Polyphosphoinositide Inclusion in Artificial Lipid Bilayer Vesicles Promotes Divalent Cation-Dependent Membrane Fusion

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ABSTRACT Recent studies suggest that phosphoinositide kinases may participate in intracellular trafficking or exocytotic events. Because both of these events ultimately require fusion of biological membranes, the susceptibility of membranes containing polyphosphoinositides (PPIs) to divalent cation-induced fusion was investigated. Results of these investigations indicated that artificial liposomes containing PPI or phosphatidic acid required lower Ca^{2+} concentrations for induction of membrane fusion than similar vesicles containing phosphatidylserine, phosphatidylinositol, or phosphatidylcholine. This trend was first observed in liposomes composed solely of one type of phospholipid. In addition, however, liposomes designed to mimic the phospholipid composition of the endofacial leaflet of plasma membranes (i.e., liposomes composed of combinations of PPI, phosphatidylethanolamine, and phosphatidylcholine) also required lower Ca^{2+} concentrations for induction of aggregation and fusion. Liposomes containing PPI and phosphatidic acid also had increased sensitivity to Mg^{2+}-induced fusion, an observation that is particularly intriguing given the intracellular concentration of Mg^{2+} ions. Moreover, the fusogenic effects of Ca^{2+} and Mg^{2+} were additive in vesicles containing phosphatidylinositol bisphosphate. These data suggest that enzymatic modification of the PPI content of intracellular membranes could be an important mechanism of fusion regulation.

INTRODUCTION

Membrane fusion is necessary for a number of biological processes including exocytotic release of neurotransmitters, enzymes, and hormones and intracellular trafficking of proteins. Random occurrences of such fusion events are inhibited by steric and hydration forces that prevent membranes from getting into close approximation (Helm and Israelachvili, 1993). The molecular mechanisms necessary for overcoming these forces and allowing two separate membranes to form a continuous bilayer are largely unknown.

Several components are likely to be involved in regulation of intracellular membrane fusion events, including phospholipids (Schewe et al., 1992; Santini et al., 1990), proteins (Zimmerberg et al., 1993; Plattner, 1989), and nucleotides (Plattner, 1989). In particular, research with artificial liposomes over the past 15 years has revealed that a membrane's phospholipid composition greatly alters its susceptibility to divalent cation-induced fusion (Duzgunes et al., 1981; Sundler et al., 1981; Sundler and Papahadjopoulos, 1981). Phosphatidylserine (PS) (Duzgunes et al., 1981; Meers et al., 1991) and phosphatidic acid (PA) (Sundler et al., 1981; Sundler and Papahadjopoulos, 1981; Meers et al., 1991; Ohki and Zschornig, 1993) reportedly increase the fusogenic potential of membranes while phosphatidylcholine (PC) decreases their fusogenicity (Duzgunes et al., 1981). Moreover, in situ analysis later revealed that membranes become enriched in the destabilizing phospholipids PA and phosphatidylethanolamine (PE) just before fusion in cultured myoblasts; this enrichment occurs at the expense of membrane-stabilizing phospholipids such as PC (Santini et al., 1990). Although it is unknown whether manipulation of membrane phospholipids is sufficient for initiation of membrane fusion, control of these components could clearly be important for fusion regulation.

The studies presented here, using the methodologies of Sundler et al. (1981), Sundler and Papahadjopoulos (1981), and Duzgunes et al. (1981), have attempted to extend this previous research in order to investigate the fusogenic potential of a unique group of anionic phospholipids, the polyphosphoinositides (PPIs). Several pieces of evidence contribute to the hypothesis that these uniquely polyvalent phospholipids may help promote divalent cation-induced fusion. 1) The unobstructed phosphate groups on PPI are oriented similarly to the phosphate group in PA; this charged species is reportedly accountable for PA's fusogenic nature (Meers et al., 1991). 2) Phosphoinositide 3-kinases are implicated in specific intracellular trafficking events, which ultimately involve fusion of intracellular vesicles with target membranes (Kanai et al., 1993; Stack et al., 1993; Jones and Clague, 1995). 3) Phosphoinositide 4-kinases and their phosphorylated products are located in a number of fusion-susceptible membranes including mast cell granules (Kurosawa and Parker, 1986), chromaffin granules (Husebye and Flatmark, 1988), glucose transporter vesicles (Del Vecchio and Pilch, 1991), lysozomes (Collins

Received for publication 27 November 1995 and in final form 13 September 1996.

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Abbreviations used: PPI, polyphosphoinositide; PIP_2, phosphatidylinositol bisphosphate; PIP, phosphatidylinositol monophosphate; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; pyrene-PC, 1-hexadecanoyl-2-(1-pyrene hexanoyl)-*sn*-glycero-3-phosphocholine; DCY, *N,N'*-Dioctadecyloxacarbocyaninine-*p*-toluene sulfonate; LUV, large unilamellar vesicle; PLD, phospholipase D.

0006-3495/96/12/3199/08 $2.00

and Wells, 1983), and the Golgi apparatus (Tooke et al., 1984).

To test the fusogenic potential of PPIs, artificial liposomes were used to measure the relative efficacy of different phospholipids in facilitating divalent cation-induced membrane fusion. Fusion experiments with these simple model membranes allow investigation of the specific components involved in this complex process. In this study, the dramatic sensitivity of membranes containing PPIs to divalent cation-dependent fusion is described.

EXPERIMENTAL

Materials

N,N'-Dioctadecyloxacarbocyaninine-*p*-toluene sulfonate (DCY) was obtained from Eastman Kodak. 1-Hexadecanoyl-2-(1-pyrene hexanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC) was obtained from Molecular Probes, Inc. Phospholipids were purchased from Sigma Chemical Co. and were stored as stock solutions in chloroform (10 mg/ml) with the exception of the phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2), which were stored as stock solutions in chloroform/methanol/water (5:10:2, v/v) (1.25 mg/ml). Type I phospholipase D purified from cabbage and all other chemicals were purchased from Sigma.

Preparation of large unilamellar vesicles for fusion assays

Large unilamellar vesicles (LUVs) were prepared using the Liposofast device from Avestin, Inc. by the method of MacDonald et al. (1991). Briefly, 1.5 mg phospholipid ($\sim 2 \times 10^{-3}$mmol) in solution (see above) and the appropriate amount of fluorescent probe (see below) were dried under nitrogen. This residue was then resuspended in 300 μl fusion buffer (110 mM KCl, 5 mM NaCl, 10 mM MOPS, pH 7.4) containing additionally 0.1 mM EDTA (for assays of Ca^{2+}) or EGTA (for assays of Mg^{2+}). Resuspension required brief sonication in a bath sonicator. The solution was then subjected to 10 rounds of freeze-thawing (1-min freeze, 2-min thaw) in a solid CO_2/ethanol bath and the resulting multilamellar vesicles were extruded 19 times through polycarbonate filters mounted in the mini-extruder. The resulting solution of LUVs was diluted to 4 ml in fusion buffer containing the EDTA or EGTA.

Because LUVs composed partially of PIP_2 have not been characterized, LUVs generated were evaluated by electron microscopy. PE/PIP_2 (3:1) LUVs were prepared as described and mixed with 1% uranyl acetate. Vesicles were then placed on 400-mesh copper grids pretreated with 0.1% bovine serum albumin and examined in a Phillips EM 300 electron microscope operated at 60 kV. Evaluation of 100 liposomes revealed that they were heterogenous in size with an average diameter of 119.6 ± 41.3 nm (standard deviation), a mode of 85.5 nm, and a median of 112.2 nm. These parameters are similar to those described for PA, PC, PS, and phosphatidylinositol (PI) LUVs generated using the same method (MacDonald et al., 1991).

Membrane fusion experiments

Two fluorimetric assays were used to measure the mixing of lipids that occurs between liposomes during the fusion event. This lipid mixing event should be distinguished from a true fusion event, which in these liposomes is experimentally defined as the mixing of aqueous contents. Both assays were claimed previously to represent true bilayer amalgamation, however, and not mere vesicle aggregation (Stegmann et al., 1993; Spungin et al., 1992). Moreover, these two assays generated results consistent with those published previously, which used a Tb^{3+}/dipicolinic acid fusion assay (Duzgunes et al., 1981; Sundler et al., 1981; Sundler and Papahadjopoulos, 1981). Additionally, the assays described below utilize hydrophobic fluorescent probes, which should have less interaction with the head group of the anionic PPIs than the Tb^{3+} used in the Tb^{3+}/dipicolinic acid assay. Finally, the pyrene-PC assay described below was previously reported to have no spontaneous transfer of the probes between donor and acceptor membranes, unlike another lipid-mixing assay using octadecylrhodamine (Stegmann et al., 1993).

Pyrene-labeled PC fusion assay

Membrane fusion results reported here utilized the fluorescent phospholipid derivative pyrene-PC (Stegmann et al., 1993). In these experiments, one population of LUVs was labeled with the pyrene-PC by drying 1 μl (20 mM ethanolic stock solution) of the probe with the lipids during LUV preparation (see above); a second preparation of LUVs received no label. When located in a membrane at sufficient concentrations, pyrene-PC forms fluorescent excimers containing molecules in the ground and excited state. When liposomes containing a small amount of these probes are fused with unlabeled liposomes, the concentration of probes, and consequently the fluorescence of the excimer, decreases. Excimer fluorescence was determined at an excitation wavelength of 345 nm and an emission wavelength of 480 nm using an Aminco Bowman Spectrofluorometer. Fusion extents were calculated by the following formula: $F = 100(F_0 - F_t)/(F_0 - F_{C12E8})$, where F_t and F_0 represent the fluorescence of the separately labeled vesicles at time (t) and time (0) after divalent cation addition, and F_{C12E8} is the fluorescence of the vesicles after the addition of octaethylene glycol *n*-dodecyl monoether (C12E8, 10 mM final concentration). Initial velocity of fusion was defined as the percent of maximum fusion at the first measurable time point, 15 s. In some experiments, light scattering was observed in unlabeled liposomes by measuring fluorescence at the same excitation and emission wavelength (345 nm).

Chlorophyll/DCY fusion assay

Membrane fusion determinations were reproduced using another lipid mixing assay (Gibson and Strauss, 1984; Spungin et al., 1992). Although no results are listed in this manuscript, the work with the chlorophyll/DCY method generated determinations of cation thresholds necessary for fusion initiation that were nearly identical to those produced using the pyrene-PC fusion assay.

RESULTS

PPIs promote Ca^{2+}-dependent fusion of homogenous LUVs

Studies on the fusogenic properties of phospholipids often investigate unilamellar vesicles composed solely of one type of phospholipid (Duzgunes et al., 1981; Sundler and Papahadjopoulos, 1981; Ohki and Zschornig, 1993; Stack et al., 1993). In such studies PS (Duzgunes et al., 1981) and PA (Sundler and Papahadjopoulos, 1981; Ohki and Zschornig, 1993) were identified as the most fusogenic phospholipids. The experiments described in Fig. 1 compared the Ca^{2+} requirement for fusion of LUV composed solely of PIP to the requirement of similar vesicles composed of PA, PS, PI, or PC. Vesicles composed of PIP and PA clearly began fusing at lower Ca^{2+} concentrations than all other phospholipids reaching threshold at concentrations less than 1 mM (threshold is defined as the concentration of divalent cation necessary to reach 15% of maximum fusion within 1 min after Ca^{2+} addition). Threshold determinations for PS, PI, PA, and PC agreed closely with values obtained previously by other investigators (Sundler and Papahadjopoulos, 1981; Ohki and Zschornig, 1993).

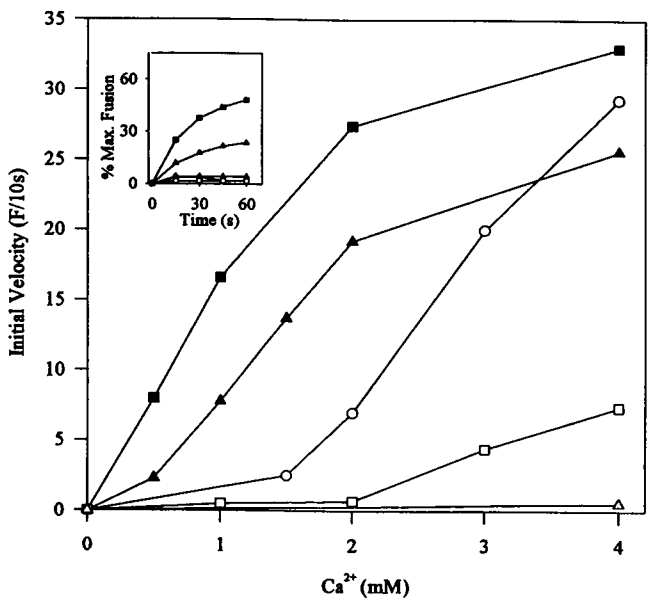

FIGURE 1 LUVs composed of PIP or PA fused at lower Ca^{2+} concentrations than other phospholipids. Homogenous LUVs were prepared of different phospholipids: ■, PIP; ▲, PA; ○, PS; □, PI; △, PC. The initial velocity of fusion was determined at several different Ca^{2+} concentrations. This graph is representative of three sets of independent measurements. *Inset*: In this representative time course 1 mM Ca^{2+} was added at time 0 and fusion was monitored for 1 min. Points are representative of three independent measurements.

PPIs promote divalent Ca^{2+}-dependent fusion of heterogeneous LUVs

The experiments described above comparing the fusogenic properties of liposomes prepared solely from one type of phospholipid have two disadvantages: 1) LUVs prepared solely of one type of phospholipid may have structural characteristics that may not compare with structural features in endogenous bilayers; and 2) the head groups of fusogenic lipids may function differently in an environment rich in other types of phospholipids. To overcome these limitations, the majority of the studies described in this paper analyzed lipids composed predominantly of a neutral phospholipid. Inasmuch as PE is the most abundant lipid in the cytoplasmic leaflet of a number of membranes, including cardiac sarcolemma (Post et al., 1988), erythrocyte plasma membranes (Zachowski, 1993), and hepatocyte contiguous surface plasma membranes (Higgins and Evans, 1978), the majority of the experiments in this study were done with liposomes composed predominantly of PE. In these studies the percent of test phospholipid (i.e., the PPIs, PS, PI, or PA) was never more than 25%.

PE inclusion in bilayers containing PA, PI, or PS slightly raises the Ca^{2+} requirement for initiation of liposome aggregation and fusion (Duzgunes et al., 1981; Sundler et al., 1981). In accordance with these observations, LUVs containing 75% PE in addition to 25% of the phospholipid being tested required more Ca^{2+} than the homogenous LUVs for initiation of liposome fusion. However, the fusogenic nature of liposomes containing PIP or PA was still observed; PIP/PE or PA/PE (1:3) LUVs fused at lower Ca^{2+} concentrations than those containing PS, PI, or PC. Additionally, the use of these predominantly PE LUVs afforded the opportunity to measure the fusogenic efficacy of PIP_2. In this and all subsequent studies, liposomes containing PIP_2 required lower divalent cation concentrations for fusion induction. Threshold determinations in these studies were 1 and 2 mM for PIP_2 and PIP, respectively (Fig. 2), while thresholds for PA, PS, PI, and PC were 2, 3, 4, and $\gg$10 mM, respectively. The latter four thresholds were in close agreement with values obtained previously by others (Duzgunes et al., 1981; Sundler et al., 1981; Sundler and Papahadjopoulos, 1981; Meers et al., 1991). The fusogenic nature of the PPIs seems to be a function of the extent of phosphorylation of their inositol ring; PIP_2 is more sensitive to Ca^{2+}-induced fusion than PIP, which is, in turn, more fusogenic than PI.

The addition of Ca^{2+} at concentrations sufficient to induce lipid mixing is accompanied by an increase in light scattering and the subsequent appearance of visible aggre

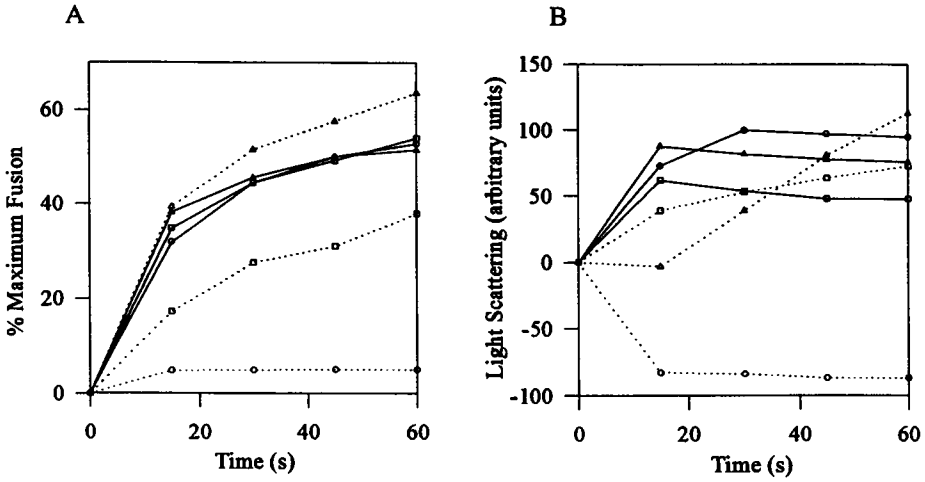

FIGURE 2 Heterogenous LUVs containing PIP_2 aggregate and fuse in the presence of 1 mM Ca^{2+}. Heterogenous LUVs were prepared of 75% PE and 25% test phospholipids: (——), PIP_2; (· · · · ·), PIP. One (○), two (□), or four (△) mM Ca^{2+} was added at time 0, and fusion (*A*) or scattering (*B*) was monitored for 1 min. Points are representative of two sets of independent measurements.

gates (Sundler et al., 1991; Bentz and Duzgunes, 1985). This is presumably due to the collapse of the fusing vesicle and the subsequent formation of an anhydrous Ca^{2+}-phospholipid complex with a different refractive index (Duzgunes et al., 1981; Bentz and Duzgunes, 1985). This proved to be the case in the studies with PIP_2/PE (1:3) and PIP/PE (1:3) LUVs. The onset of increased scattering correlated with the fusion of PPI, and under no conditions using LUVs containing PIP_2/PE (1:3) or PIP/PE (1:3) were increases in scattering observed in the absence of fusion. The decrease in scattering observed when subthreshold concentrations of divalent cation were added was presumably due to the formation of large unfused liposome aggregates, which tend to clear the light path (Duzgunes et al., 1981; Bentz and Duzgunes, 1985). Light scattering experiments performed using liposomes containing PA, PS, and PC correlated with those obtained previously (Sundler et al., 1991; Duzgunes et al., 1991).

PE is substantially less inhibitory to vesicle fusion than another predominant neutral lipid, PC (Duzgunes et al., 1981). PC comprises about 10% of the endofacial leaflet of plasma membranes of human erythrocytes (Zachowski, 1993) and rat hepatocytes (Higgins and Evans, 1978). When PE was replaced by up to 25% PC in LUVs containing PS (25%) as their test phospholipid, the LUVs became refractory to fusion at all concentrations tested. However, in LUVs containing PIP_2 as test phospholipid, this inhibitory effect on fusion by PC was much less dramatic, except at the highest concentration (data not shown).

Effect of magnesium, manganese, and phosphate on membrane fusion of heterogenous LUVs containing PIP_2

Although most studies on membrane fusion analyze the fusogenic effects of Ca^{2+} ions, Mg^{2+} ions are present in much higher concentrations in mammalian cells (Ortiz et al., 1990). PIP_2/PE, PIP/PE, or PA/PE (1:3) LUVs were only slightly less sensitive to Mg^{2+}-induced fusion than they were to fusion induced by Ca^{2+} (Fig. 3). Specifically, the vesicles containing PIP_2, PIP, or PA required only 2 mM (PIP_2) or 3 mM (PIP and PA) Mg^{2+} for near-maximal fusion responses. In contrast, PS/PE (1:3) LUVs were particularly less sensitive to Mg^{2+}-induced fusion, requiring 6 mM Mg^{2+} for maximal fusion responses.

Unlike Ca^{2+}, few cellular fusion events are initiated by a sudden influx of Mg^{2+}. To determine whether Ca^{2+} potentiates the effects of Mg^{2+}, LUVs were prepared as before and incubated with "subthreshold" Mg^{2+} concentrations (1 mM). Ca^{2+} was then added at 250 or 500 μM final concentrations and fusion extents recorded (Fig. 4). While these concentrations of Ca^{2+} were not sufficient to initiate fusion in the absence of Mg^{2+} (data not shown), PIP_2/PE (1:3) LUVs underwent Ca^{2+} dependent fusion at both concentrations in the presence of Mg^{2+}. Conversely, LUVs containing PA or PS in place of PIP_2 did not fuse appreciably in these conditions (data not shown).

Manganese is more fusogenic than Ba^{2+}, Sr^{2+}, Ca^{2+}, or Mg^{2+} for membranes containing PS (Ohki and Duzgunes, 1979). In separate experiments (data not shown), fusion thresholds for Mn^{2+} were determined to be ~1 mM for LUVs containing PIP_2, PIP, PA, or PS (test lipid/PE, 1:3). Additionally, phosphate ions enhance fusion rates between both liposomes (Duzgunes et al., 1981; Fraley et al., 1980) and erythrocyte membranes (Schewe et al., 1992), putatively by the formation of calcium phosphate complexes at the surface of the membrane, allowing the two membranes to come into close apposition. In other experiments (data not presented) exogenous phosphate (10 mM) had no effect on the fusogenic potential of LUVs containing the already phosphorylated PIP_2.

Enzymatic activation of fusion

PIP_2, PIP, and PA can be generated enzymatically at the expense of less fusogenic phospholipids. For example, PIP_2 and PIP are generated by the sequential phosphorylation of PI by PI-kinases, and PA is generated by hydrolysis of

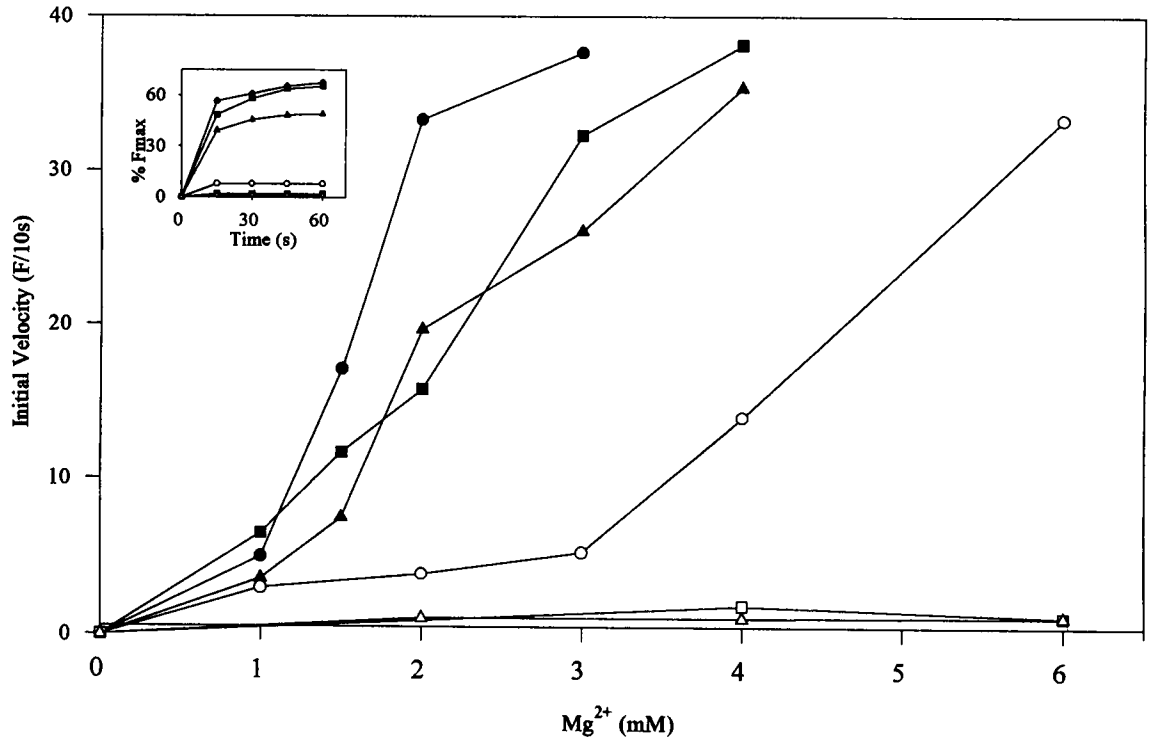

FIGURE 3 Heterogenous LUVs containing PIP_2, PIP, or PA fuse in response to Mg^{2+}. Heterogenous LUVs were prepared with 75% PE and 25% test phospholipid: ●, PIP_2; ■, PIP; ▲, PA; ○, PS; □, PI; △, PC. The initial velocity of fusion was determined at several different Mg^{2+} concentrations. Points are representative of three sets of independent measurements. *Inset:* In this representative time course 1 mM Mg^{2+} was added at time 0, and fusion was monitored for 1 min. Points are representative of three independent measurements.

membrane phospholipids by phospholipase D (PLD). Both PI-kinases (Kanai et al., 1993; Stack et al., 1993) and PLD (Kahn et al., 1993) are implicated in intracellular trafficking and exocytotic events. To test the hypothesis that these enzymes may regulate the occurrence and/or location of membrane fusion events, specifically by creating local domains of fusogenic phospholipids, the fusion experiments described above were conducted in the presence of PLD. Purified PLD (Sigma Chemicals) was used to initiate fusion of pure PC LUVs. When these nonfusogenic liposomes were incubated in the presence of Ca^{2+}, fusion between PC LUVs was initiated within seconds after the addition of 25 or 50 units of PLD (data not shown).

DISCUSSION

Usefulness of fusion experiments with liposomes

To simplify characterization of the components that regulate biological membrane fusion events, simple model systems using artificial membranes are often utilized (for review see Szoka, 1987). Results from liposome-liposome or liposome-planar membrane fusion experiments have provided important information about fusion mechanisms including the role of divalent cations (Ohki and Duzgunes, 1979; Duzgunes et al., 1987), phospholipid head groups (Duzgunes et al., 1981; Sundler et al., 1981; Sundler and Papahadjopoulos, 1981), surface hydrophobicity (Ohki and Zschornig, 1993), hydration and phase transition events (Duzgunes et al., 1987), temperature (Bentz et al., 1985), phosphate ions (Fraley et al., 1980), and integral (Boggs et al., 1977) or cytosolic (Meers et al., 1991) proteins. A number of these studies used LUVs ~100 nm in diameter as model membranes (Duzgunes et al., 1981; Sundler et al., 1981; Sundler and Papahadjopoulos, 1981; Fraley et al., 1980; Bentz et al., 1985; Bentz and Duzgunes, 1985). Unlike smaller liposomes (generally <50 nm in diameter), fusion between LUVs is specific for divalent cations (Bentz and Duzgunes, 1985). Furthermore, LUVs approximate the size of dense core synaptic vesicles (Zhu et al., 1986) and chromaffin granules (Scheuermann, 1993). Finally, LUVs of consistent size can be prepared quickly and easily regardless of the type of phospholipid used (MacDonald et al., 1991). For these reasons LUVs were ideal for these initial studies investigating the fusogenic potential of PPIs.

Mechanisms of membrane fusion

Many mechanisms have been proposed to describe how Ca^{2+}/phospholipid interactions initiate membrane fusion. Such interactions could increase the surface tension of lipid bilayers or alter the curvature of membranes, both of which can promote membrane amalgamation (Zimmerberg et al.,

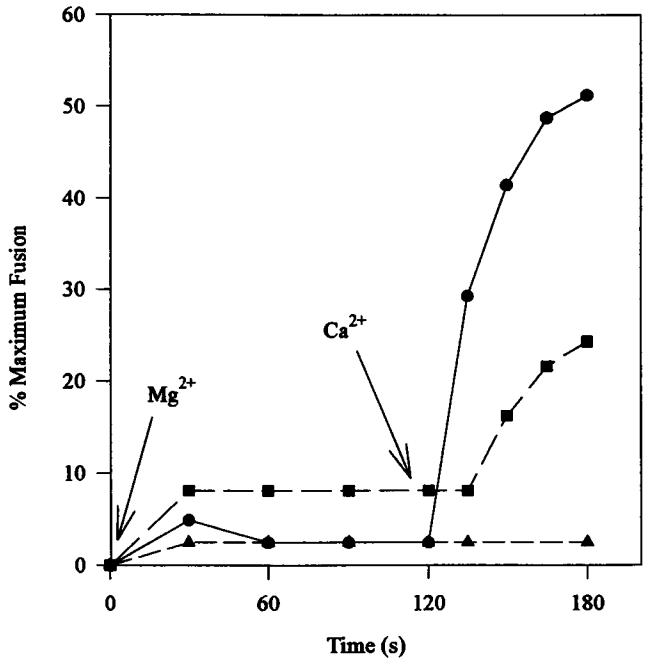

FIGURE 4 The fusogenic effects of Ca^{2+} and Mg^{2+} are cooperative. Heterogenous LUVs were prepared with 75% PE and 25% PIP_2. 1 mM Mg^{2+} was added at time 0 and either 0 (▲), 250 (■), or 500 (●) μM Ca^{2+} was added at 120 s. Fusion was monitored for a total of 3 min. This experiment is representative of three independent measurements.

1993). However, the ability of calcium to dehydrate lipid head groups and subsequently allow membranes to get into close approximation is perhaps the most important driving force for initiation of membrane fusion (Zimmerberg et al., 1993; Meers et al., 1991; Feigenson, 1986). Millimolar Ca^{2+} concentrations can completely dehydrate the space between PS bilayers (Feigenson, 1986) and the anionic PS serves as an aggregation promoter in this type of model (Meers et al., 1991; Feigenson, 1986). Other elements, such as free fatty acids (Creutz, 1992) or increased temperature (Bentz et al., 1985) serve as fusion promoters. The polyanionic nature of PPIs suggests that they could also serve as aggregation promoters.

In accordance with this mechanism, the data presented here unequivocably demonstrate that LUVs containing PPIs are more susceptible to Ca^{2+}-induced fusion than bilayers composed of lipids with less negative charge. More specifically, however, these data suggest a role for the exposed phosphates on PPI head groups in stimulation of membrane fusion. The most fusogenic phospholipids, the PPIs and PA, all contain phosphate groups that are extended away from the membrane and unobstructed by other charged groups. Furthermore, PS, whose phosphate group does not have easy access to divalent cations, becomes much more fusogenic in environments rich in phosphate ions. This supposedly occurs because of formation of calcium phosphate bridges between the two membranes (Fraley et al., 1980). Exogenous phosphate does not affect the fusogenic properties of liposomes containing the already phosphorylated PIP_2, however.

These phosphorylated phospholipids also have the ability to fuse in response to low concentrations of Mg^{2+}, a trait not seen for PS or other phospholipids (Duzgunes et al., 1981). This phenomenon was previously observed for small unilamellar vesicles composed solely of PA and was described on the basis that the unobstructed phosphate group in PA is more accessible to the smaller Mg^{2+} ion than anionic groups of other phospholipids (Ohki and Zschornig, 1993). This hypothesis would also explain why bilayers containing PIP and PIP_2 fuse efficiently in response to Mg^{2+}. Not only are the phosphate groups in PPIs not obstructed by polar regions on their own head group, but by being extended away from the membrane by the inositol ring they are also less hindered by polar groups of surrounding phospholipids. As a consequence of this observed fusogenic potential of phosphorylated lipids, enzymatic control of their phosphorylation state with kinases, phosphatases, or lipases could prove to be an important mechanism for regulation of membrane fusion events.

Relationship to physiological conditions

The largest criticism of in vitro analysis of membrane fusion centers on previous observations that millimolar Ca^{2+} concentrations are necessary to initiate membrane amalgamation. Similarly, most liposomes do not fuse at concentrations of Mg^{2+} below 6 mM. Given these observations and the concentrations of Mg^{2+} found intracellularly (0.5–1 mM free ion concentration) (Ortiz et al., 1990), the susceptibility of vesicles containing PIP_2, PIP, or PA to Mg^{2+}-induced fusion is particularly intriguing. Moreover, since Ca^{2+} potentiates the fusogenicity of Mg^{2+}, small changes in intracellular Ca^{2+} concentrations could provide further regulation of fusion events.

A second argument could also be made against a role for PPIs in membrane fusion events based on the fact that the PPI content of the vesicles in this model system is much higher than would be found in endogenous membranes (~1%) (Gascard et al., 1993). However, the local concentration of PPIs in the region of an activated phosphoinositide kinase could be quite high. Moreover, other proteins could contribute to the confinement of these phospholipids to specific intracellular locations. Because only a 10-nm stretch of membrane is thought to be involved in most fusion events (Plattner and Knoll, 1993) a small increase in the local concentration of these lipids could have a significant effect on regulated intracellular fusion.

Implications

Several components are likely to be necessary for both induction and regulation of physiologically important membrane fusion events. The fusogenic nature of phosphory-

lated phospholipids makes them ideal candidates for regulated components in these processes. In particular, PPIs and PA can be rapidly produced enzymatically at the expense of less fusogenic phospholipids. Perhaps it is no coincidence that activation of such enzymes has been associated with fusion-susceptible membranes and membrane fusion events. For example, the products of PI-3-kinase have been implicated in trafficking glucose transporter vesicles to the plasma membrane (Kanai et al., 1993) and late Golgi-derived vesicles to lysozomelike vacuoles (Stack et al., 1993), and in early endosome fusion (Jones and Clague, 1995). The lipid products of this enzyme are insensitive to hydrolysis by PI-PLC (Serunian et al., 1989), suggesting that either the intact phospholipids or some metabolite other than inositol phosphates are important for these trafficking steps. Although the possibility is strong that PPIs regulate proteins such as ADP-ribosylation factor and synaptotagmin, which are components of putative fusion machines (Schiavo et al., 1995; Kahn et al., 1993), the studies presented in this manuscript suggest that these lipids could additionally be acting to produce an altered or activated membrane domain that is more susceptible to membrane fusion. Similarly, the phosphoinositide 4-kinase, which is found in glucose transporter vesicles (Del Vecchio and Pilch, 1991), mast cell granules (Kurosawa and Parker, 1986), chromaffin granules (Husebye and Flatmark, 1988), lysozomes (Collins and Wells, 1983) and the Golgi apparatus (Tooke et al., 1984) could also create fusogenic domains on the cytoplasmic surface of these fusion-susceptible membranes. Moreover, phospholipase D, an enzyme that creates PA from less fusogenic lipids, is also implicated in membrane trafficking (Kahn et al., 1993) and exocytotic (Lin et al., 1991) events. Finally, a PI/PC transfer protein that translocates PI to the cytosolic leaflet of the Golgi membrane at the expense of PC is essential for proper functioning of this organelle (Cleves et al., 1991).

The question remains whether the lipid bilayer phospholipids are passively or actively involved in regulation of membrane fusion events. These data suggest that regulation of the PPI content of membranes could have a significant effect on the induction of divalent cation-induced membrane fusion.

We would like to express our gratitude to Dr. Ronald Browning for the use of his spectrofluorometer, and to Dr. Lonnie Russell for his assistance with electron microscopy.

This work was supported by Grants-in-Aid for Research from the American Heart Association, Illinois Affiliate (M.S.), and from Sigma Xi, the Scientific Research Society (S.S.).

Mr. Summers is a recipient of a predoctoral student stipend from the American Heart Association, Illinois Affiliate.

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