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CONTROL OF MEMBRANE FUSION BY PHOSPHOLID HEAD GROUPS

I. PHOSPHATIDATE/PHOSPHATIDYLINOSITOL SPECIFICITY

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We have studied the characteristics of fusion of large unilamellar vesicles composed of phosphatidate and phosphatidylinositol alone and in mixtures with other naturally occurring phospholipids. Fusion was induced by the addition of Ca^{2+} or Mg^{2+} and was monitored by detecting the mixing of aqueous vesicle contents. Release of vesicle contents was measured by dequenching of carboxyfluorescein fluorescence. Aggregation was monitored by 90° light scattering. The results indicated striking differences with respect to the fusion capacity of the different vesicles. Phosphatidate vesicles fuse in the presence of both Ca^{2+} and Mg^{2+} at threshold concentration ranges of 0.03–0.1 mM (Ca^{2+}) and 0.07–0.15 mM (Mg^{2+}) depending on the pH of the medium, 8.5–6.0, respectively. In contrast, phosphatidylinositol vesicles do not fuse with either Ca^{2+} or Mg^{2+} even at 50 mM concentrations, in spite of aggregation induced by both cations in the range of 5–10 mM. A large difference in terms of fusion capacity is retained even when these two phospholipids are mixed with phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine in 2 : 2 : 4 : 2 molar ratios. The results are discussed in terms of the molecular mechanism of membrane fusion and the possible role of the metabolic interconversion of phosphatidylinositol to phosphatidate as an on-off control system for membrane fusion phenomena involved in secretion.

Introduction

Divalent cation-induced fusion of lipid bilayer vesicles presently constitutes the most accessible and best studied model for membrane fusion. It is also an experimental system in which functional properties of membrane lipids can be studied and compared. The recent introduction of a quantitative, fluorimetric, assay for vesicle fusion [1] has greatly facilitated such studies. The fusion characteristics of phosphatidylserine (PS) and its mixtures with neutral phospholipids have been studied extensively [1–8]. Phosphatidate and phosphatidylglycerol vesicles also undergo fusion at characteristic threshold divalent cation concentrations [9]. Sonicated vesicles prepared from mixtures of phosphatidate with phosphatidylcholine appear to grow to a limited size after a few rounds of fusion in the presence of Ca^{2+} [10, 11]. In the present and accompanying [12] paper we report studies on the fusion of large unilamellar lipid vesicles containing phosphatidate and phosphatidylinositol, alone or in mixtures with other naturally occurring phospholipids.

Phosphatidate and phosphatidylinositol are of particular interest with respect to the cellular phenomenon called the 'phospholipid effect', i.e. an increased metabolic turnover of the polar part of phosphatidylinositol [13]. The phenomenon occurs in many cells and tissues following stimulation to secretion [14] and has been shown to consist, ini-

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Abbreviation: Tes, tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

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tially, of an extensive net conversion of phosphatidylinositol to phosphatidate [15–17]. Here we emphasize the effect of this particular head-group replacement on the fusion competence of lipid vesicles.

Materials and Methods

Phosphatidylserine from bovine brain and phosphatidylcholine from egg yolk were isolated as described [18]. Egg yolk phosphatidylethanolamine, soy bean phosphatidylinositol and sodium phosphatidate prepared from egg yolk phosphatidylcholine were purchased from Avanti Polar Lipids, Birmingham, AL. All lipids showed only one component upon thin-layer chromatography in chloroform/methanol/water and chloroform/methanol/ammonia systems and were stored in chloroform solution at −50°C under argon. Carboxyfluorescein was obtained from Eastman Kodak, and was recrystallised before use [19].

Unilamellar vesicles (approximately 0.1 μm diameter) were prepared by reverse-phase evaporation, using diethylether as solvent, followed by extrusion [20] through a polycarbonate membrane (Unipore, 0.1 μm pore diameter, Bio-Rad). Encapsulation of $TbCl_3$, dipicolinic acid and carboxyfluorescein were carried out as described [2] except for the following modifications, undertaken to prevent Tb binding to phosphatidate: Nitrilotriacetic acid, sodium salt at 100 mM was used instead of sodium citrate as the Tb chelator and the osmolarity of other encapsulated solutions and the external medium was adjusted by raising the concentration of NaCl. Encapsulated solutions thus had the following composition: 2.5 mM $TbCl_3$, 100 mM nitrilotriacetic acid, 2 mM L-histidine, 2 mM tris (hydroxymethyl)methyl-2-aminoethanesulphonate (Tes), pH 7.4; 100 mM dipicolinic acid, 2 mM L-histidine, 2 mM Tes, pH 7.4; and 50 mM carboxyfluorescein, 100 mM NaCl, 0.1 mM EDTA, 2 mM L-histidine, 2 mM Tes, pH 7.4, respectively. As external medium, 0.2 M NaCl, 0.1 mM EDTA, 2 mM L-histidine, 2 mM Tes with pH 7.4 or as indicated, was used.

Vesicles (in 1 ml) were freed of external Tb-nitrilotriacetic acid, dipicolinic acid and carboxyfluorescein by passage through a column of Sephadex G-75 (1 × 20 cm) using the above mentioned buffer (dipicolinic acid and carboxyfluorescein vesicles) or the same buffer containing 1 mM EDTA (Tb-nitrolotriacetic acid vesicles) as eluent. Tb-nitrilotriacetic acid vesicles used for calibration of maximum response in the fluorimetric assay for fusion were freed of EDTA by passage through a second column of Sephadex G-75, eluted with buffer containing no EDTA.

Assays for fusion and release of vesicle content were carried out in 1 ml of buffer containing 0.1 mM EDTA in a quartz cuvette maintained at 25°C and equipped with a magnetic stirring device [2]. Assays for fusion contained equimolar amounts of Tb-nitrilotriacetic acid vesicles and dipicolinic acid vesicles (25 μM lipid each, except where indicated) while release of vesicle content was measured using carboxyfluorescein loaded vesicles (50 μM lipid). Fluorescence and 90° light scattering were measured, and continuously registered, using an SLM-4000 fluorimeter (SLM Instruments, Champaign-Urbana, IL). In the assay for fusion excitation and registration of light scattering was at 276 nm while fluorescence (Tb-dipicolinic acid complex formation) was measured above 520 nm using a Corning 3-68 cut-off filter and no monochromator. For carboxyfluorescein release, the same conditions were used except that the wavelength for excitation and registration of 90° light scattering was 430 nm.

Chelation of Tb with nitrilotriacetic acid was shown to prevent significant binding of Tb to phosphatidate by the following criteria: (1) Infusion of Tb-nitrilotriacetic acid (5 μM–2 mM Tb and a Tb : nitrilotriacetic acid molar ratio equal to that used for encapsulation) did not induce aggregation of large unilamellar or small unilamellar (sonicated) phosphatidate vesicles, as shown by 90° light scattering. (2) Basal light scattering at 430 nm as well as Ca^{2+} induced changes in light scattering were the same for phosphatidate vesicles containing encapsulated Tb-nitrilotriacetic acid, dipicolinic acid or carboxyfluorescein. (3) The amount of Tb and dipicolinic acid associated with Tb-nitrilotriacetic acid and dipicolinic acid vesicles, respectively, corresponded to virtually identical volumes of encapsulated solution.

To validate a direct comparison of the present results with those obtained using citrate as a Tb chelator [2], phosphatidylserine vesicles were prepared using the protocol described above for phosphatidate vesicles. The concentration dependence and kinetics of Ca^{2+}-induced fusion of these vesicles was the same as that previously described [2].

When the pH was varied, vesicles prepared at pH 7.4 were added to buffer pretitrated to give the desired pH in the final assay mixture. The fluorescence yield of Tb-dipicolinic acid was constant in the pH-range (5.0–9.0) studied. The concentrations of added divalent ions reported represent those added in excess of 0.1 mM, since this concentration of EDTA was present in the external medium in all incubations.

Results and Discussion

Aggregation and fusion of phosphatidate vesicles

A mixture of phosphatidate vesicles, containing encapsulated dipicolinic acid and Tb ions, respectively, underwent aggregation and fusion (Fig. 1 A–F) when exposed to sufficient concentrations of Ca^{2+} or Mg^{2+} as seen by increases in light scattering *, with subsequent appearance of visible aggregates and by the formation of the highly fluorescent Tb-dipicolinic acid complex. At pH 7.4 (Fig. 1B) significant aggregation and fusion was observed at 0.2 mM Ca^{2+} or 0.4 mM Mg^{2+}. However, the concentration of these ions needed to induce fusion was extremely dependent on pH (Fig. 1A–C). This dependency is summarized in Fig. 2. At pH 6, fusion started at about 1 mM Ca^{2+} while at pH 8.5, significant fusion ** occurred already at 30 μM added Ca^{2+}, which is similar to the concentration of phosphatidate exposed on the outer surface of the vesicles, or half the total phosphatidate concentration. This relationship between phosphatidate concentration and the Ca^{2+} concentration required for fusion is further illustrated in Table 1, where the vesicle concentration has been varied. The results clearly indicate that, at pH 8.5 and in the range of vesicle concentrations investigated, a substantial fraction of the added calcium was bound by the vesicles and that the concentration of (free) Ca^{2+} in the bulk solution must have been in the micromolar range.

It is well known that phosphatidic acid undergoes protolysis to its doubly ionized state with a pK (in terms of pH of the bulk solution) of 8.5–9 in the absence of divalent ions and at low ionic strength [21,22]. The resulting increase in charge density of phosphatidate vesicles is likely to contribute significantly to the higher sensitivity towards Ca^{2+} and Mg^{2+} that we observed at alkaline pH. The results of Fig. 2 seem to indicate a lower pK than 8.5–9 in our system containing 0.2 M NaCl and Ca^{2+} or Mg^{2+}. This is not unexpected since both mono- and divalent cations have been shown to release protons and increase the ionization of phosphatidate bilayers [21].

Another event in the series of events induced by Ca^{2+} and Mg^{2+} was found to be strongly pH dependent, namely the release into the medium of entrapped vesicle contents. In phosphatidylserine vesicles, the temporal relationship between Ca^{2+}-induced fusion and the release of contents is such that extensive fusion takes place before significant release is observed [1,2]. A situation similar to this was found with phosphatidate vesicles below neutral pH where the fluorescent Tb-dipicolinic acid complex was well retained within the vesicles initially (Fig. 1A) and the rate of carboxyfluorescein release was much lower than the rate of fusion (Fig. 3A). However, as the pH was raised, the rate of carboxyfluorescein release relative to fusion increased (Fig. 3B and C) and the amount of fluorescent Tb-dipicolinic acid complex registered at any given time was drastically reduced (Fig. 1 and 4). If fusion always preceeded release of vesicle contents, the very rapid release at higher pH (Fig. 3) would certainly lead to an underestimation of the initial rate of fusion in the present assay, since the Tb-dipicolinic acid complex dissociates rapidly when released into the external medium [2]. Although our results are consistent with a mere shortening of the time period between fusion and release as a cause for the low Tb-dipicolinic acid fluorescence at alkaline pH, the possibility of ion-induced release without fusion cannot be ruled out under these conditions. In any case, the results clearly indicate that drastic structural alterations, probably related to fusion are induced very rapidly following

* Factors other than vesicle aggregation and fusion (e.g. change of refractive index due to ion binding or change of vesicle shape) could contribute to changes in light scattering. The pattern of light scattering changes observed with phosphatidate vesicles varies with pH and differs from the tri-phasic pattern observed with phosphatidylserine vesicles.

** Registered fluorescence was attributed to vesicle fusion since (a) it could be fixed by the addition of an excess of EDTA and (b) it did not develop when vesicles containing encapsulated dipicolinic acid or Tb-nitrilotriacetic acid were incubated separately rather than in mixture.

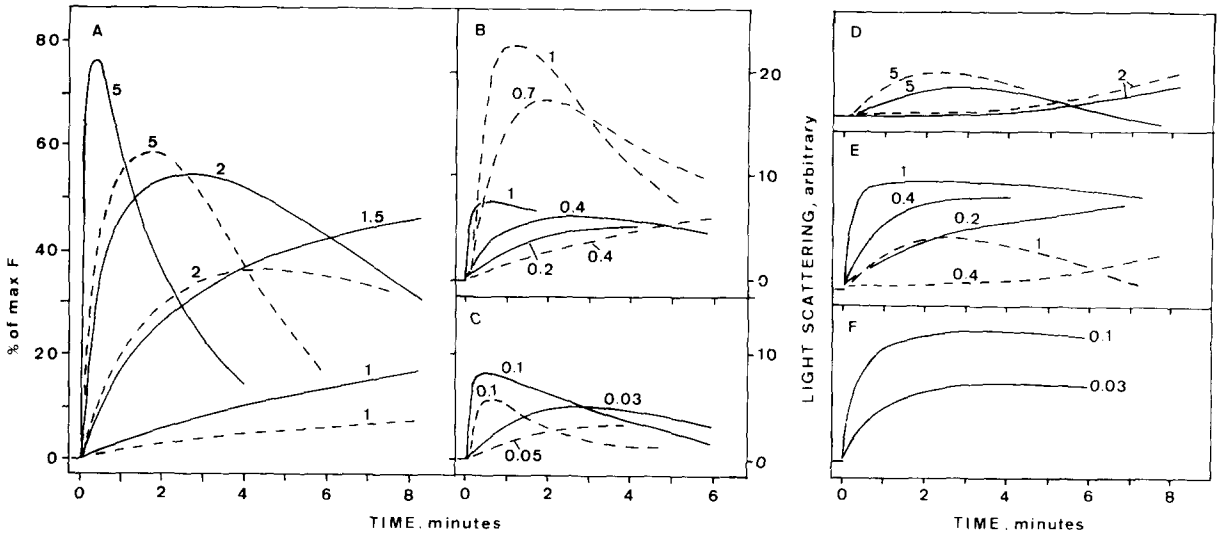

Fig. 1. Kinetics of Ca^{2+}- and Mg^{2+}-induced fusion (A–C) and aggregation (D–F) of phosphatidate vesicles at pH 6.0 (A, D); pH 7.4 (B, E) and pH 8.5 (C, F). Fusion is presented as relative Tb-dipicolinic acid fluorescence, F. Aggregation was followed as changes in 90° light scattering at 276 nm. Solid lines, Ca^{2+}; broken lines, Mg^{2+}. Divalent cation concentrations (mM) are indicated in the figure.

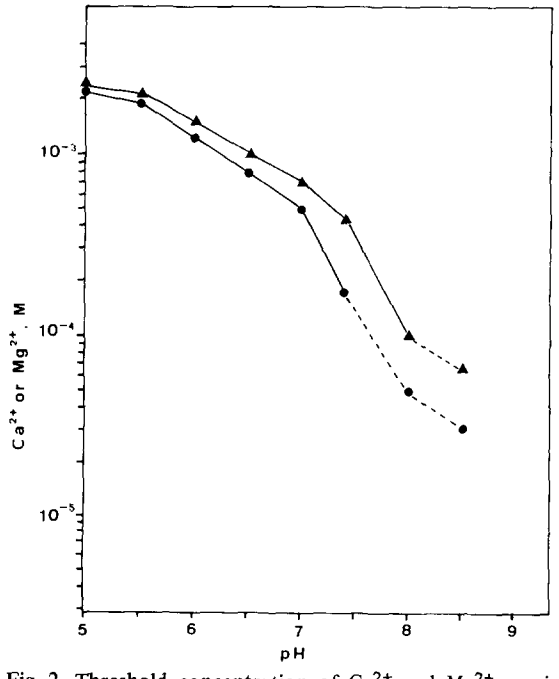

Fig. 2. Threshold concentration of Ca^{2+} and Mg^{2+} required for fusion of phosphatidate vesicles as function of pH. The threshold is defined as the concentration of divalent ion required for a rate of fusion of 5% of maximal Tb-dipicolinic acid fluorescence per min. Broken lines are used in the concentration range where values are significantly affected by the vesicle concentration. Symbols: •, Ca^{2+}; ▲, Mg^{2+}.

Ca^{2+} or Mg^{2+} addition to doubly ionized phosphatidate vesicles.

In agreement with previous results [9], Mg^{2+} was almost as effective as Ca^{2+} in inducing aggregation, fusion and release of contents in phosphatidate vesicles. There was, however, a difference in the pH dependance of Ca^{2+} and Mg^{2+}-induced fusion and, particularly, in the retention of vesicle contents as a function of pH (Fig. 4). This might be explained by a

TABLE I

RELATIONSHIP BETWEEN PHOSPHATIDATE (VESICLE) CONCENTRATION AND Ca^{2+} 'THRESHOLD' FOR VESICLE FUSION AT pH 8.5

The threshold is defined as the concentration of Ca^{2+} required for an initial rate of fusion of 5% of maximum Tb-dipicolinic acid fluorescence per min.

Vesicle concentration (μM phosphatidate)	Ca^{2+} threshold (μM)
25	13
50	30
100	55
250	135

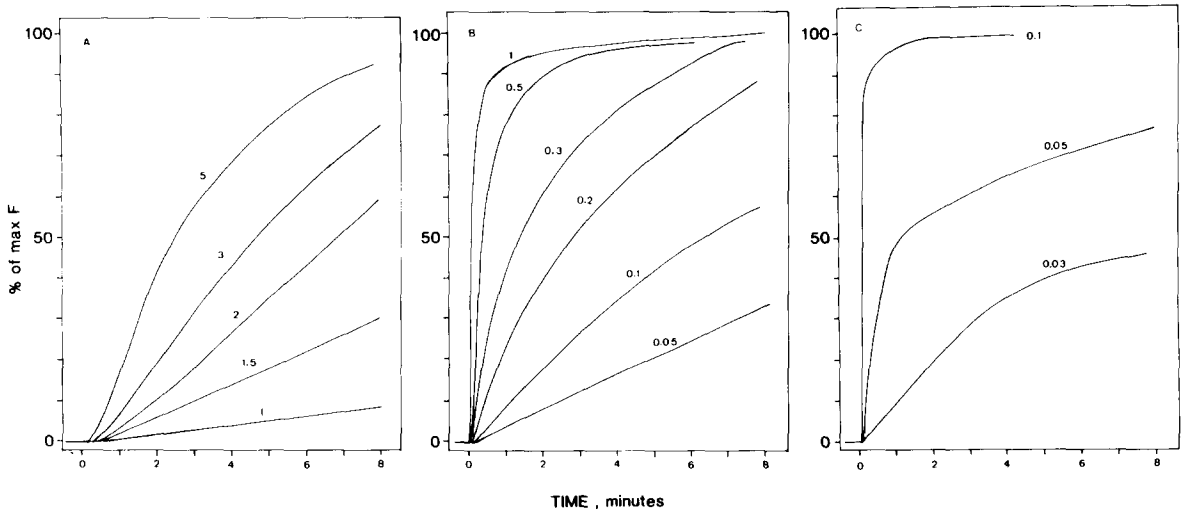

Fig. 3. Kinetics of Ca^{2+}-induced release of vesicle content (carboxyfluorescein) from phosphatidate vesicles at pH 6.0 (A), pH 7.4 (B) and pH 8.5 (C). Concentrations of Ca^{2+} (mM) are indicated in the figure.

more effective displacement of protons from phosphatidate by Ca^{2+} than by Mg^{2+} as has been observed previously [21].

Aggregation but no fusion of phosphatidylinositol vesicles

As shown in Fig. 5, unilamellar vesicles of phosphatidylinositol underwent Ca^{2+}- and Mg^{2+}-induced changes in 90° light scattering that were indicative of vesicle aggregation [2]. Large, visible aggregates also developed in these samples within 2–3 min after addition of divalent ion. Despite heavy aggregation,

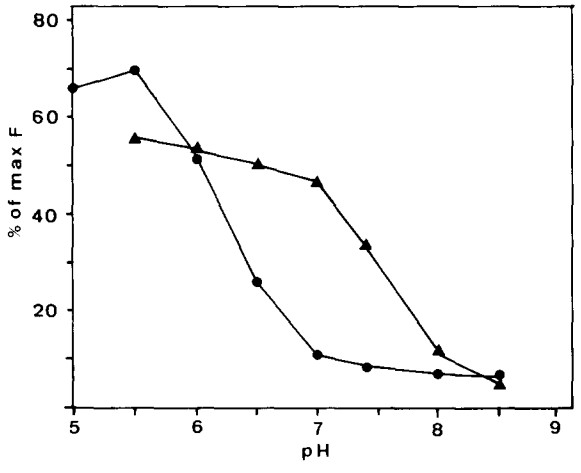

Fig. 4. Retention of vesicle contents during Ca^{2+}- and Mg^{2+}-induced fusion of phosphatidate vesicles, expressed as the peak Tb-dipicolinic acid fluorescence observed at each pH. The concentration of Ca^{2+} (•) or Mg^{2+} (▲) used at each pH was 10-times the threshold concentration shown in Fig. 2.

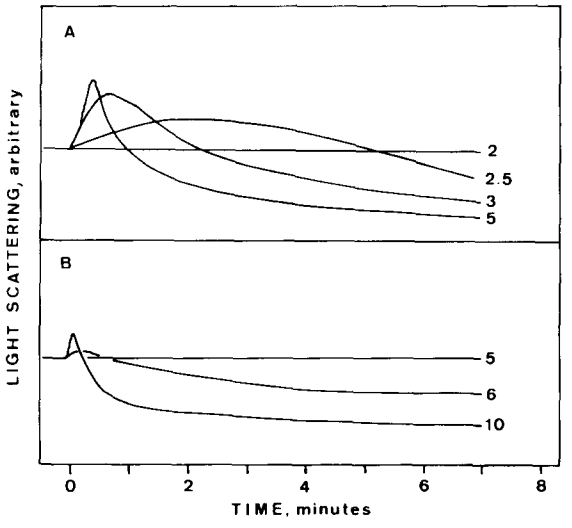

Fig. 5. Aggregation of phosphatidylinositol vesicles by Ca^{2+} (A) or Mg^{2+} (B) as shown by 90° light scattering at 430 nm. pH was 7.4. Light scattering changes at 276 nm were similar except that the initial increase in light scattering was not observed. Divalent ion concentrations (mM) are shown in the figure.

however, no fusion was observed in the fluorimetric assay used, even at 50 mM Ca^{2+}. Varying the pH between 5.5 and 9.0 or the temperature of incubation between 10 and 40°C did not change the results significantly and no fusion could be detected; thus pure phosphatidylinositol vesicles appear unable to undergo Ca^{2+}- or Mg^{2+}-induced fusion. There was also no significant release of encapsulated carboxyfluorescein following ion-induced aggregation of the vesicles or during subsequent disaggregation induced by adding an excess of EDTA. Precipitation of calcium phosphate on the surface of phosphatidylserine vesicles has previously been shown to enhance their fusion [23], but phosphatidylinositol vesicles were resistant to fusion also under those conditions.

On the basis of these results, one might expect phosphatidylinositol to be inhibitory to Ca^{2+}-induced fusion when incorporated in mixed lipid vesicles with a fusion-susceptible lipid, such as phosphatidylserine.

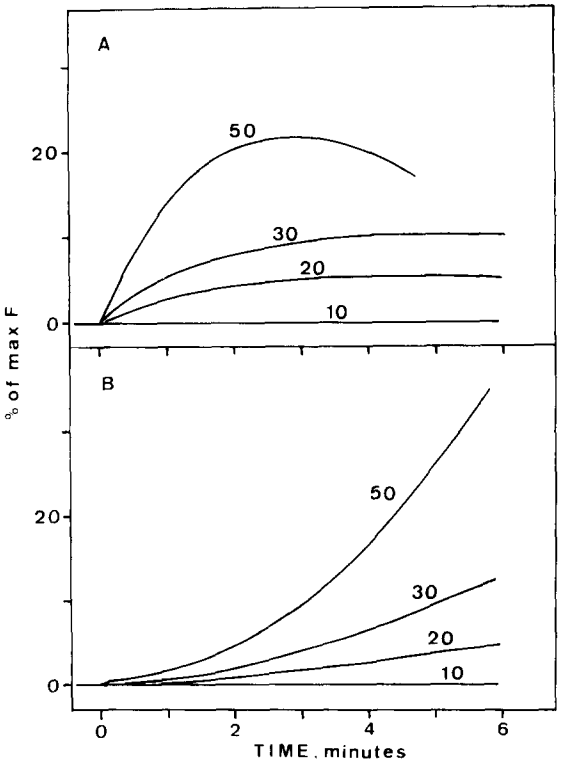

Fig. 6. Kinetics of Ca^{2+}-induced fusion (A) and release of contents (B) of phosphatidylinositol-phosphatidylserine (50 : 50) vesicles at pH 7.4. Concentrations of Ca^{2+} (mM) are indicated.

This was indeed the case, as shown in Fig. 6. Vesicles containing only phosphatidylserine are known to undergo aggregation and fusion starting at 2.5 mM Ca^{2+} [2]. Vesicles composed of equimolar amounts of phosphatidylserine and phosphatidylinositol also aggregated at 2.5 mM Ca^{2+} but fusion did not occur unless the Ca^{2+} concentrations exceeded 10 mM (Fig. 6). Phosphatidylinositol is, therefore, not as inhibitory to fusion as phosphatidylcholine since equimolar mixtures of phosphatidylserine and phosphatidylcholine only aggregate but do not fuse with up to 50 mM Ca^{2+} [7].

The present results further illustrate that vesicle aggregation and vesicle fusion are processes that may be dissociated even when both are induced by the same ion. A dissociation of these processes has previously been obtained using different ions since Ca^{2+} induces aggregation and fusion of large phosphatidylserine vesicles while Mg^{2+} induces aggregation but not fusion [2,24]. The results with the mixed phosphatidylinositol-phosphatidylserine vesicles also confirm the previous conclusion [1,5,7] that release of vesicle contents is a consequence of vesicle fusion and not related to vesicle aggregation per se.

Phosphatidate is normally a very minor component of cellular membranes but has been shown to accumulate at the expense of phosphatidylinositol in several cells and tissues following exposure to secretory stimuli [14–17, 25–27]. For example, in blood platelets about one half of the cellular phosphatidylinositol is converted in this way within seconds of thrombin stimulation [16,17,25]. If the conversion is not uniformly spread among the cellular membrane systems, it would be even more extensive in those membranes where it occurs. We have, therefore, studied the effect of substitution of phosphatidate for phosphatidylinositol in vesicles with a complex lipid composition resembling that of cellular membranes (phosphatidylserine : phosphatidylethanolamine : phosphatidylcholine) and in which phosphatidate (phosphatidylinositol) was a relatively minor component (Fig. 7). Vesicles containing phosphatidate were found to be much more susceptible to Ca^{2+}-induced fusion (at 1–5 mM concentration) than corresponding vesicles containing phosphatidylinositol, which fused only at a concentration above 10 mM Ca^{2+}. If phosphatidylinositol is a membrane component that helps stabilizing bilayer structure and

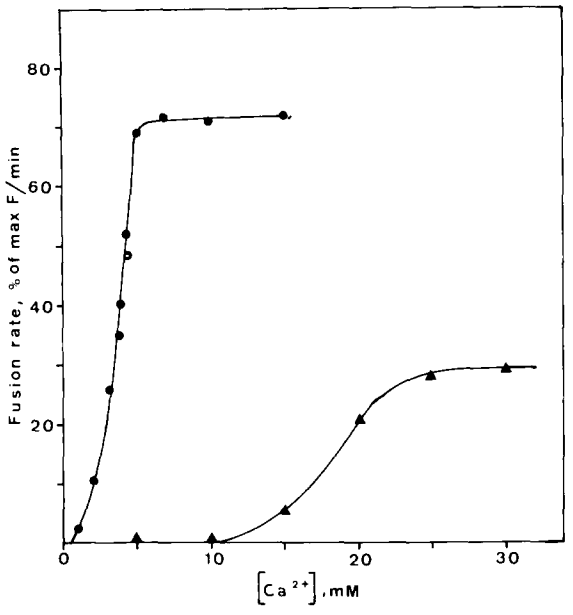

Fig. 7. Effect of substitution of phosphatidate (•) for phosphatidylinositol (▲) on the ion concentration dependence of Ca^{2+}-induced fusion among mixed lipid vesicles composed of phosphatidate (or phosphatidylinositol)/phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine (20 : 20 : 40 : 20). pH was 7.4.

counteract bilayer fusion, then its extensive conversion into phosphatidate in cellular membranes might facilitate, or even trigger, membrane fusion at sites of close contact between membrane bilayers, particularly since this phenomenon seems to occur in conjunction with a mobilization of cellular Ca^{2+} [28].

Concluding remarks

The studies presented in this paper have demonstrated a dramatic difference in fusion characteristics between phosphatidate and phosphatidylinositol both as single component vesicles and in mixed vesicles approximating the composition of biological membranes. While phosphatidate is induced to undergo fusion at low concentrations of Ca^{2+} ($\leqslant$1 mM) phosphatidylinositol is completely resistant to fusion even at high Ca^{2+} concentrations. We therefore propose that metabolic interconversion of phosphatidylinositol to phosphatidate which is known to occur during exposure to secretory stimuli could represent an on-off mechanism for Ca^{2+}-induced phenomena at the cellular level. As a mechanistic explanation for the widely different characteristics in fusion between these two phospholipids we propose that the bulky hydrated inositol group inhibits fusion by not allowing the formation of dehydrated complexes between (phosphate groups from two) closely apposed membranes.

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