Phospholipase C-Promoted Membrane Fusion. Retroinhibition by the End-Product Diacylglycerol†

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ABSTRACT: The catalytic activity of phospholipase C induces fusion of pure lipid vesicles. When large unilamellar liposomes composed of phosphatidylcholine/phosphatidylethanolamine/cholesterol (2:1:1 mole ratio) are treated with phospholipase C, in the presence of 10 mM Ca^{2+}, two enzyme effects can be distinguished: a fast one (half-time on the order of seconds) consisting mainly of vesicle–vesicle fusion and a slow one (half-time on the order of minutes) representing bulk lipid hydrolysis. The fast fusion process is inhibited by the end-product diacylglycerol, as well as by lysophosphatidylcholine and by low Ca^{2+} concentrations. The temperature dependence of enzyme activity (phospholipid hydrolysis), vesicle aggregation, and vesicle fusion (mixing of aqueous contents) has been separately studied. Enzyme activity and vesicle aggregation rates increase monotonically with temperature, while an optimum temperature is found for vesicle fusion, depending on liposome composition and assay conditions. The presence of diacylglycerol incorporated to the membrane (up to 10 mol %) does not produce any fusion effect even at temperatures as high as 80 °C: in situ diacylglycerol production by the enzyme appears to be required. The data are interpreted in support of a hypothesis according to which a "fusion intermediate" would be required, depending (among others) on bilayer composition, temperature, and Ca^{2+} concentration, for vesicle fusion to occur.

The crucial role of phospholipid metabolism in cell activation has been clearly established (Pelech & Vance, 1989). In particular, secretory processes are known to occur as a result of phospholipid cleavage and diacylglycerol (DG)[1] production in the membrane. Direct evidence has been produced coupling production of diacylglycerols (DG) arising from PC, via a specific phospholipase C, to exocytosis in neutrophils (Haines et al., 1991); also, DG accumulation during the fusion of rough endoplasmic reticulum membranes has been reported (Lavoie et al., 1991). Moreover, studies using model membranes have shown that DG are potent modulators of membrane structure (Das & Rand, 1986).

By combining fluorescence spectroscopy and liposome technology, Nir et al. (1980) and Wilschut et al. (1980, 1985) described the fusion reaction of vesicles composed of negatively charged lipids in the presence of divalent cations, and, after modeling the process, were able to distinguish two steps with characteristic rate constants: membrane aggregation or apposition, and fusion or destabilization of the membranes in apposition, the latter including mixing of aqueous vesicle contents (Nir et al., 1983; Bentz et al., 1983). A different situation was encountered in studies with liposomes containing lipids such as PE, that exhibit thermotropic transitions to nonlamellar phases (Gagné et al., 1985; Ellens et al., 1986). In these cases, fusion appears to occur preferentially near or above the hexagonal transition temperature; it has been suggested that under those conditions nonlamellar intermediates would be formed, which in turn would allow the mixing of aqueous contents (Siegel et al., 1989; Burger & Verkleij, 1990). When this is the case, the aggregation and fusion processes cannot be separated, since the conditions allowing aggregation also produce the mixing of lipids and or vesicle contents (Allen et al., 1990).

In a previous paper (Nieva et al., 1989), it was demonstrated that the catalytic activity of phospholipase C from *Bacillus cereus* was able to induce aggregation, and then fusion, of liposomes. The enzyme source was chosen in view of its availability, and also because of the immunological and functional similarities between the *B. cereus* and mammalian phospholipase C (Clark et al., 1986; Larrodera et al., 1990; García de Herreros et al., 1991). Further work (Burger et al., 1991) has provided ultrastructural evidence for large vesicle aggregates induced by phospholipase C activity: the aggregates appear surrounded by a continuous membrane, presumably the result of many fusion events, and the constituent vesicles appear less structured the nearer the aggregate core. In the present study, the existence of two enzyme-dependent membrane destabilization processes is shown, one of which occurs at low DG concentrations in the membrane, and is saturated and inhibited by high levels of DG. It is suggested that this process is responsible for vesicle fusion, and may be modulated by intermediates of a defined nonlamellar structure.

MATERIALS AND METHODS

Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was supplied by Boehringer-Mannheim. Egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were from Lipid Products (South Nutfield, England); cholesterol (CHOL) was from Sigma (St. Louis, MO). Diacylglycerol (DG), obtained by phospholipase C hydrolysis of egg PC, was purchased from Lipid Products. 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS) and p-xylylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Eugene, OR).

Substrate Preparation. Large unilamellar vesicles (LUV) prepared by the extrusion method of Mayer et al. (1986) were used as the phospholipase C substrate. The lipid composition of these liposomes was PC/PE/CHOL (2:1:1 mole ratio).

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[1] Abbreviations: CHOL, cholesterol; DG, diacylglycerol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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When required, 5% or 10% DG was included in the original formulation, to give PC/PE/CHOL/DG (47:23:25:5) or PC/PE/CHOL/DG (43:22:25:10), respectively; note that, in the latter formulations, phospholipid but not cholesterol was substituted by DG. The aqueous lipid suspensions were extruded through Nuclepore filters, 0.1-μm pore diameter. Freeze-fractured electron microscopic measurements indicated an apparent average vesicle diameter of 114 nm.

Fusion Assays. Mixing of aqueous vesicle contents, as well as vesicle leakage, was estimated using the ANTS/DPX fluorescent probe system described by Ellens et al. (1985). Three liposome preparations were used, loaded with (a) 50 mM ANTS, 90 mM NaCl, 10 mM $CaCl_2$, and 10 mM HEPES, pH 7.0; (b) 180 mM DPX, 10 mM $CaCl_2$, and 10 mM HEPES, pH 7.0; or (c) 25 mM ANTS, 90 mM DPX, 45 mM NaCl, 10 mM $CaCl_2$, and 10 mM HEPES, pH 7.0. When appropriate, 2 mM $CaCl_2$ instead of 10 mM $CaCl_2$ was used. Nonencapsulated material was removed from the vesicles using a Sephadex G-75 column, with 10 mM HEPES, 200 mM NaCl, and 10 mM $CaCl_2$ (or 2 mM $CaCl_2$), pH 7.0, as the elution buffer. This buffer was also used in all the fusion and enzyme assays. The osmolalities of all solutions were measured in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany) and adjusted to 0.4 osm/kg by adding NaCl. Lipid concentration in the assays was usually 0.3 mM; the process was started by adding 1.6 units/mL enzyme, corresponding approximately to the optimum figure of 10 enzyme molecules/vesicle (Nieva et al., 1989). Both phospholipids used in this study, PC and PE, showed equal susceptibility to phospholipase action when present in the same vesicle preparation. When the enzyme was preincubated with 50 mM *o*-phenanthroline (a specific inhibitor of phospholipase C; Little & Otnaess, 1975), neither phospholipid hydrolysis nor liposome fusion was observed.

Fluorescence scales were calibrated for fusion and release assays as described previously (Nieva et al., 1989). Briefly, the 100% fluorescence level (or 0% fusion) was set by using a 1:1 mixture of ANTS and DPX liposomes. The fluorescence level corresponding to 100% mixing of contents was determined from 0.3 mM liposomes containing coencapsulated ANTS and DPX; the value so obtained corresponds to either 100% fusion or 0% leakage. The 100% fluorescence level for leakage was obtained by detergent lysis of the liposomes containing both ANTS and DPX. Corrections for differences in the amount of entrapped solutes in the various vesicle preparations were routinely carried out after measuring the ratio of ANTS fluorescence before and after the addition of excess detergent (5 mM Triton X-100). The fluorescence change of a preparation containing 0.15 mM ANTS liposomes plus 0.15 mM "empty" liposomes (i.e., buffer-loaded) was routinely subtracted from the ANTS/DPX fluorescence signal, in order to account for scattering and other possible artifacts. Since the aggregates, under our measuring conditions, may involve a large number of vesicles, fusion rates and maximal fusion values were directly estimated from the degree of ANTS quenching at the required time point. No further corrections were made over those values. The lag times were calculated on the time-course curves as the time for the maximum slope line to intersect with the 0% effect level base line. Assays were performed in thermostated cuvettes with constant stirring, in an LS-50 Perkin Elmer spectrofluorometer. Excitation light was adjusted at 355 nm, and emission at 520 nm. An interference filter (450 nm) was used to avoid scattered excitation light. Liposome aggregation was estimated semi-

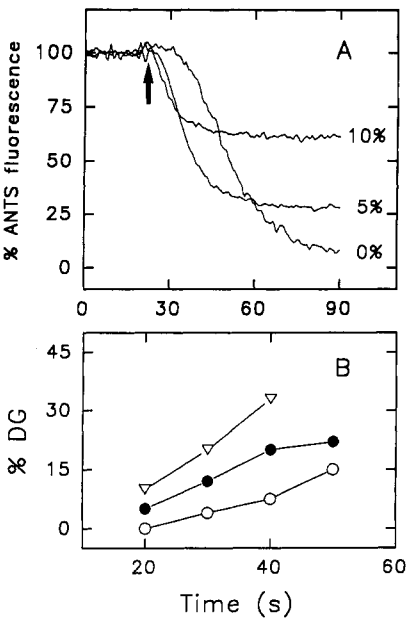

FIGURE 1: Time course of phospholipase C-induced fusion and concomitant DG production. Phospholipase C (1.6 units mL^{-1}) was added at t = 20 s (arrow); lipid concentration was 0.3 mM; temperature was 37 °C; Ca^{2+} concentration was 10 mM. (A) Traces corresponding to the mixing of contents as detected from the quenching of the fluorescent probe ANTS by DPX. Liposome formulation (mole ratio) was 0% (PC/PE/CHOL, 50:25:25), 5% (PC/PE/CHOL/DG, 47:23:25:5), or 10% (PC/PE/CHOL/DG, 43:22:25:10). (B) DG contents expressed in mole percent: (○) initial 0%; (●) initial 5%; (▽) initial 10%.

quantitatively as an increase in scattered light, by fixing the excitation and emission monochromators at 520 nm.

Enzyme Assays. Phospholipase C activity was assayed by determining phosphorus contents in the aqueous phase of an extraction mixture (chloroform/methanol 2:1) after addition of aliquots from the reaction mixture at different times. Occasionally, simultaneous measurements of phosphate release and diacylglycerols present in enzyme-treated liposomes were carried out, always with good correlation. In these cases, the enzyme activity was stopped at the appropriate times by increasing the pH to ca. 10; enzyme-treated vesicles were then collected by centrifugation, and diacylglycerols were quantitated, using the radioenzymatic assay for diacylglycerol kinase (Amersham) essentially following the method of Preiss et al. (1986). Phosphorus contents of the samples were determined by the method of Bartlett (1959). Protein concentration was assayed according to Lowry et al. (1951).

RESULTS

Phospholipase C from *Bacillus cereus* is highly effective in promoting the fusion of large unilamellar liposomes composed of PC/PE/CHOL (2:1:1), in the presence of 10 mM Ca^{2+}. As shown in Figure 1, the fusion process may be modified by substituting for DG some of the phospholipids in the initial liposome formulation. At 37 °C, when no DG is present in the substrate, fusion starts about 10 s after enzyme addition, when the amount of generated DG is about 4% of the total lipid. The reaction proceeds for another 20 s (up to ≈15% DG), before end-product inhibition becomes apparent; still, although at declining rates, the process continues until virtually 100% mixing occurs, without any significant vesicle leakage. When the initial liposome composition is modified, so that 5% of the phospholipids is substituted by DG, the fusion process becomes faster. The lag time after enzyme addition is shorter: 10 s after the addition, the total DG content is 12%,

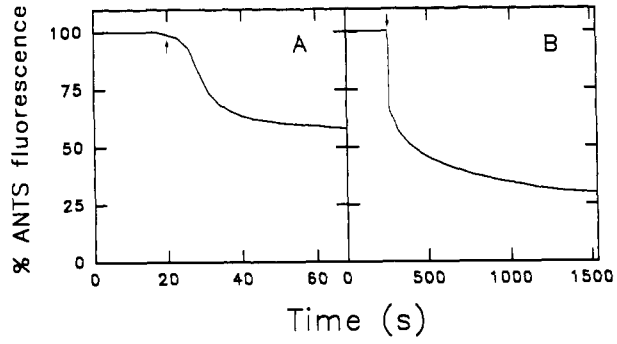

FIGURE 2: Short- and long-time kinetics of liposome fusion. Phospholipase C was added at the time indicated by the arrow; fusion was assayed as mixing of aqueous contents. Liposome formulation (mole ratio) was PC/PE/CHOL/DG (43:22:25:10). (A) Short-time kinetics. (B) Long-time kinetics.

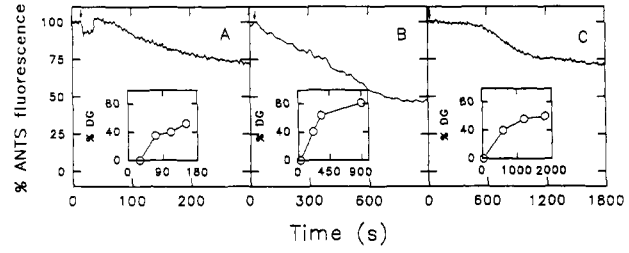

FIGURE 3: Inhibition of the fast component of phospholipase C-induced vesicle fusion. Conditions as in Figure 1, except (A) liposomes composed initially of PC/PE/CHOL/lyso-PC (43:22:25:10); (B) liposomes composed of PC/PE/CHOL (2:1:1) but 2 mM Ca^{2+}; or (C) liposomes composed of pure PC + 16 units mL^{-1} phospholipase C. Inserts: DG contents, expressed as mole percent. Enzyme addition is indicated by an arrow.

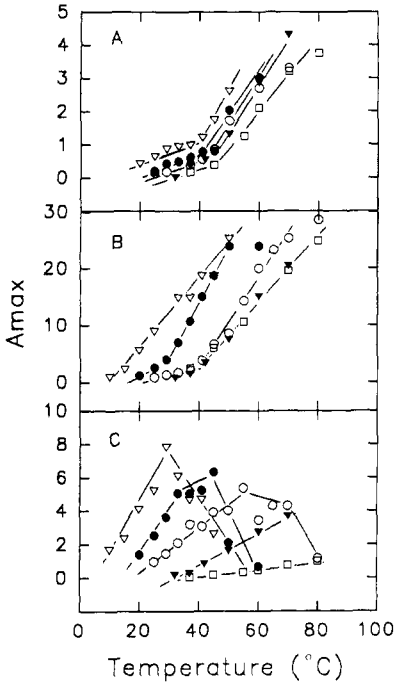

FIGURE 4: Maximum activities (rates) measured after addition of phospholipase C, as a function of temperature. (A) Maximum rates of phospholipase C activity, as percent total lipid hydrolysis per second. (B) Maximum rates of vesicle aggregation, as percent total change in light scattering per second. (C) Maximum rates of vesicle fusion (mixing of aqueous contents), as percent total change in fluorescence intensity per second. Liposome formulation (mole ratio): (○) PC/PE/CHOL (2:1:1); (●) PC/PE/CHOL/DG (47:23:25:5); (▽) PC/PE/CHOL/DG (43:22:25:10); (▼) PC/PE/CHOL/lyso-PC (43:22:25:10); (□) PC/PE/CHOL (2:1:1) but 2 mM Ca^{2+}.

and the reaction rate is already maximal. Saturation occurs at ≈20 s after enzyme addition, when DG makes up to 20% of the total lipids. In this case, vesicle contents are partially released, in a process that occurs simultaneously with contents mixing (data not shown). When the substrate liposomes contain 10% of the phospholipids substituted by DG, fusion starts as soon as phospholipase C is added, and the process reaches a plateau after 10 s, when the total amount of DG is ≈20%. These results appear to indicate that, at 37 °C, the enzyme catalyzes efficiently the fusion of liposomes containing PC/PE/CHOL (2:1:1) plus DG when the proportion of the latter is in the range 5–20% of the total lipid. At higher DG proportions, fusion becomes inhibited by this end-product. Leakage in liposomes originally containing 5% DG indicates that the membrane destabilization event that is at the origin of vesicle fusion may also lead to release of contents when it occurs in the periphery of an aggregate. Leakage is only detected with 5% DG in the original liposome formulation, probably because this represents a compromise situation: in the absence of DG, the process is too slow to produce significant spillage of contents [see Burger et al. (1991)], and in the presence of 10% DG, the extent of fusion is too small.

End-product inhibition is not complete; at least in samples containing initially 10% DG, a nonleaky process of mixing of vesicle contents proceeds slowly, eventually reaching saturation at a much later stage (Figure 2). Thus, two superimposed kinetics appear to exist: a fast one ($t_{1/2} \approx 10$ s) that reaches a plateau when the system contains ≈20% DG and a slow one ($t_{1/2} \approx 3.2$ min).

The fast process is abolished when lyso-PC is incorporated into the liposomes at a 10% mole ratio (Figure 3A) or when Ca^{2+} concentration is reduced from 10 to 2 mM (Figure 3B) though the hydrolytic activity of the enzyme remains unchanged. In these samples, mixing of aqueous contents occurs only at DG concentrations higher than 30%. Fast fusion is also absent in pure PC liposomes, where a slow process of contents mixing occurs in the 40–60% range of DG concentrations, until saturation takes place (Figure 3C).

A detailed analysis considering the various liposome preparations as a function of temperature shows that fusion varies with temperature in a different way than enzyme activity, or vesicle aggregation (Figure 4). A good correlation is observed, at all temperatures, between aggregation and enzyme activity for the liposomes not originally containing DG; both phenomena appear to be activated at ≈45 °C. For DG-containing liposomes, the enzyme activity appears to be higher at all temperatures, but the temperature dependence is otherwise similar to the other substrates. However, aggregation is much more efficient in the presence of DG than in its absence, particularly at low temperatures, suggesting that DG confers the vesicles some degree of adherence.

The temperature dependence of vesicle fusion (as mixing of contents) shows maxima whose absolute values are higher, and occur at lower temperatures, the higher the initial DG contents. The presence of lyso-PC, or low Ca^{2+} concentrations, specifically inhibits this process. The increased fusion in the DG-containing samples could be due to increased vesicle adherence. In contrast with aggregation, fusion does not increase monotonically with temperature, and each liposome composition exhibits a peculiar pattern as a function of temperature. These data suggest a certain independence between the fusion and aggregation phenomena.

The independent behavior of those two processes is also confirmed by the fact that the lapse of time required to reach a maximum of fusogenic activity is always longer than (or, at high temperatures, equal to) the corresponding time for

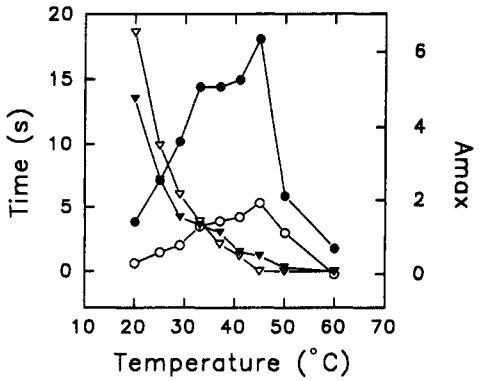

FIGURE 5: Vesicle fusion and vesicle leakage: a comparison of maximum rates and lag times. Liposome composition was PC/PE/CHOL/DG (47:23:25:5). Maximum rates are expressed as in Figure 4; lag times are measured with respect to vesicle aggregation. (●) Maximum fusion rates; (○) maximum leakage rates; (▼) lag time for fusion; (▽) lag time for leakage.

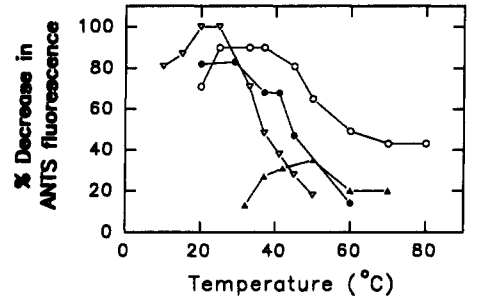

FIGURE 6: Maximum fusion values plotted as a function of temperature. Values were measured from experiments as shown in Figure 1, once apparent steady-state conditions were reached after 5 min. Liposome formulation: (○) PC/PE/CHOL (2:1:1); (▽) PC/PE/CHOL/DG (43:22:25:10); (▲) PC/PE/CHOL/lyso-PC (43:22:25:10); (●) PC/PE/CHOL/DG (47:23:25:5).

attaining the maximum aggregation rate (data not shown). In other words, under most conditions, there is a lag time between aggregation and fusion. In addition, the lag time is dependent on temperature and liposome formulation. It should be noted that, in samples originally containing DG, the lag between the two phenomena is sufficient to ensure that fusion is not limited by aggregation (e.g., 15 s for samples containing 10% DG at 20 °C).

The small amount of vesicle leakage that has been observed with some liposome formulations has also been studied as a function of temperature, for liposomes initially containing 5% DG. The data are shown in Figure 5, together with those on vesicle fusion (the latter replotted from Figure 4). Both fusion and leakage display parallel patterns as a function of temperature, suggesting that both processes are closely related and may share a common mechanism.

When the maximum fusion values (extent of fusion after 5 min) are also plotted as a function of temperature (Figure 6), it is seen that they are shifted to lower T values when DG is originally included in the liposome formulation. Again, lyso-PC is seen to inhibit the fusion process.

DISCUSSION

Phospholipase C-induced liposome fusion may be considered to consist of three elementary processes: DG generation in the bilayer, vesicle aggregation, and fusion of apposed membranes. In view of the high degree of coupling shown by the phospholipase reaction and the subsequent processes under our experimental conditions, the "mass action" kinetic model developed by Nir et al. (1983) and Bentz et al. (1983) for Ca^{2+}-induced fusion of negatively-charged liposomes has to be extended for our case. The slow enzymic reaction is the rate-limiting step in the whole process, and, consequently, we cannot dissect the fusion reaction into two discrete consecutive steps, as in the case of the reacting vesicles with high affinity for cations. Instead, we can qualitatively approach the mechanism of the fusion process; our studies as a function of temperature reveal that the hydrolase, aggregating, and fusogenic properties of phospholipase C should be considered separately.

According to our results, the enzyme activity, and not the mere presence of DG in the bilayer, is responsible for the fusion process. Liposomes containing 10% DG were stable even at 80 °C in the absence of the enzyme. This may suggest that the localized action of the enzyme on the outer monolayer is crucial for the initial aggregation steps; even allowing for a transbilayer movement of DG, the asymmetric enzyme activity will tend to keep a steady-state gradient of DG concentration between the monolayers. Das and Rand (1986) demonstrated that the interbilayer equilibrium was not modified in the presence of added DG, which correlates well with the lack of effect observed in the absence of enzyme. We have also shown that the fusion process can be decomposed into two components; one is fast and very effective, and occurs when the DG concentration in the bilayer is between 5 and 20%. This fast component becomes saturated at higher DG concentrations, and is also inhibited by temperature, by lyso-PC, and by low Ca^{2+} concentrations. The fast fusion component is occasionally accompanied by the release of vesicle contents. The second component is much slower, and is easily detected in samples in which the fast process is either saturated or inhibited; it proceeds even with high DG concentrations and could reflect the gradual loss of lamellar structure within the aggregates.

The temperature dependence of the fast fusion process is different from that of aggregation or enzyme activity (Figure 4). At low temperatures, fusion proceeds at a slow rate, but it is very efficient (plateau values near 100%); when temperature is increased, the rate increases, but the saturation point decreases. Finally, at the highest temperatures tested, both values are decreased. This inhibitory effect, at temperatures at which both enzyme activity and vesicle aggregation are enhanced, may reflect the existence of a structural intermediate whose formation would be influenced both by temperature and by DG concentration. The fact that lyso-PC also inhibits the fast fusion process suggests that the intermediate may have a nonlamellar structure (Epand, 1985), as in the case of temperature-dependent fusion in systems containing methyl-DOPE (Ellens et al., 1986). The existence of such an intermediate would be in accordance with the destabilizing properties of DG, described for a variety of systems (Das & Rand, 1986; Siegel et al., 1989a), and with the rich thermotropic polymorphism exhibited by PC/PE/CHOL mixtures (Cullis & de Kruijff, 1978; Tilcock et al., 1982). Our recent ultrastructural data obtained with this system (Burger et al., 1991) also support the idea of a nonlamellar intermediate in the fusion process. The effect of Ca^{2+} as a specific modulator of the fusion process is difficult to explain at the moment, and deserves further experimentation. Our results also open the way for independently exploring vesicle aggregation and fusion as a result of phospholipase C activity.

In summary, phospholipase C acts both as an aggregating and as a fusogenic agent in the system, but the mechanism is different from the cases in which fusion occurs as a necessary

consequence of aggregation (Allen et al., 1990). There is no aggregation unless DG is generated by the enzyme, and there is no fusion unless the required proportion of DG exists in the bilayer; thus, the enzyme activity is necessary for both processes. Our results show for the first time that DG, described so far in different systems as a potent fusion promoter, could have the opposite effect and inhibit fusion when present at high proportions in the membrane. The model system described in this work mimics the physiological process of membrane fusion in a closer way than other liposome-based systems: the enzyme activity is responsible for both vesicle aggregation and bilayer fusion; in addition, fusion is specifically inhibited by the end-product of the enzyme reaction, which suggests a possibility of controlling the process under physiological conditions.

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