Articles

Leaky Vesicle Fusion Induced by Phosphatidylinositol-Specific Phospholipase C: Observation of Mixing of Vesicular Inner Monolayers†

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ABSTRACT: Large unilamellar vesicles containing phosphatidylinositol (PI), neutral phospholipids, and cholesterol are induced to fuse by the catalytic activity of phosphatidylinositol-specific phospholipase C (PI-PLC). PI cleavage by PI-PLC is followed by vesicle aggregation, intervesicular lipid mixing, and mixing of vesicular aqueous contents. An average of 2−3 vesicles merge into a large one in the fusion process. Vesicle fusion is accompanied by leakage of vesicular contents. A novel method has been developed to monitor mixing of lipids located in the inner monolayers of the vesicles involved in fusion. Using this method, the mixing of inner monolayer lipids and that of vesicular aqueous contents are seen to occur simultaneously, thus giving rise to the fusion pore. Kinetic studies show, for fusing vesicles, second-order dependence of lipid mixing on diacylglycerol concentration in the bilayer. Varying proportions of PI in the liposomal formulation lead to different physical effects of PI-PLC. Specifically, 30−40 mol % PI lead to vesicle fusion, while with 5−10 mol % PI only hemifusion is detected, i.e., mixing of outer monolayer lipids without mixing of aqueous contents. However, when diacylglycerol is included in the bilayers containing 5 mol % PI, PI-PLC activity leads to complete fusion.

Membrane fusion is currently the object of active research, because of its implication in physiological processes (e.g., neurotransmission, fecundation) as well as in pathological events (e.g., cell infection by human immunodeficiency or influenza viruses). Moreover, other important cellular mechanisms (cell fission, budding, and certain aspects of transmembrane macromolecular transport) may share part of their molecular machinery with membrane fusion (*1*−*3*). An extensive series of studies have been carried out with the aim of unraveling the molecular mechanism of membrane fusion using model membranes, particularly liposomes. These semisynthetic lipidic vesicles mimic very well many of the properties of cell membranes, and they have been instrumental, in conjunction with fluorescence spectroscopic techniques, in deciphering many problems related to membrane fusion, mainly the role of lipid phase transitions and of structural intermediates in the process (*4*−*9*).

Some contributions from this laboratory have dealt with the system of liposome fusion induced by phosphatidylcholine-preferring phospholipase C (PC-PLC),[1] a protein of bacterial origin. This was the first example ever described of a model system in which fusion was induced through a catalytic agent (*10*). A review of our work in this field can be found in Goñi et al. (*11*). PC-PLC-induced liposomal fusion takes place through the experimentally dissectionable steps of phospholipid hydrolysis, with diacylglycerol (DAG) formation, vesicle aggregation, intervesicular lipid mixing, and mixing of aqueous contents. The process is nonleaky, presumably because the fusion products remain surrounded by a single membrane, the result of multiple fusion events (*12*). Diacylglycerol produced by PLC has a key role in facilitating vesicle-vesicle aggregation and lamellar-to-nonlamellar lipid phase transitions (*13*).

The present work describes a novel model system of membrane fusion, induced by the catalytic activity of phosphatidylinositol-specific phospholipase C (PI-PLC). This enzyme is quite unlike PC-PLC, particularly because its mechanism of action is different (*14*). A bacterial PI-PLC has been used in our study for reasons of convenience, but the molecule has mechanistic and structural similarities with the mammalian isozymes (*15*). The enzyme appears to act

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; BSA, bovine serum albumin; Ch, cholesterol; DAG, diacylglycerol; DOPC, dioleoylphosphatidylcholine; DPX, *p*-xylene-bis(pyridinium bromide); DTT, dithiothreitol; LUV, large unilamellar vesicles; NBD-PE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC-PLC, phosphatidylcholine-preferring phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; R_{18}, octadecylrhodamine B; Rh-PE, L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B-sulfonyl) (egg); SUV, small unilamellar vesicles.

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in the "scooting mode", one enzyme molecule binding to one vesicle and hydrolyzing all the accessible PI molecules (*16*). The presence of a negatively charged lipid, PI, in our bilayers leads to conditions and circumstances that were not observed in our other studies. PI-PLC-induced fusion is accompanied by extensive release of liposomal contents, in common with what appears to be the case at least in some viral fusion events (*17*). A new procedure for the assay of intervesicular mixing of inner monolayers has been developed in the course of this study and applied to our system. The ensemble of data constitute a detailed description of a model system of leaky fusion, including hitherto untreated aspects of the transition of hemifusion to fusion states by addition of DAG, as monitored by both mixing of vesicular contents and mixing of inner monolayer lipids.

MATERIALS AND METHODS

Materials. PI-PLC (EC 3.1.4.10) from *Bacillus cereus*, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS), *p*-xylenebis (pyridinium bromide) (DPX), and octadecylrhodamine B (R_{18}) were supplied by Molecular Probes Inc. (Eugene, OR). Dioleoylphosphatidylcholine (DOPC), [1,2 dimyristoyl-*sn*-glycero-3-phosphorylethanolamine-*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)] (NBD-PE), and [L-α-phosphatidylethanolamine-*N*-(lissamine-rhodamine-B-sulfonyl) (egg)] (Rh-PE), were purchased from Avanti Polar Lipids (Alabaster, AL). Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), phosphatidylinositol (PI), and 1,2-diacylglycerol (DAG) derived from egg PC were grade I from Lipid Products (South Nutfield, UK). Cholesterol (Ch), Triton X-100, bovine serum albumin (BSA) (essentially free from fatty acids), and DL-dithiothreitol (DTT) were from Sigma (St. Louis, MO). All other materials (salts and organic solvents) were of analytical grade or better.

Vesicle Preparation and Characterization. The appropriate lipids were mixed in organic solution, and the solvent was evaporated to dryness under N_2. Solvent traces were removed by evacuating the lipids for at least 2 h. The lipids were then swollen in 10 mM Hepes, 150 mM NaCl, pH 7.5 buffer. Large unilamellar vesicles (LUV) were prepared from the swollen lipids by extrusion and sized by using 0.1-μm pore-size Nuclepore filters, as described by Mayer et al. (*18*). The average size of LUV was measured by quasi-elastic light scattering, using a Malvern Zeta-sizer instrument. When required, small unilamellar vesicles (SUV) were prepared by sonication as described previously (*10*). Lipid concentration, determined by phosphate analysis (*19*), was 0.3 mM in all experiments.

Hydrolysis and Aggregation Assays. All assays were carried out at 39 °C with continuous stirring, in 10 mM Hepes, 150 mM NaCl buffer (pH 7.5), in the presence of 0.1% BSA for optimum catalytic activity. Enzyme concentration was 0.16 U/mL, and liposomal concentration was 0.3 mM. Enzyme activity was assayed by determination of water-soluble phosphorus contents in 50-μL aliquots removed from the reaction mixture at defined intervals. Extraction with 250 μL of chloroform/methanol/hydrochloric acid (200:100:3, by volume) stops the reaction. Two phases separate that can be assayed for phosphorus. Lipid aggregation was monitored in a Cary Varian UV-vesicle spectrometer as an increase in turbidity (absorbance at 450 nm) of the sample.

Lipid Mixing Assays. Two methods were followed to detect, respectively, total and inner monolayer lipid mixing. For total lipid mixing 0.6% NBD-PE, 0.6% Rh-PE vesicles were mixed with probe-free liposomes at a 1:4 ratio (*20*). NBD-PE emission was followed at 530 nm (excitation wavelength at 465 nm) with a cut-off filter at 515 nm. One hundred percent mixing was set after addition of 1 mM Triton X-100.

For the inner lipid mixing assay DTT and BSA (to a final concentration of 0.2% BSA and 10 mM DTT in the cuvette) were added to abolish the fluorescence from probes in the outer monolayer. Through the action of BSA, NBD-PE is extracted from liposomes by back-exchange (*21*), while DTT reduces BSA disulfide bonds and decreases its membrane perturbing capacity. As a result of this combination, fluorescence from the outer liposome monolayer is abolished while maintaining vesicle integrity. All fluorescence assays were performed in a Perkin-Elmer LS-50B luminescence spectrometer at 39 °C with continuous stirring.

Leakage and Fusion Assays. Intervesicular mixing of aqueous contents and vesicle contents leakage were assayed according to Ellens et al. (*22*), with the ANTS/DPX system. Liposomes contained either (i) 25 mM ANTS and 100 mM NaCl, (ii) 90 mM DPX and 60 mM NaCl, or (iii) 12.5 mM ANTS, 45 mM DPX, and 85 mM NaCl. A Sephadex G-75 (Pharmacia) chromatography column was used to separate liposomes from nonencapsulated probes, using 10 mM Hepes, 150 mM NaCl, pH 7.5 buffers as eluent. Osmolality was checked using an Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). Solutions were corrected for perfect isotonicity inside and outside the vesicles by adding small volumes of concentrated NaCl up to 0.310 (Osm/kg). The 100% and 0% contents mixing levels were set according to Nieva et al. (*10*, *23*). The 100% leakage signal was obtained by adding Triton X-100 to mark liposome lysis. Two correction factors were applied to contents mixing curves: light scattering by the fused vesicles and dissociation factor. Fusion scattering was monitored using a 1:1 mixture of vesicles containing 25 mM ANTS, 100 mM NaCl, and probe-free liposomes, at a 0.3 mM final lipid concentration. The dissociation factor intends to compensate for the leakage of aqueous contents that occurs shortly after the start of fusion. It is estimated by the sum of "F + 0.5D" (F: fusion fluorescence signal, D: dissociation signal from the preencapsulated probes), as described by Bentz et al. (*24*). Thus, "corrected contents mixing" corresponds to the amount of mixing that would be measured if leakage had not occurred. Latency periods, or "lag times", when occurring, were measured at the intersection of the curve maximum slope with the $x = 0$ line, as shown in ref *25*.

RESULTS

Vesicle Aggregation and Fusion. Preliminary experiments had indicated that PI/PE/PC/Ch (40:30:15:15, mole ratio) was a most effective composition for PI-PLC to show its fusogenic properties. Therefore large unilamellar vesicles (LUV) of that composition were used in all experiments, unless otherwise stated. A number of effects of PI-PLC on these vesicles can be seen in Figure 1. Panel A shows the parallel cleavage of PI and increase in turbidity. Over 80% of the PI present in the bilayers is hydrolyzed in ca. 10 min,

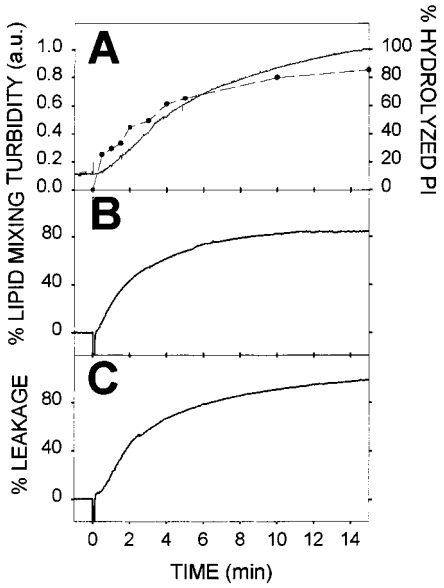

FIGURE 1: Kinetics of PI-PLC effects on large unilamellar vesicles consisting of PI/PE/PC/Ch (40:30:15:15). (A) Dots, PI hydrolysis; continuous line, increase in turbidity; (B) intervesicular lipid mixing; (C) leakage of vesicular aqueous contents. Lipid and enzyme concentrations are, respectively, 0.3 mM and 0.16 U/mL.

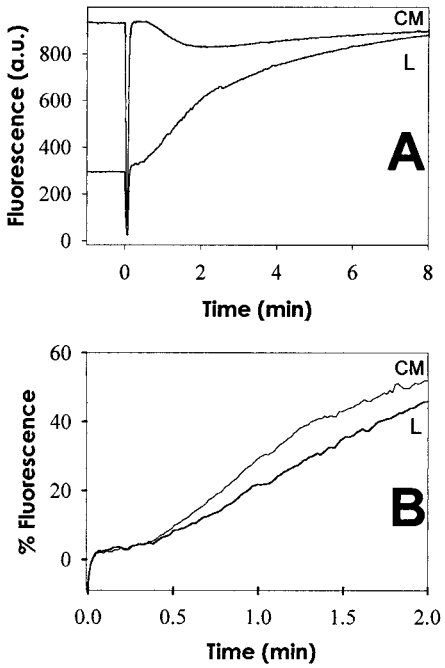

FIGURE 2: Kinetics of PI-PLC effects on large unilamellar vesicles consisting of PI/PE/PC/Ch (40:30:15:15). (A) Changes in ANTS fluorescence corresponding to vesicle contents mixing, uncorrected (CM) and leakage (L). (B) Vesicle contents mixing after correction for leakage and rescaling (CM), and vesicle leakage (L). The corrected contents mixing is the amount of mixing that would be observed in the absence of leakage.

and longer periods of time do not lead to further hydrolysis. Most of the PI hydrolysis, and concomitant increase in turbidity, occurs in the first 5 min after enzyme addition.

The increased turbidity is reflecting a phenomenon of vesicle aggregation (*25*). In Figure 1, panel A, the change in turbidity parallels phospholipid hydrolysis. Indeed, this is the rule when PC-PLC is involved (*26*, *27*), but with PI-PLC, after examining several dozens of binary and ternary lipid mixtures containing PI, increased turbidities were found to accompany enzyme activity only in very few cases (A. V. Villar, unpublished). This naturally low tendency to aggregation is most probably due to electrostatic repulsions of the negatively charged PI-containing bilayers. The obvious consequence is that, with most lipid compositions, PI-PLC cannot be used to induce fusion.

PI hydrolysis produces concomitant intervesicular lipid mixing (Figure 1, panel B) and leakage of liposomal aqueous contents (Figure 1, panel C). Heat-denatured PI-PLC did not elicit any of the effects seen in Figure 1, thus confirming the enzymic nature of the observed phenomena.

Almost simultaneously with lipid mixing, mixing of vesicular aqueous contents occurs, as shown in Figure 2. In Figure 2, panel A, the fluorescence signals corresponding to both contents mixing and leakage curves have been plotted. Leakage is certainly perturbing the contents mixing signal. The latter shows an initial decrease in ANTS fluorescence, as expected when it combines with DPX from a different liposome population, but then the slope sign changes and the fluorescence actually increases after ca. 2 min, due to the competing leakage effect. The signal for contents mixing can be corrected for leakage (*24*), and the result is shown in Figure 2, panel B, for the initial 2 min, when correction can be most accurately performed. The joint observation of lipid and contents mixing is a clear proof of vesicle fusion (*5*). Leakage coexisting with mixing of vesicular aqueous contents is a property of PI-PLC-induced fusion that did not occur in the PC-PLC-induced process (*10*).

As a complement to the time-resolved observations reported in Figures 1 and 2, equilibrium measurements were carried out of the average particle diameter before and after enzyme treatment, using quasi-elastic light scattering (QELS). LUV obtained by extrusion through 100-nm pore filters had an average diameter of 114.2 ± 1.3 nm (polydispersity 0.075 ± 0.051). After allowing the enzyme to act for 15 min, particle size had increased to 167.8 ± 1.4 nm average diameter (polydispersity 0.078 ± 0.071). The low polydispersity is an indication of very narrow distribution of particle sizes. The observed increase in average diameter would correspond to an average of 2.3 of the original vesicles fusing into a new one.

Mixing of Inner Monolayer Lipids. At least in principle a phenomenon may occur in which vesicles come into a kind of contact such that the lipid molecules in the outer monolayers of each vesicle intermix, without mixing of inner monolayer lipids, thus without formation of a fusion pore and without mixing of contents. This has been called "hemifusion" or "close apposition", and it has indeed been found in certain systems (*28*, *29*). In the present case, since complete fusion occurs, mixing of inner monolayer lipids is expected to accompany the mixing of vesicular aqueous contents.

To directly observe and measure the intervesicular mixing of inner monolayer lipids we have developed a simple, single-step procedure, based on the ability of bovine serum albumin (BSA) to bind and remove from the outer monolayer the fluorescent probes NBD-PE and Rh-PE. BSA at 0.1% (w/v) is routinely used in PI-PLC assays (*30*). At that concentration, BSA does not modify in any way the fluorescent probes in the vesicles. However, at 0.2% (w/v), very significant changes occur, with removal of virtually all

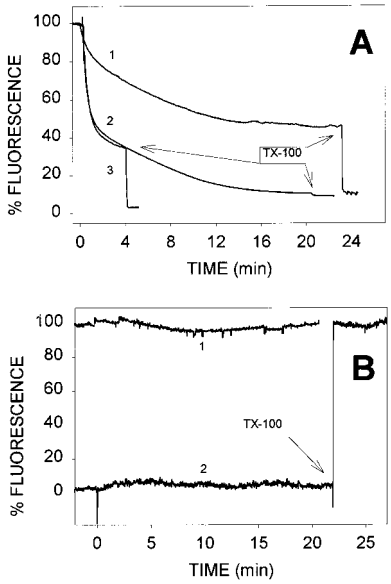

FIGURE 3: Quenching of fluorescence of NBD-PE and Rh-PE in the outer monolayer of LUV. (A) Curve 1: Time course of fluorescence after addition at time 0 of 0.2% BSA and 10 mM DTT. Curves 2 and 3: Time course of fluorescence after addition at time 0 of sodium dithionite, as indicated in ref *32*. (B) Intervesicular mixing of aqueous contents (curve 1) and leakage of vesicular aqueous contents (curve 2), during BSA + DTT treatment. TX-100: in each case the arrow indicates addition of Triton X-100, final concentration 1 mM.

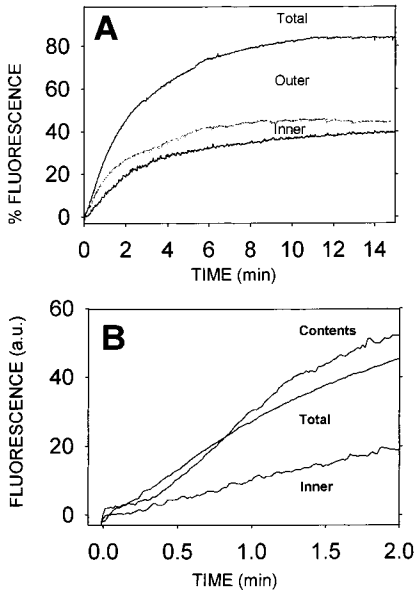

FIGURE 4: Different aspects of the PI-PLC effects on large unilamellar vesicles composed of PI/PE/PC/Ch (40:30:15:15). (A) A comparison of intervesicular lipid mixing corresponding to both monolayers (Total), inner and outer monolayers. Mixing of outer monolayer lipids is obtained as a difference between the experimentally measured Total − Inner curves. (B) The early stages of contents mixing, total and inner monolayer lipid mixing, superimposed to show the coincident lag time of inner monolayer lipid mixing and contents mixing. Note that the scales for contents and lipid mixing need not be the same under our conditions, so that direct comparison of ordinate values between different curves may not be possible.

the fluorescent molecules and leakage of contents (not shown). BSA-derived peptides have been previously shown to destabilize lipid bilayers (*31*). We have found that the combination of 0.2% BSA and 10 mM dithiothreitol (DTT) (the latter probably reducing some disulfide bridges in the BSA molecule) has milder effects than 0.2% BSA alone.

The BSA/DTT mixture quenches by about one-half the fluorescence of the vesicle suspension, without causing any leakage or mixing of vesicular contents (Figure 3). Fluorescence decreases gradually for about 15 min, and then it becomes stabilized at about 45% of the original value. Addition of Triton X-100 (1 mM) leads to immediate quenching of >90% of the original fluorescence (Figure 3, panel A). These data suggest the selective removal/quenching of the outer monolayer probes by the BSA/DTT. An alternative procedure for the removal of fluorescence from the outer lipid monolayer, based on fluorescent probe reduction by dithionite, has been proposed (*32*). Under our conditions, however, the dithionite procedure of McIntyre and Sleight (*32*) leads to a very extensive quenching (>80% of the original fluorescence after 15 min), presumably due to dithionite diffusion across the bilayer (Figure 3, panel A). In a comparative test of both procedures, carried out on DOPC SUV, i.e., the system used by McIntyre and Sleight (*32*), very similar results were obtained with both methods. After dithionite addition, a plateau in fluorescence intensity was reached after ca. 2.5 min, corresponding to 49% of the original value. On the same system, BSA/DTT caused a gradual decrease in fluorescence for about 6 min, after which an apparent equilibrium was attained, when fluorescence intensity was 53% of the initial value (plots not shown). Moreover, using yet another fusion model system, namely, fusion of PC/PE/Ch LUV induced by PC-preferring PLC (*10*), both the dithionite and the BSA/DTT methods gave virtually superimposable results in an assay of mixing of inner monolayer lipids (not shown). In view of these data, and of those shown in Figure 3, we consider that our procedure provides a reliable and, for PI-based liposomes, more accurate method for removing the fluorescence of NBD-PE and Rh-PE located in the outer monolayer of lipidic vesicles.

When vesicles treated with the BSA/DTT mixture for 15 min are incubated with PI-PLC, the enzyme activity is the same as recorded in 0.1% BSA alone (data not shown). The lipid mixing assay under the new conditions reveals specifically the mixing of the lipids in the inner monolayers. Figure 4, panel A, shows the mixing of total lipids and the mixing of inner monolayers, measured in separate experiments on the same LUV preparation, respectively, in the absence and presence of BSA/DTT. In the same panel, the mixing of outer monolayer lipids (dotted line) can be observed as the difference between the "total" minus "inner" curves.

As mentioned above, mixing of inner monolayer lipids is an essential prerequisite for mixing of aqueous contents to occur. The superimposed plots for total lipid mixing, inner lipid mixing, and contents mixing, at the early stages of the process, are shown in Figure 4, panel B. Inner lipid mixing is seen to start with a lag time of about 0.25 min. Exactly the same lag period is observed for the contents mixing curve, which demonstrates, as expected, that inner lipid mixing is required for aqueous contents mixing to be observed.

Lipid Concentration Dependence of Fusion. To get further insights into the mechanism of PI-PLC-dependent liposome fusion, vesicle aggregation and intervesicular lipid mixing were assayed at varying lipid concentrations, in the 30−600 μM range for total lipid. The observed rates of

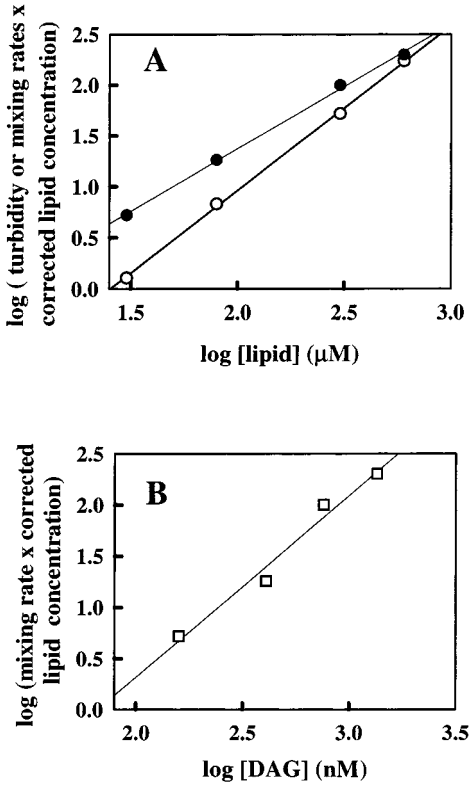

FIGURE 5: Influence of lipid concentration on the rate of vesicle aggregation and lipid mixing induced by PI-PLC. Rates are multiplied by the "corrected lipid concentration", i.e., relative to the lower tested (30 μM) lipid concentration (*33*). (A) Effect of varying the concentration of total lipid at a constant enzyme concentration of 0.16 U/mL. (○) Vesicle aggregation. The regression line has a slope of 1.8; the correlation coefficient is $r^2 = 0.999$. (●) Lipid mixing. Slope: 1.2; $r^2 = 0.998$. (B) Rates of lipid mixing plotted versus DAG concentrations. DAG concentrations are measured 1 min after the onset of lipid mixing. Slope: 1.8; $r^2 = 0.98$.

aggregation or lipid mixing were corrected for the lipid concentration relative to 30 μM and plotted in a double-logarithmic manner against the total lipid concentration, as described by Wilschut et al. (*33*). The resulting plots can be seen in Figure 5, panel A. The corresponding slopes indicate the reaction order with respect to lipid (or vesicle) concentration. Vesicle aggregation is expected to be second-order with respect to vesicle concentration, since it depends on vesicle−vesicle contacts. A slope of 1.8 is found experimentally, in agreement with the prediction (Figure 5, panel A, open circles). Lipid mixing between the initially separate vesicles would also be expected to be second-order with respect to total lipid (or vesicle) concentration; however, the experimental value found is 1.2 (Figure 5, panel A, filled circles), indicating that factors other than the frequency of vesicle−vesicle collisions would be the limiting factor in the rate of intervesicular mixing of lipids.

In our previous work with PC-PLC (*11*), we had proposed that the enzyme reaction product DAG would be instrumental in causing the structural changes leading to fusion. The validity of this hypothesis for PI-PLC-induced fusion was tested by plotting the rates of lipid mixing not against total lipid but against DAG concentration in the system. For this purpose, DAG concentrations were measured 1 min after the onset of lipid mixing. This time was selected arbitrarily, as a point at which measurements can be conveniently performed, while mixing rates are still at their maximum. The double-logarithmic plot of lipid mixing rates versus DAG concentration (Figure 5, panel B) has a slope of 1.8, thus supporting the idea that DAG concentration in the bilayers is the limiting factor for intervesicular lipid mixing under these conditions.

Lipid Composition Dependence of Fusion. The current ideas on the mechanism of vesicle−vesicle fusion involve the transient presence of a nonlamellar lipidic intermediate, the "stalk" (*7*, *9*). Formation of nonlamellar structures is quantitatively dependent on the proportion of lipids that induce negatively curved monolayers. Phospholipases C are believed to induce fusion by generating a typical negative curvature-inducing lipid, diacylglycerol (*34*, *35*), in this case from the lamellar-forming PI. On the basis of these ideas, a number of experiments have been designed in which LUV composition has been changed. The proportion of the DAG-generating PI has been varied from 40 to 5 mol %, while the mole ratio of the other three components has been kept at ≈2:1:1, PE/PC/Ch. Total lipid and enzyme concentrations were kept constant in all cases, at 0.3 mM and 0.16 U/mL, respectively. The results are summarized in Table 1. Lowering PI concentration leads naturally to a decrease in hydrolysis rate, and aggregation decreases almost in parallel, together with lipid mixing and leakage. Contents mixing, however, is abolished at PI concentrations below 30% mole ratio, even if under these conditions lipid mixing proceeds at measurable rates. This is an indication that "hemifusion", i.e., interbilayer lipid mixing without fusion pore formation, is taking place in these cases. As expected, no mixing of inner monolayer lipids could be detected in LUV's containing less than 30 mol % PI (data not shown).

Diacylglycerol and Fusion. As discussed in the introduction, DAG, the lipid end-product of PI-PLC, is a key element in phospholipase C-promoted fusion. In studies with PC-PLC (*23*), experiments in which DAG was included, at different proportions in the bilayer composition prior to enzyme addition, revealed two different roles for DAG: one as an inducer of nonlamellar phases and the other as the trigger of the fusion process, only the latter fraction of DAG having to be necessarily generated in situ by the enzyme. With the aim of improving our understanding of PI-PLC-induced fusion, a series of LUV preparations were performed, with the basic compositions containing either 40 or 5 mol % PI (as in Table 1) plus an additional 10% DAG in each case.

The presence of DAG in the liposome formulation had an important effect on the lag time of the intervesicular mixing of aqueous contents (Figure 6). When LUV composed of PI/PE/PC/Ch (40:30:15:15, mole ratio) were used, contents mixing occurred only after a lag period of 22 s. When additional 5 or 10 mol % DAG were present in the bilayer before enzyme addition, the lag times were reduced to, respectively, 13 and 0 s. The same phenomenon was observed with PC-PLC (*23*), and the explanation in the present case is probably the same as in the former one, namely, that a certain proportion of DAG (in our case higher than 5 mol %) is required for the nonlamellar fusion intermediate to be formed (*36*, *37*).

This is confirmed by directly measuring the proportion of DAG in the sample in Figure 6 precisely at the start of the contents mixing event. In all three cases, fusion starts when

Table 1: Effect of LUV Composition on the PI-PLC-Induced Vesicle Fusion[a]

lipid composition PI/PE/PC/Ch (mole ratios)	increase in size after 15 min (nm)[b]	hydrolysis rate (nmol of PI/min mL)	aggregation rate (min^{-1})	lipid mixing rate (min^{-1})	contents mixing rate (min^{-1})	contents leakage rate (min^{-1})
40:30:15:15	53 ± 1.4	5.5 ± 0.29	5.3 ± 0.14	10.0 ± 0.13	2.2 ± 0.09	2.2 ± 0.04
30:36:17:17	97 ± 3.5	1.4 ± 0.28	9.5 ± 0.13	9.9 ± 0.31	0.11 ± 0.10	2.1 ± 0.28
20:40:20:20	nd	0.96 ± 0.18	2.5 ± 0.30	1.8 ± 0.5	0.00 ± 0.01	2.0 ± 0.81
10:46:22:22	12 ± 4.0	0.24 ± 0.00	0.40 ± 0.05	1.7 ± 0.40	0.00 ± 0.01	0.90 ± 0.03
5:47:24:24	nd	0.12 ± 0.00	0.21 ± 0.02	0.56 ± 0.04	0.00 ± 0.01	0.70 ± 0.10

[a] Rates given are maximum rates, they may not be the initial rates if a lag period occurs. Contents mixing rates have been corrected for leakage. Total lipid concentration was 0.3 mM. Average values ± SEM ($n = 3$). [b] Initial vesicle diameter was 114.2 ± 1.3 nm.

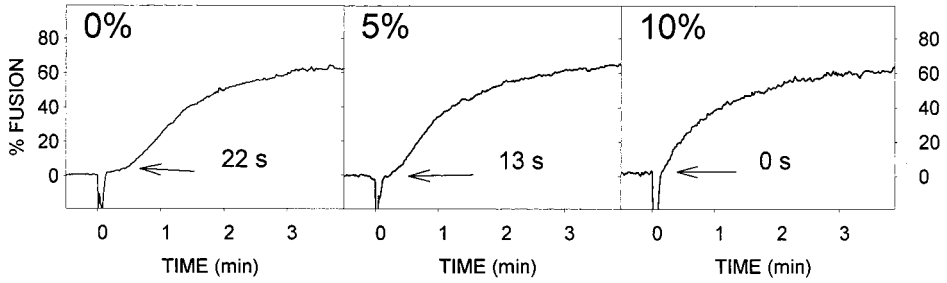

FIGURE 6: Effect of adding diacylglycerol on the kinetics of contents mixing in LUV composed of PI/PE/PC/Ch (40:30:15:15). (A) Control; (B) + 5 mol % DAG in the liposomal composition; (C) + 10 mol % DAG. The figures by the arrow indicate the lag time of aqueous contents mixing, in seconds, for each case.

Table 2: Effect of Diacylglycerol in LUV Composition on the PI-PLC-Induced Vesicle Lipid Mixing and Contents Mixing[a]

lipid composition PI/PE/PC/Ch (mole ratio)	extent of total lipid mixing (%)[b]	extent of inner monolayer lipid mixing (%)[b]	ratio internal/total lipid mixing	contents mixing[b]
5:47:24:24	20 ± 2	4 ± 2	0.20	0 ± 2
id + 10% DAG	41 ± 8	19 ± 4	0.46	27 ± 5
40:30:15:15	83 ± 9	37 ± 5	0.44	76 ± 1
id + 10% DAG	27 ± 3	13 ± 4	0.48	74 ± 0

[a] Average values ± SEM ($n = 2-3$). [b] Values observed at equilibrium, 15 min after enzyme addition.

DAG occurs at 10 ± 1 mol % in the bilayer. This would suggest that the mixture containing originally 40 mol % PI requires about 10 mol % DAG to undergo a localized lamellar to nonlamellar transition at one or more points in the bilayer, with the ensuing formation of structural fusion intermediates. (It should be stressed that even the highly fusogenic LUV containing 10% DAG were stable for days at 4 °C in the absence of enzyme).

In connection with the results in Table 1, it was shown above that the mixture containing 5% PI leads, upon treatment with PI-PLC, to a phenomenon of hemifusion, in which only lipids in the outer monolayer intermix. Table 2 shows effectively that, under those conditions, both contents mixing and mixing of inner monolayer lipids, as measured with the BSA/DTT method, are very low. However the presence of 10% DAG in the original composition leads to a different outcome of the experiment, with significant amounts of contents and inner lipid mixing. Table 2 summarizes as well studies performed with LUV containing 40% PI. The presence of additional 10% DAG in bilayers containing 40% PI decreases the extent of lipid mixing, but inner lipid mixing decreases more or less in parallel, so that the ratio inner/total lipid mixing remains at near 0.5. Under these conditions, a fusion pore is being formed, the inner vesicle compartments are connected, and mixing of contents ensues. For the mixture containing 5% PI, however, inner lipid mixing is negligible, and the inner/total lipid mixing ratio is well below 0.5. Correspondingly, no mixing of aqueous contents is detected. A lipid mixing ratio near 0.5 is restored by adding 10% DAG to the original bilayer composition, and consequently contents mixing is achieved under the latter conditions. Thus the presence of DAG at a certain proportion in the membrane may turn a process of hemifusion into one of complete fusion.

DISCUSSION

PI-PLC fusion of pure lipid vesicles, as characterized in Results, displays features, namely, its leaky character and the generation of hemifusion states, that have not been found or described in detail in other enzymatically driven fusion processes. In addition, the present system provides an opportunity to examine more rigorously the role of diacylglycerol in PLC-induced fusion.

A Leaky Process. Part of the aqueous contents in the LUV leak out of the vesicles as a consequence of the enzyme activity (Figure 1). Leakage occurs concomitantly with mixing of aqueous contents (Figure 2), but it is observed as well under circumstances in which no contents mixing takes place, e.g., in compositions with 5 or 10% PI (Table 1). The mechanism of leakage appears thus to be not identical to that of fusion pore formation, although release of vesicular contents should also be due to a destabilizing effect of DAG on the bilayer (*13*, *38*). Enzymatic production of ceramide by sphingomyelinase in sphingomyelin bilayers also induces extensive leakage (*39*). The reason PC-PLC-induced fusion is nonleaky is probably the formation of large aggregates surrounded by a single membrane, the result of multiple fusion events, as discussed previously (*12*). PI-containing vesicles are not prone to aggregation due to electrostatic

repulsion, fusion products are much smaller (the result of 2−3 vesicles coming together), and vesicle contents may leak through membranes once a certain proportion of DAG is formed. It should be noted in this respect that some cell-virus fusion events may also be accompanied by leakage, as suggested by Shangguan et al. (*17*).

Hemifusion. While the joint observation of lipid and contents mixing is a clear proof of vesicle fusion, lipid mixing in the absence of contents mixing is a sign of what is called "hemifusion" or "close apposition" (*28*, *29*). In the latter case only the lipids in the outer monolayers intermix. The possibility of assaying mixing of inner monolayer lipids together with mixing of aqueous contents (Figures 3 and 4) allows a clear distinction between hemifusion and true fusion. Tables 1 and 2 show examples of each of these two phenomena, respectively for LUV containing 5, 10, or 20% PI in the absence of additional DAG (hemifusion) or LUV containing 40 or 30% PI or just 5% but in the presence of 10% added DAG (true fusion). In the absence of added DAG, the slow enzymic generation of this lipid is enough to produce some degree of vesicle aggregation and lipid mixing, the latter involving mostly the outer monolayer. However, the evolution of the "diafragms" or "*trans*-monolayer contacts" (*6*, *35*), typical of hemifusion, to fusion pores occurs only in the presence of higher proportions of DAG. DAG concentrations in the early stages of the process appear to be crucial in determining the fate, hemifusion or fusion, of the aggregated vesicles (Figures 5 and 6). To the authors' knowledge, this is the first report of stable hemifusion structures resulting from liposome−liposome interaction.

Roles of Diacylglycerol. We have proposed that DAG plays a dual role in phospholipase C-promoted fusion: bulk DAG, that can be premixed with the other lipids in the process of liposome preparation, without leading to vesicle destabilization (*23*), allows the formation of nonlamellar lipidic structures, while enzyme-generated, localized DAG-rich domains provide the initial destabilization (*13*). This is confirmed by several observations in this paper. (i) In particular, the reduction in fusion lag-time upon inclusion of DAG in the liposome composition (Figure 6) and the transition from hemifusion to fusion of the 5% mixture upon addition of DAG (Table 2) speak clearly of the requirement of a given proportion of DAG in the mixture for the fusion pore to form. [Unfortunately, no data are available on the phase behavior of mixtures containing PI as used in this work, so that our reasoning is based on the published phase diagrams for PC-based systems (*36*)]. (ii) The kinetic studies presented in this paper (Figure 5) also support the role of DAG as a bilayer destabilizer and/or fusogenic lipid. (iii) Moreover, the fact that our vesicles, including those based on the highly fusogenic mixture containing 10% DAG (Figure 6), are stable for days at 4 °C unless PI-PLC is added supports the previously published (*11*) idea that the rapid, localized, asymmetric generation of a small amount of DAG by the enzyme is an essential trigger for the whole process to develop.

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