS phosphate complex occurs only above physiological pH.

[4] Since changes in light scattering are also associated with both vesicle aggregation and fusion (Wilschut et al., 1980), the amorphous to crystalline transition was also followed by monitoring a decrease in the free Ca^{2+} concentration which occurs during crystallization (Meyer & Eanes, 1978). The crystalline transformation was monitored with a Ca^{2+}-selective electrode, and its onset is denoted in Figures 2a and 4a by an arrow.

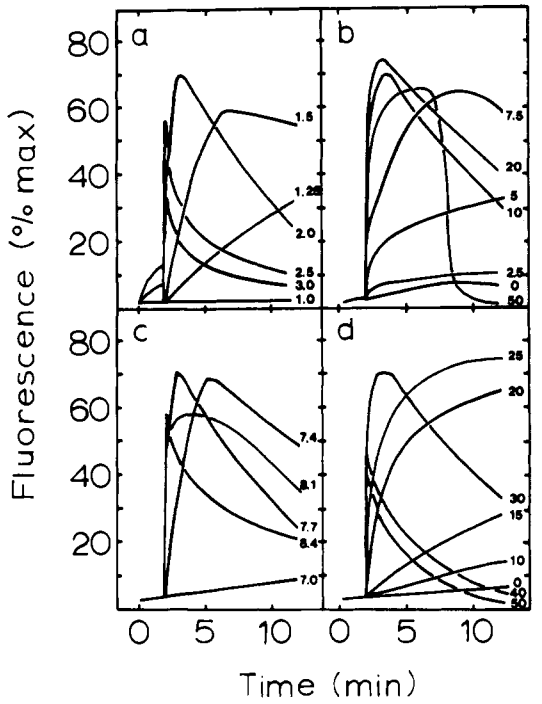

FIGURE 5: Dependence of calcium phosphate induced PS/Chol vesicle fusion on calcium and phosphate concentrations, incubation temperature, and pH. All incubations were performed in a volume of 1 mL at a phospholipid concentration of 100 μM. (a) Vesicles were preincubated (30 °C) with the levels of $CaCl_2$ shown in the figure for 2 min prior to the addition of 10 mM phosphate (pH 7.7); (b) vesicles were preincubated (30 °C) with 2.0 mM $CaCl_2$ for 2 min prior to the addition of phosphate (pH 7.7) at the concentrations shown in the figure; (c) vesicles were preincubated (30 °C) with 2 mM $CaCl_2$ for 2 min prior to the addition of 10 mM phosphate (pH is shown in figure); (d) vesicles were preincubated at the temperatures shown in the figure with 2 mM $CaCl_2$ for 2 min prior to the addition of 10 mM phosphate (pH 7.7). All solutions were corrected for temperature-induced pH changes.

The extent of vesicle fusion is critically dependent on the incubation temperature (Figure 5d). The highest level of sustained Tb fluorescence is reached between 25 and 30 °C. Above 30 °C, the rapid formation of apatite causes extensive leakage and results in decreased Tb fluorescence.

In summary, the optimal conditions for fusion of PS/Chol vesicles are achieved by preincubation of vesicles with 2 mM Ca^{2+} at 30 °C, followed by addition of 10 mM phosphate (pH 7.7). The percent enhancement of total Tb fluorescence is increased slightly by raising the lipid concentration in the range from 0.05 to 0.5 μm/mL. The substitution for NaCl of either KCl or glucose had no effect on the level of Tb fluorescence (data not shown). It should be emphasized that maximal enhancement of Tb fluorescence occurs under conditions of minimal leakiness accompanying fusion; while increasing Ca^{2+} or phosphate concentrations, pH, or temperature results in an increase of the actual rate and extent of vesicle fusion, the greater leakiness of vesicles under such conditions results in lower levels of Tb fluorescence. Reversing the order of Ca^{2+} and phosphate addition always resulted in greater leakage of vesicle contents, although the actual rate of vesicle fusion was also greater under these conditions (Figures 1 and 2); it is likely that this situation (e.g., phosphate present initially) may be more biologically relevant, and it will be examined in greater detail later.

The specificity of Ca^{2+} and phosphate in enhancing the fusion of PS/Chol vesicles is demonstrated in Figure 6. Mg^{2+}, Sr^{2+}, and Ba^{2+} are much less effective than Ca^{2+} in inducing vesicle fusion following the addition of 10 mM phosphate.

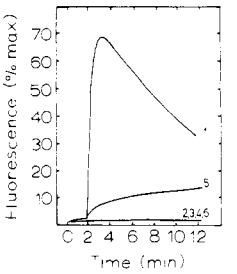

FIGURE 6: Specificity of calcium phosphate induced PS/Chol vesicle fusion. All incubations were performed at 30 °C, pH 7.7, at a phospholipid concentration of 100 μM. Vesicles were preincubated with divalent metals (2 mM) for 2 min prior to the addition of phosphate or other anions (10 mM). Ca^{2+} followed by the addition of phosphate (curve 1), oxalate (curve 2), or arsenate (curve 3). Mg^{2+} (curve 4), Ba^{2+} (curve 5), or Sr^{2+} (curve 6) followed by the addition of phosphate.

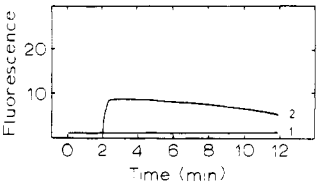

FIGURE 7: Calcium phosphate induced fusion of PS/DPPC vesicles. Incubations were at 25 °C (pH 7.7) at a phospholipid concentration of 100 μM. (Curve 1) 50 mM $CaCl_2$; (curve 2) preincubation with 2 mM $CaCl_2$ for 2 min followed by addition of 10 mM phosphate.

Similarly, Ba^{2+}, Sr^{2+}, Mn^{2+}, and Zn^{2+} were shown to be poor substitutes for Ca^{2+} in promoting the fusion of erythrocyte ghosts in phosphate buffer (Zakai et al., 1976). Other divalent anions, including arsenate and oxalate (Figure 5), were unable to enhance Tb fluorescence when added to vesicles preincubated with 2 mM Ca^{2+}.

All the above demonstrations of calcium phosphate induced vesicle fusion have employed phospholipid mixtures which are susceptible to fusion by Ca^{2+} alone, although much higher Ca^{2+} concentrations are required in the absence of phosphate. As a result, it is not clear whether calcium phosphate itself promotes fusion or whether the aggregation of vesicles by calcium phosphate precipitation simply permits close apposition of vesicle membranes and therefore allows Ca^{2+}-induced fusion to occur at subthreshold levels of Ca^{2+}.

In order to distinguish between these two possibilities, PS vesicles composed of 50 mol % dipalmitoylphosphatidylcholine (DPPC) were prepared since it is known that PS vesicles containing such high levels of PC are not susceptible to fusion by Ca^{2+} alone (Papahadjopoulos et al., 1974; Miller & Racker, 1976; Gad et al., 1979). This is illustrated clearly in Figure 7 where the addition of up to 50 mM (curve 1) $CaCl_2$ to PS/DPPC (1:1) vesicles does not result in a detectable increase in Tb fluorescence. However, the addition of 10 mM phosphate to PS/DPPC vesicles which were preincubated with only 2 mM Ca^{2+} (Figure 7, curve 2) results in a substantial (10%) increase in Tb fluorescence.

A possible explanation for the dramatic effect of phosphate in inducing the fusion of PS/DPPC vesicles (Figure 7) could be that formation of calcium phosphate/PS complexes facilitates segregation of PS molecules into larger domains which could then serve as fusion sites. It has been shown that under certain conditions (Ohnishi & Ito, 1974; Papahadjopoulos et al., 1974) Ca^{2+} can promote phase separations in phospholipid mixtures, presumably by segregation of PS into distinct domains (Papahadjopoulos et al., 1978). In this respect, the multivalent calcium phosphate/PS complex may be even more effective in promoting lipid phase separations because of its

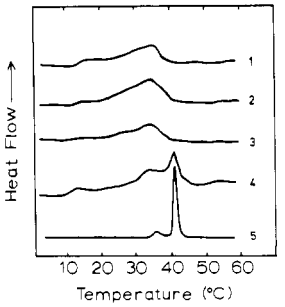

FIGURE 8: Differential scanning calorimetry on the effect of calcium phosphate on PS/DPPC vesicles. All incubations (5 mL) were at 25 °C for 1 h at a phospholipid concentration of 1 mM. The vesicle preparations were centrifuged (150 000*g* for 3 h at 25 °C), and the pellet was transferred to a calorimeter pan for analysis. The scans shown were obtained at a heating rate of 5 °C/min (sensitivity, 1 mcal/s). (Curve 1) Control PS/DPPC vesicles; (curve 2) vesicles incubated with 3 mM $CaCl_2$; (curve 3) vesicles incubated with 10 mM $CaCl_2$; (curve 4) vesicles incubated with 3 mM $CaCl_2$ plus 10 mM phosphate; (curve 5) control DPPC vesicles.

ability to undergo extensive cross-linking. Therefore, the ability of calcium phosphate to induce phase separations in PS/DPPC vesicles was examined by differential scanning calorimetry (Figure 8). As has been reported earlier (Stewart et al., 1979), PS/DPPC (1:1) vesicles undergo a broad phase transition centered at 30 °C (Figure 8, curve 1). Incubation of the vesicles for 1 h at 25 °C in the presence of 3 mM (curve 2) or 10 mM (curve 3) $CaCl_2$ produces only a slight broadening of the transition peak. In contrast, incubation of the vesicles under the same conditions with 3 mM $CaCl_2$ and 10 mM phosphate resulted in a new peak appearing at 40 °C (curve 4), suggesting that phospholipid phase separation had occurred and resulted in the formation of PC-enriched domains.

In order to extrapolate the above results to the possible effect of phosphate in enhancing the Ca^{2+}-induced fusion of biological membranes, experiments must be performed under conditions more closely resembling those found in biological systems. For instance, the fraction of PS seldom exceeds 10% of the total lipid in most biological membranes, although its asymmetric distribution in certain membranes may increase its local concentration in the bilayer (Bergelson & Barsukov, 1977; Op den Kamp, 1979). In addition, phosphate is present intracellularly and in most biological fluids at concentrations of 1–5 mM (Altman & Dittmer, 1974).

Therefore, we have examined the effect of Ca^{2+} in promoting the fusion of phospholipid vesicles comprised of only 10 mol % PS (PS/PE/Chol 1:4:5) in the presence of low levels of phosphate (Figure 9). The values for Tb, DPA, and CF entrapment by PS/PE/Chol vesicles are in close agreement with the calculated entrapped volume (3.0 μL/μmol of phospholipid) for 0.1 μm vesicles (Table I), indicating that the high percentage of PE does not affect vesicle stability. The addition of 2 mM Ca^{2+} to PS/PE/Chol vesicles (curve 1) does not result in detectable vesicle fusion (Figure 9a) or CF release (Figure 9b). However, when the incubation is repeated with 10 mM phosphate present (curve 2), the addition of Ca^{2+} results in a transient increase in Tb fluorescence (Figure 9a) followed by a decline to lower values. CF release under such conditions is 90% after 10 min (Figure 9b). Lowering the initial phosphate concentration to 5 mM (curve 3) prevents the decline in the Tb signal, and the level of fluorescence remains constant at 30–35%; in a parallel experiment, CF release is reduced to 70% (Figure 9b). Further lowering of the initial phosphate concentration reduces the extent of vesicle fusion (curve 4). The value of 30–35% for Tb fluorescence

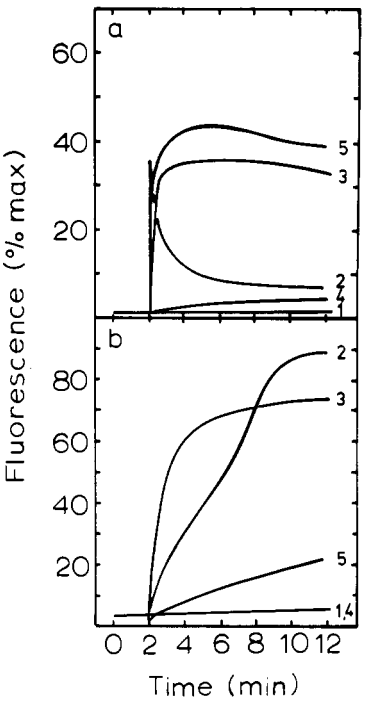

FIGURE 9: Calcium phosphate induced fusion of PS/PE/Chol vesicles and release of vesicle contents. Incubations were at 30 °C (pH 7.7) at a phospholipid concentration of 100 μM. (a) Fusion and (b) CF release. (Curve 1) 2 mM $CaCl_2$ alone; (curve 2) preincubation with 10 mM phosphate for 2 min followed by addition of 2 mM $CaCl_2$; (curve 3) preincubation with 5 mM phosphate for 2 min followed by addition of 2 mM $CaCl_2$; (curve 4) preincubation with 2.5 mM phosphate for 2 min followed by addition of 2 mM $CaCl_2$; (curve 5) preincubation with 2 mM $CaCl_2$ for 2 min followed by addition of 10 mM phosphate.

obtained with 5 mM phosphate is higher than that obtained for either PS or PS/Chol vesicles under identical conditions (data not shown), indicating that lowering the amount of PS in the vesicle reduces leakiness promoted by calcium and phosphate.

Reversing the order of addition of 2 mM Ca^{2+} and 10 mM phosphate (curve 5) results in a sustained level of 45% of the total Tb fluorescence (Figure 9a), which is not accompanied by a large increase in vesicle leakiness since the extent of CF release (Figure 9b) is relatively low (~20%). This low leakage is similar to that observed for PS/Chol vesicles (Figure 1c) although the Tb fluorescence with the latter vesicles is almost 2-fold higher. This apparent disparity may be explained if PS/PE/Chol vesicles undergo fewer multiple fusion events that do vesicles which contain a higher percentage of PS.

Discussion

Studies of the interaction of Ca^{2+} with PS vesicles have revealed that millimolar Ca^{2+} concentrations cause extensive vesicle aggregation and fusion, followed by subsequent leakage of vesicle contents and ultimate collapse of bilayers to form cochleate lipid cylinders (Papahadjopoulos et al., 1977; Portis et al., 1979; Wilschut et al., 1980). The fusion of PS vesicles by Ca^{2+} may be mediated through the formation of a specific anhydrous "trans" complex between Ca^{2+} and the PS head groups on two apposing vesicles (Portis et al., 1979). Formation of the complex may be a prerequisite for vesicle fusion since such a structure represents a local, anhydrous destabilization of two closely apposed (anhydrous) membranes.

In the present paper, we have demonstrated that the presence of phosphate can lower significantly the threshold for Ca^{2+}-induced fusion of PS-containing vesicles. The results (Figures 2 and 4) indicate that significant vesicle fusion occurs

only when calcium phosphate precipitation is initiated in the presence of PS vesicles, suggesting that crystal nucleation on the vesicle membrane surface is required for vesicle aggregation and fusion. Numerous reports (Irving & Wuthier, 1968; Boskey & Posner, 1977) have implicated acidic phospholipids as nucleation sites for the formation of HAP; it is assumed that PS acts as a tridentate ligand with Ca^{2+}, so that the remaining coordinate bonds are free to undergo bonding with phosphate. After the initial calcium phosphate/PS complex has formed, crystal growth proceeds by the accumulation of additional Ca^{2+} and phosphate ions. The rapid formation of calcium phosphate bridges would then promote vesicle aggregation and provide membrane contacts for fusion at subthreshold Ca^{2+} concentrations.

The observation that Ca^{2+} and phosphate can promote fusion and phase separations in mixtures of PS and PC under conditions where Ca^{2+} alone is ineffective (Figures 7 and 8) indicates that the calcium phosphate/PS complexes are segregated more efficiently to form PS-enriched domains. Formation of these PS domains may be a prerequisite for the fusion of PS/DPPC vesicles by Ca^{2+} and phosphate in a manner analogous to that proposed by Papahadjopoulos et al. (1974) and Sun et al. (1979) for the fusion of PS/PC vesicles by Ca^{2+}. It is not possible to determine which of the calcium phosphate phases promotes vesicle aggregation, fusion, or phase separation since the crystal phase present at the vesicle surface (nucleation site) may be different from that monitored in the bulk solution.

Under conditions where the transformation to an apatite-like phase is delayed (Figure 4), extensive fusion can occur without appreciable leakage of vesicle contents. However, the transition to an apatic phase is accompanied by a dramatic increase in membrane leakiness. Wuthier & Eanes (1975) have shown that during HAP formation PS vesicles are disrupted to form membranous sheets.

The ability of apatite crystals to disrupt membrane structure may provide insight into the mechanism by which calcium phosphate crystals, which are formed inside matrix vesicles during cartilage maturation, are released in the process of mineralization. The accumulation of high levels of Ca^{2+} and phosphate in the interior of the matrix vesicles results in their spontaneous precipitation and subsequent formation of ACP (Anderson, 1973). Conceivably, the interaction of ACP with PS, which is localized preferentially in the inner bilayer of these vesicles (Majeska et al., 1979), could stabilize this transient crystal phase (Figures 2 and 4). Ultimately, the formation of apatite crystals would cause destabilization of the matrix vesicle membrane and release of the calcium phosphate deposits into the surrounding interstitial matrix. As a result, it may not be necessary to involve the action of phospholipases (Wuthier, 1973) or other lipolytic enzymes in degrading matrix vesicles in order for calcification to proceed.

The demonstration that fusion occurs between phospholipid vesicles containing only 10 mol % PS (Figure 9) and that it is dramatically enhanced in the presence of physiological levels of phosphate indicates the possible involvement of this ion combination in biological fusion events. The ability of Ca^{2+} and phosphate to induce phospholipid phase separation and formation of PS-enriched domains may be important in the fusion of biological membranes, such as synaptic vesicles and chromaffin granules, which contain only 10–20 mol % PS (Nagy et al., 1976; Buckland et al., 1978). The molecular segregation of PS (and other acidic phospholipids) into pure domains in the presence of Ca^{2+} and phosphate may provide areas free of membrane proteins where fusion between lipid bilayers could occur. In this respect, Zakai et al. (1977) have shown that the formation of calcium phosphate crystals on the cytoplasmic surface of human erythrocyte ghost membranes results in freeze–fracture surfaces which are nearly devoid of intramembranous particles. In addition, the extreme rapidity of fusion induced in the presence of phosphate may be of consequence in natural membrane systems; it has been calculated that the time for presynaptic vesicle fusion to occur following activation of the Ca^{2+} channels is on the order of 100 μs (Kelly et al., 1979). It is interesting to note that at 37 °C the rate of PS/Chol vesicle fusion induced by 2 mM Ca^{2+} is increased approximately 1000-fold in the presence of phosphate (Figure 5d).

We have shown that the rate and extent of fusion are critically dependent on pH, temperature, and the concentrations of Ca^{2+} and phosphate (Figure 5). In addition, a variety of other biological compounds, including ATP, amino acids, proteins, Mg^{2+}, and organic acids, are known to influence the formation of calcium phosphate crystals (Irving, 1973; Walton et al., 1967). The above factors provide new and attractive mechanisms for regulating membrane fusion. It should be emphasized that while the present study has employed metastable calcium phosphate solutions to promote spontaneous crystal nucleation and therefore is limited to the use of ion concentrations in the millimolar range, lower Ca^{2+} concentrations may be effective in biological systems because of the existence of discrete sites for Ca^{2+} sequestration (Bohan et al., 1973; Hillman & Llinas, 1974) and/or release (Heuser et al., 1974; Plattner et al., 1977).

An example of how the combined action of Ca^{2+} and phosphate ions may promote membrane fusion occurs during trichocyst discharge in *Paramecium*. The secretory vesicles which contain the trichocysts in *Paramecium* are attached at the plasma membrane to arrays of membranous particles (Matt et al., 1978). Trichocyst expulsion is known to be triggered by a Ca^{2+}-mediated stimulus, and it has been shown recently that a Ca^{2+}-transport ATPase is present at the vesicle attachment site (Plattner et al., 1977). Ion-capture experiments indicate that local high concentrations of Ca^{2+} and phosphate are generated in the area between the two apposed membranes during trichocyst discharge. The magnitude of these local ion concentrations can be inferred from calculations indicating that total cellular ATP levels in *Paramecium* drop from 1.25 to 0.5 mM during triggering of trichocyst discharge (Matt et al., 1978); presumably, the concentrations of Ca^{2+} and phosphate at the secretory vesicle–plasma membrane junction would be sufficient to promote crystal nucleation and membrane fusion at these restricted sites. It has also been suggested (Bilinski et al., 1979) that precipitation of phosphate by Ca^{2+} ions may be necessary in order for the decondensation and discharge of the matrix proteins from the secretory vesicles to occur. The well-documented requirement for both Ca^{2+} and ATP in most biological membrane fusion events (Poste & Allison, 1973) may indicate that similar mechanisms could be operating in these cases.

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