Morphological Changes Induced by Phospholipase C and by Sphingomyelinase on Large Unilamellar Vesicles: A Cryo-Transmission Electron Microscopy Study of Liposome Fusion

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ABSTRACT Cryo-transmission electron microscopy has been applied to the study of the changes induced by phospholipase C on large unilamellar vesicles containing phosphatidylcholine, as well as to the action of sphingomyelinase on vesicles containing sphingomyelin. In both cases vesicle aggregation occurs as the earliest detectable phenomenon; later, each system behaves differently. Phospholipase C induces vesicle fusion through an intermediate consisting of aggregated and closely packed vesicles (the "honeycomb structure") that finally transforms into large spherical vesicles. The same honeycomb structure is also observed in the absence of enzyme when diacylglycerols are mixed with the other lipids in organic solution, before hydration. In this case the sample then evolves toward a cubic phase. The fact that the same honeycomb intermediate can lead to vesicle fusion (with enzyme-generated diacylglycerol) or to a cubic phase (when diacylglycerol is premixed with the lipids) is taken in support of the hypothesis according to which a highly curved lipid structure ("stalk") would act as a structural intermediate in membrane fusion. Sphingomyelinase produces complete leakage of vesicle aqueous contents and an increase in size of about one-third of the vesicles. A mechanism of vesicle opening and reassembling is proposed in this case.

INTRODUCTION

Membrane fusion may be analyzed on at least two different levels, the kinetic and the structural, with the aim of discovering, respectively, the rate-limiting stage in the process and factors influencing that stage, and the structural intermediates that may exist in fusion (Bentz and Ellens, 1988). Kinetic analysis of vesicle fusion was considerably improved by the development of a variety of fluorescence assays that allowed the separate study of vesicle leakage, bilayer lipid mixing, and mixing of liposomal aqueous contents (Wilschut and Papahadjopoulos, 1979; Düzgünes and Bentz, 1988). Despite various efforts, similar progress has, however, not been made in the structural studies.

For fusion of two membranous compartments to occur, it is first necessary to bring about the apposition of both membrane surfaces, and this requires in turn overcoming the bilayer hydration barriers. Then some kind of bilayer destabilization is required, so that the bilayer structure is modified, allowing the formation of aqueous channels between apposed vesicles without loss of vesicular contents (Verkleij, 1984; Siegel, 1986). In the last decade, lipid polymorphism has often been related to the destabilization of model membranes. Formation of inverted hexagonal lipid phases has been associated to release of aqueous vesicular contents (Bentz et al., 1985; Ellens et al., 1986a,b, 1989; Allen et al., 1990). Other studies have indicated a correlation between liposomal fusion and lamellar to nonlamellar phase transitions of lipids (Ellens et al., 1986b, 1989; Siegel, 1986; Siegel et al., 1989b; Nieva et al., 1995; Basáñez et al., 1996a,b). Finally, in some cases the existence of common intermediates for both leakage and fusion has been proposed (Siegel et al., 1989, 1994; Frederik et al., 1991).

The most widely accepted model of membrane fusion implies the formation of a lipidic intermediate, semitoroidal in shape (the "stalk"), between the membranes in apposition (Chernomordik and Zimmerberg, 1995d; Chernomordik et al., 1995c; Siegel, 1993). This transient intermediate would rapidly evolve toward a more stable structure, the fusion pore, connecting both sides of the membranes in apposition. The "stalk" intermediate, which should make hemifusion possible, could also, because of its high curvature, be related to inverted nonlamellar phases (Siegel, 1993). However, direct physical evidence of these transient intermediates has not yet been produced, and although some experimental data support their existence (Chernomordik et al., 1993, 1995a,b; Kemble et al., 1994; Melikyan et al., 1995; Vogel et al., 1993), the molecular mechanism of membrane fusion is still the object of controversy.

In past years several studies on the fusion of liposomes promoted by phospholipase C have been performed in this laboratory (Nieva et al., 1989, 1993, 1995; Burger et al., 1991; Basáñez et al., 1996a). These investigations have suggested the relevance of diglycerides, the hydrophobic product of the enzymatic action, in the fusion process. It is thus relevant to examine the morphological effects of diglycerides, either cosolubilized with other lipids or produced in situ through the action of phospholipase C. Furthermore,

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more recent studies from this laboratory have shown that in situ production of ceramides, through the action of sphingomyelinase on sphingomyelin-containing bilayers, leads to extensive leakage of vesicular contents (Ruiz-Argüello et al., 1996). This indicates the pertinence of also carrying out morphological observations of vesicles containing sphingomyelin and ceramides.

Cryotransmission electron microscopy (Dubochet et al., 1988; Almgren et al., 1996) makes it possible to obtain images that are two-dimensional projections of all of the particles in suspension without the need for treating the sample (apart from vitrification) with fixation reagents or other chemicals. Thus this technique appeared to be appropriate for following the morphological changes that lead to vesicle fusion and vesicle leakage in the presence of phospholipase C and sphingomyelinase, respectively. In fact, our images show liposome fusion in both cases, although different mechanisms appear to operate: fusion related to nonlamellar phase formation, with phospholipase C, and fusion with release of contents and apparently unrelated to inverted lipidic phases, in the case of sphingomyelinase.

MATERIALS AND METHODS

Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was supplied by Boehringer-Mannheim. Sphingomyelinase (EC 3.1.4.12), also from *B. cereus*, was from Sigma (St. Louis, MO). Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), and diacylglycerol (DG), obtained by phospholipase C hydrolysis of egg PC, were all grade I and were from Lipid Products (South Nutfield, England). Egg sphingomyelin and egg ceramide were from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Ch) was from Sigma. Fluorescent probes were supplied by Molecular Probes (Eugene, OR).

The appropriate lipids or lipid mixtures (including diacylglycerol when required) were solubilized in chloroform-methanol, and the solvents were removed in a rotary evaporator. The resulting thin film of lipid was left in a vacuum chamber in the dark for at least 2 h to remove solvent traces. Multilamellar vesicles were prepared by swelling the lipid thin film in buffer (10 mM HEPES, 200 mM NaCl, pH 7.0). Large unilamellar vesicles (LUVs) were obtained by the extrusion method of Mayer et al. (1986), using Nucleopore filters with 0.1-μm pore diameter.

All enzyme treatments were carried out at 37°C. Lipid and enzyme concentrations are specified for each experiment in the corresponding figure legend. For measurements of enzyme activities, aliquots of the reaction mixtures were removed at prefixed times and mixed with chloroform-methanol (2:1). After phase separation, phosphorous (from phosphorylcholine) was assayed in the aqueous phase (Nieva et al., 1989). Vesicle aggregation was estimated by the increase in scattered light (Nieva et al., 1989, 1993; Ruiz-Argüello et al., 1996). Lipid mixing was measured by dilution in the bilayer of the self-quenching probe octadecylrhodamine B (R18), as described by Hoekstra et al. (1984) (see for details Nieva et al., 1989, and Basáñez et al., 1996b). Vesicle leakage and mixing of aqueous vesicle contents were estimated using the ANTS/DPX fluorescent probe system described by Ellens et al. (1986b) (details as in Nieva et al., 1989, and Basáñez et al., 1996b).

For cryo-transmission electron microscopy (cryo-TEM), samples were prepared in a controlled environment vitrification system (CEVS). The system, which comprises an improved version of CEVS described by Bellare et al. (1988), ensures good temperature control and minimizes evaporation during sample preparation. The preparation procedure has been described in detail elsewhere (Bellare et al., 1988) but consists, in short, of the following. The sample was equilibrated within the CEVS at the desired temperature (37°C) and humidity (98–99% rh). Thereafter a small (~2 μl) drop of sample solution was withdrawn and deposited on a holey polymer film-covered TEM grid (Fukami and Adachi, 1965). After careful spreading of the drop, excess liquid was blotted away with filter paper. By this technique, thin (10–500 nm) sample films, spanning the ~5 μm large holes in the polymer film, were formed. After blotting the sample was immediately plunged into liquid ethane held at its freezing point. The vitrified sample was then transferred under liquid nitrogen to a Zeiss EM 902 electron microscope. The specimen temperature was kept below 108 K, and all observations were made in zero-loss brightfield mode and at an accelerating voltage of 80 kV. The micrographs were underfocused to enhance contrast; this limits the resolution to no better than 3 nm.

RESULTS

Polymorphism of PC:PE:Ch:DG aqueous dispersions

Previous studies have shown (Nieva et al., 1993) that phospholipase C induces fusion of LUVs consisting of PC:PE:Ch (2:1:1) when 5–20% of the phospholipid in the bilayer has been converted into DG. Moreover, when PC, PE, Ch, and DG were dispersed in water after they were premixed in the appropriate proportions in organic solvents (e.g., a mixture containing 10% DG would be PC:PE:Ch:DG, 43:22:25:10), x-ray diffraction data revealed that a bicontinuous cubic phase was progressively forming (Nieva et al., 1995). The cryo-TEM studies were initiated by examining dispersions of this kind. The mixture PC:PE:Ch (50:25:25) in water gives rise to multilamellar vesicles (Fig. 1 *A*), in agreement with previous ^{31}P-NMR and x-ray data (Nieva et al., 1995). When some of the original phospholipid is substituted by DG (e.g., by mixing in organic solvent PC:PE:Ch:DG (40:20:25:15) instead of PC:PE:Ch (50:25:25)), the samples become heterogeneous inasmuch as, together with the large multilamellar vesicles mentioned above, other lipidic structures are seen. In PC:PE:Ch:DG (40:20:25:15) samples dispersed and incubated for ~5 min at 37°C, the dominant structures are dense aggregates formed by polygonal compartments of various sizes (Fig. 1 *B*). With longer incubation times the lipid dispersion becomes more viscous, and after 1 h it is semisolid in appearance. At this stage brief sonication treatments are required to make the sample amenable to cryo-TEM study. The appearance of this sample is shown in Fig. 1 *C*, where a nonlamellar phase has formed. In fact, a similar sample examined by x-ray diffraction shows reflection in the ratio $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{5}:\sqrt{6}:\sqrt{8}:\sqrt{9}$, which is typical of the Q^{224} phase (Mariani et al., 1988; Nieva et al., 1995), the cell parameter of which would be a = 141.6 Å (data not shown). In the central part of the structure in Fig. 1 *C*, there is a large area showing a clearly distinguishable repeat distance of ~148 Å. This structure has great structural similarities with the recently published cryo-TEM pictures of single crystals of another inverted cubic phase, Q^{229} (Gustafsson et al., 1996), and is very different from, e.g., particles with inverted hexagonal structure (Almgren et al., 1996). We conclude that the aggregate in Fig. 1 *C* represents a polycrystalline particle of cubic structure.

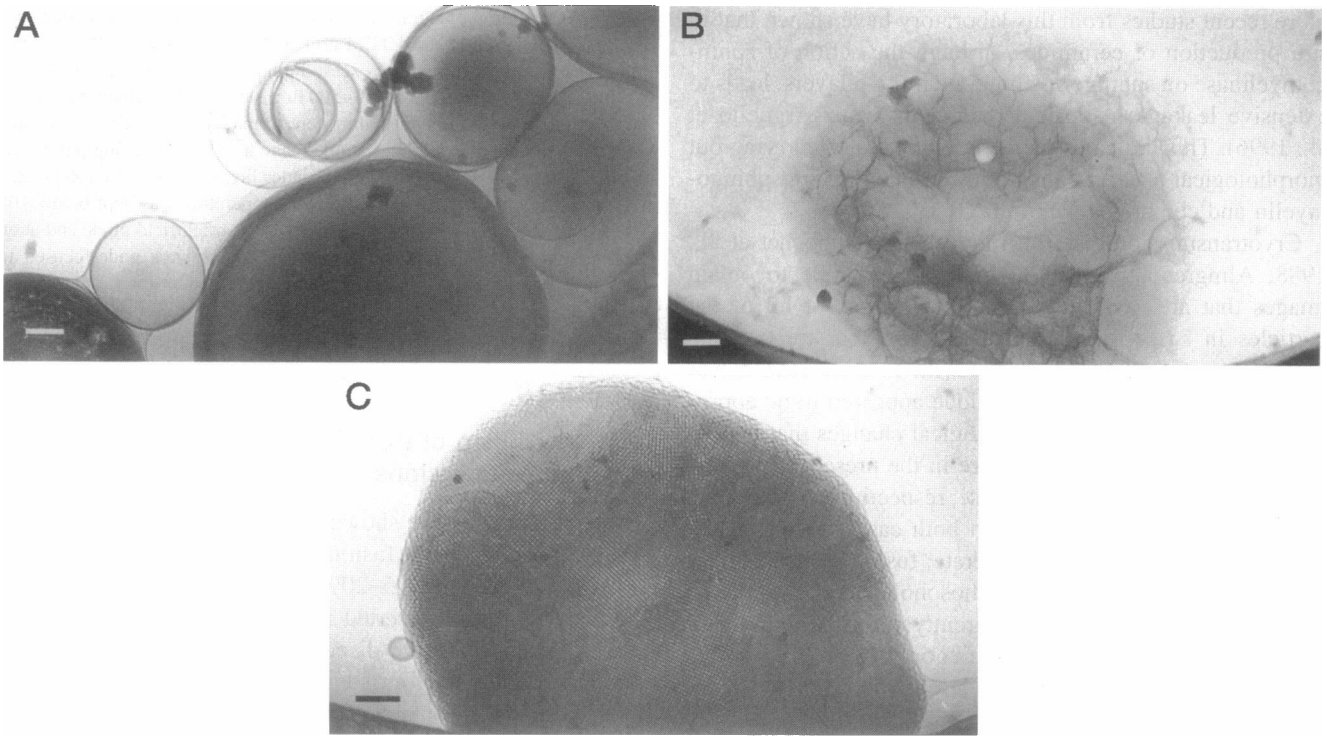

FIGURE 1 Cryo-TEM micrographs of aqueous lipid dispersions. (*A*) Multilamellar vesicles of PC:PE:Ch (50:25:25). (*B*) PC:PE:Ch:DG (40:20:25:15) after a 5-min incubation at 37°C. (*C*) PC:PE:Ch:DG (40:20:25:15) after a 60-min incubation at 37°C. Bar: 100 nm.

Phospholipase C and LUVs of PC:PE:Ch (50:25:25)

The conditions described by Nieva et al. (1989) for phospholipase C-promoted LUV fusion lead to a process that is essentially completed in ~30 s. To carry out time-resolved cryo-TEM studies, it was necessary to discover the conditions that would lead to a slower fusion process. This was achieved with 1 mM lipid and 0.16 U/ml enzyme, at 37°C. As shown in Fig. 2, lipid hydrolysis, vesicle aggregation, and mixing of aqueous contents occur at much slower rates under these conditions, e.g., content mixing starts only 20 s after enzyme addition, and it does not become saturated before 5–6 min, thus allowing ample time for sampling at various stages of the process. Note, however, that the DG concentrations marking the onset and termination of fusion are still 5% and 20%, respectively (Fig. 2) (Nieva et al., 1993). Thus the relationship between DG concentration and fusion appears to be largely independent of the DG production rate, at least under our conditions.

Fig. 3 shows cryo-TEM micrographs corresponding to a LUV preparation (PC:PE:Ch, 50:25:25) at 0, 90, 180, and 360 s after phospholipase C addition (Fig. 3, *A*, *B*, *C*, and *D*, respectively). The original liposomes are about 100 nm in diameter, and they give rise to increasingly larger aggregates as the enzyme action proceeds. Fig. 3 *B* shows the structure of an aggregate in which, according to the data in Fig. 2, fusion is taking place: the vesicles adopt polygonal shapes, without any apparent gap between the adjacent membranes. This "honeycomb" structure had already been described in identical preparations treated with the freeze-fracture technique (Burger et al., 1991), and is remarkably similar to the arrangement of PC:PE:Ch:DG (40:20:25:15) incubated for a short time at 37°C (Fig. 1 *B*). Honeycomb structures were found in virtually any phospholipase C-

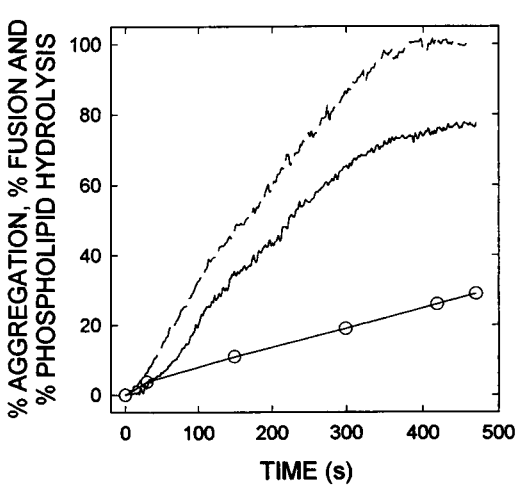

FIGURE 2 Effects of phospholipase C on large unilamellar vesicles of PC:PE:Ch (50:25:25). Circles, Percentage hydrolyzed phospholipid. Continuous line, liposome fusion (mixing of aqueous contents) assayed after the method of Ellens et al. (1986). Broken line, Vesicle aggregation, measured as absorbance at 450 nm. The total lipid concentration was 1 mM; the enzyme concentration was 0.16 U/ml. Temperature: 37°C.

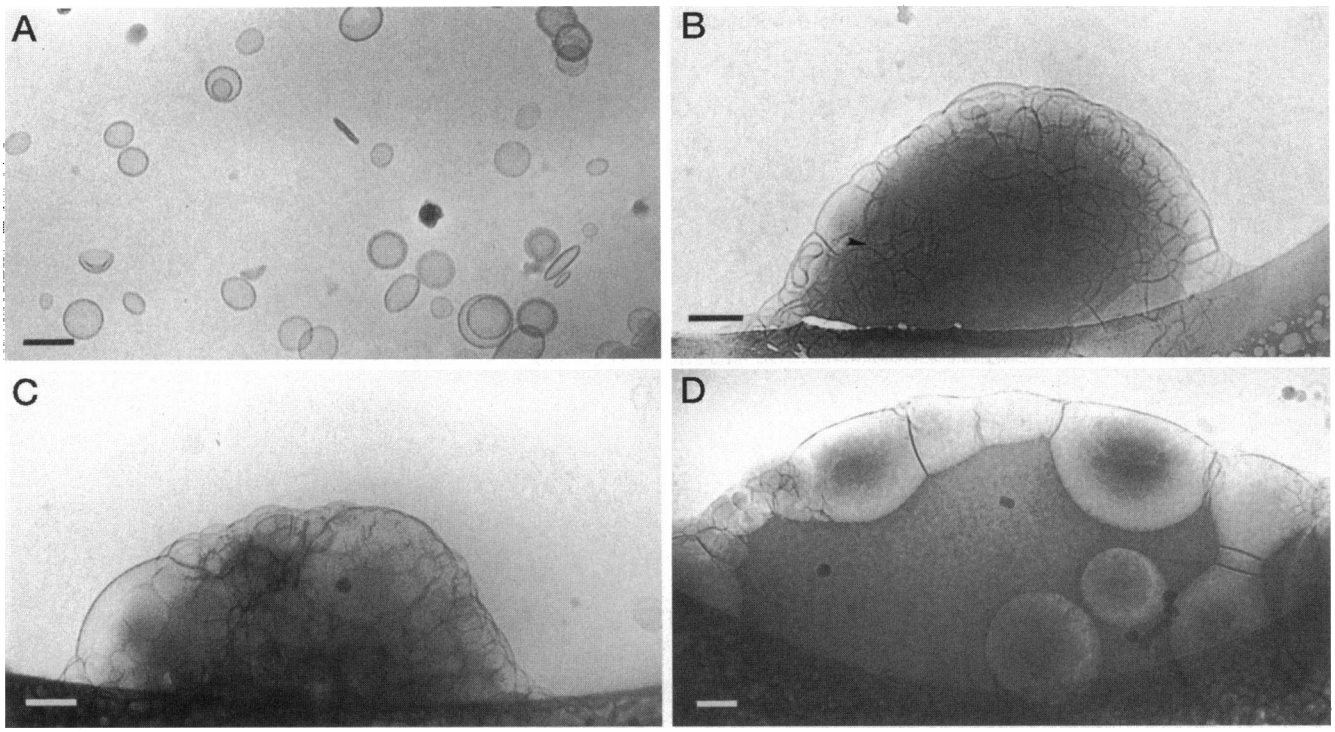

FIGURE 3 Cryo-TEM micrographs of large unilamellar vesicles of PC:PE:Ch (50:25:25) at various times after phospholipase C addition. (*A*) 0 s; (*B*) 90 s; (*C*) 180 s; (*D*) 360 s. Experimental conditions as in Fig. 2. Bar: 100 nm. In Fig. 3 B, the arrowhead points to a putative fusion pore.

treated preparation observed at times during which mixing of aqueous contents is taking place. In some cases, the polygonal compartments appear to be connected by structures that could correspond to fusion pores, although this interpretation has to be made with caution, in the absence of additional proof. At long times after enzyme addition (e.g., Fig. 3 *D*), the aggregates progressively lose their honeycomb structure and form larger compartments, presumably the end point of an extensive series of fusion events.

Phospholipase C and LUVs of pure PC

In a different series of experiments the effect of phospholipase C on LUV composed of pure egg PC was examined. With this substrate the enzyme activity displays a lag period that ends when liposome aggregation starts (Basáñez et al., 1996c). A representative experiment is shown in Fig. 4: the enzyme activity is very low in the first 5 min; then, when the proportion of DG in the membrane reaches ~5–10 mol%, the suspension turbidity increases and the phosphohydrolase activity is triggered. Fusion, however (as detected through mixing of aqueous contents), is delayed with respect to vesicle aggregation, because it starts only when DG concentration in the bilayer is ~20–25 mol%. Thus a second lag period exists between liposomal aggregation and fusion. No leakage of aqueous contents was detected during aggregation or fusion (data not shown).

The micrograph in Fig. 5 *A*, taken 240 s after enzyme addition, is in agreement with the turbidity data (Fig. 4), because the LUVs are very similar in size and shape to those in the untreated sample (compare with Fig. 3 *A*). Thus during the enzyme lag period, no vesicle aggregation is seen. The next micrograph (Fig. 5 *B*) was taken at 360 s and corresponds to the time range in which turbidity increases, but there is still no content mixing (Fig. 4). Fig. 5 *B* shows an aggregate consisting of a multitude of vesicles, each of

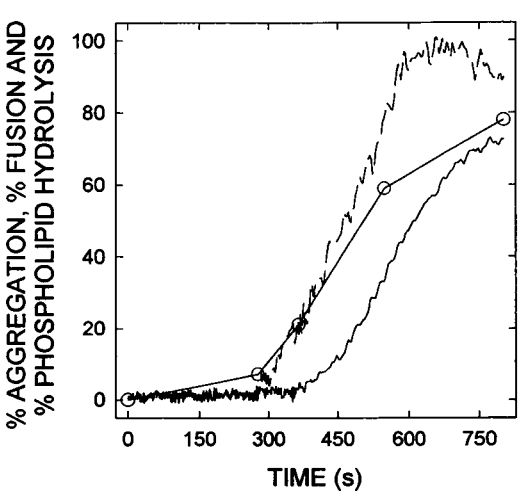

FIGURE 4 Effects of phospholipase C on large unilamellar vesicles of pure egg PC. Circle, Percentage hydrolyzed PC. Continuous line: Liposome fusion, as mixing of aqueous contents. Broken line, Vesicle aggregation. Lipid and enzyme concentrations were, respectively, 0.6 mM and 1.6 U/ml. Temperature: 37°C.

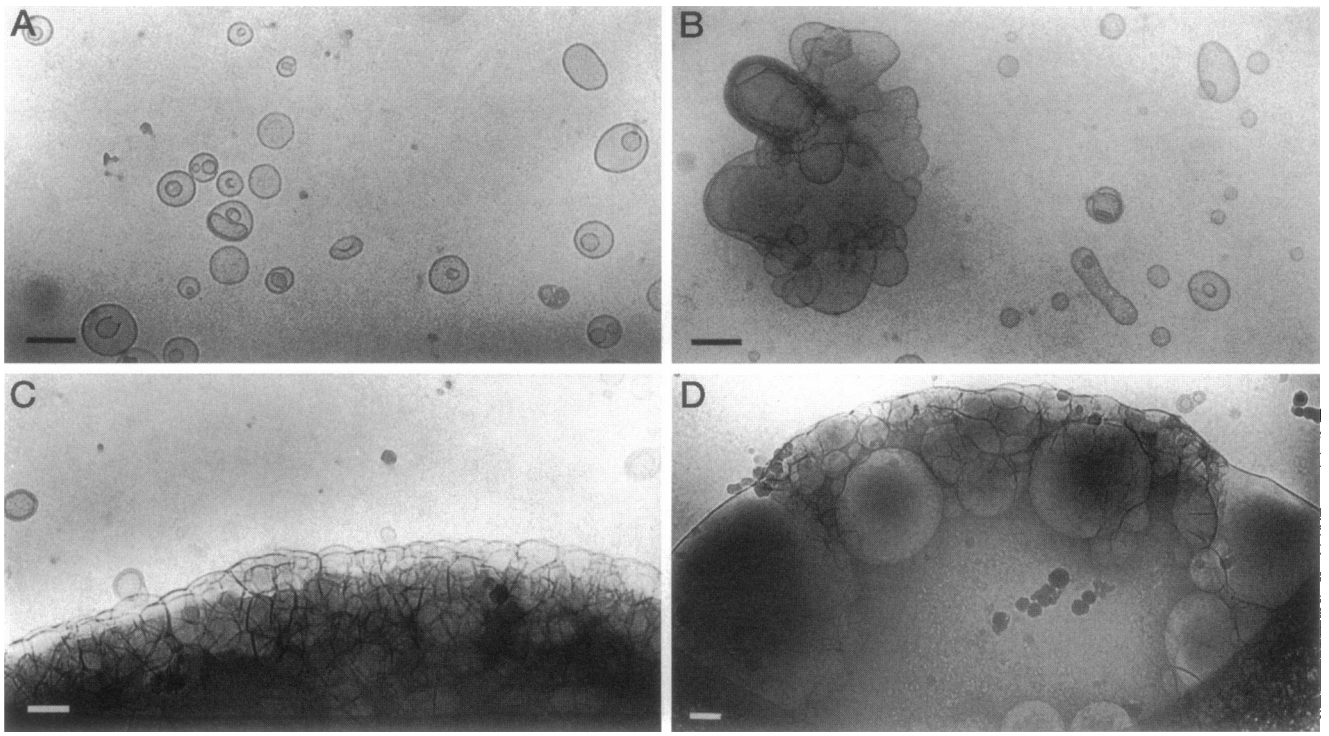

FIGURE 5 Cryo-TEM micrographs of large unilamellar vesicles of egg PC at various times after phospholipase C addition. (*A*) 240 s; (*B*) 360 s; (*C*) 480 s; (*D*) 600 s. Experimental conditions as in Fig. 4. Bar: 100 nm.

which appears to keep the integrity of its membrane, as well as a spheroidal shape (compare with the nonaggregated vesicles on the right side of the picture). At later times, the appearance of the aggregates changes to the "honeycomb" pattern (see, e.g., Fig. 5 *C*, taken 480 s after enzyme addition). The structure is very similar to what was seen in Fig. 1 *B*, Fig. 3 *B*, and in Burger et al. (1991); in all cases the honeycomb pattern is present when, according to fluorescence measurements, the rate of content mixing is highest. As observed with PC:PE:Ch bilayers (Fig. 3 *D*), when fusion of PC vesicles slows down, the micrographs reveal larger vesicles and a progressive loss of the polygonal structures (Fig. 5 *D*).

Sphingomyelinase and LUVs of SM:PE:Ch (50:25:25)

As shown by Ruiz-Argüello et al. (1996), the addition of *B. cereus* sphingomyelinase to LUVs composed of SM:PE:Ch (50:25:25) leads to extensive leakage and an increase in sample turbidity (Fig. 6). A lag is observed between these two phenomena: 2 min after enzyme addition, more than 50% leakage is detected, whereas the turbidity remains at less than 10% of its maximum value. Some lipid mixing is detected, although it is never beyond 30% when the process reaches its plateau. No mixing of vesicle aqueous contents is seen under these conditions.

Micrographs taken at 0 and 3.5 min after enzyme addition (Fig. 7, *A* and *B*) demonstrate the formation of large membranous bags together with many vesicles of the original size. The smaller vesicles appear to be inside the large ones, but it cannot be ascertained that they are not superimposed on the latter. At longer times (6 min) the size of the large vesicles appears to increase gradually, but many vesicles of ~100 nm are still seen. Thus sphingomyelinase induces an increase in size of the vesicles, but apparently in the absence of honeycomb patterns or other detectable intermediates.

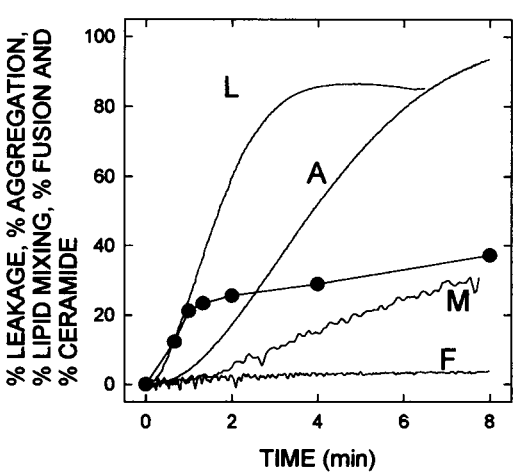

FIGURE 6 Effects of sphingomyelinase on large unilamellar vesicles of SM:PE:Ch (50:25:25). L, Vesicle leakage. A, Vesicle aggregation. M, Vesicle lipid mixing. F, Vesicle fusion (mixing of aqueous contents). ●, Sphingomyelin hydrolysis. Total lipid and enzyme concentrations were, respectively, 1.5 mM and 0.16 U/ml. Temperature: 37°C.

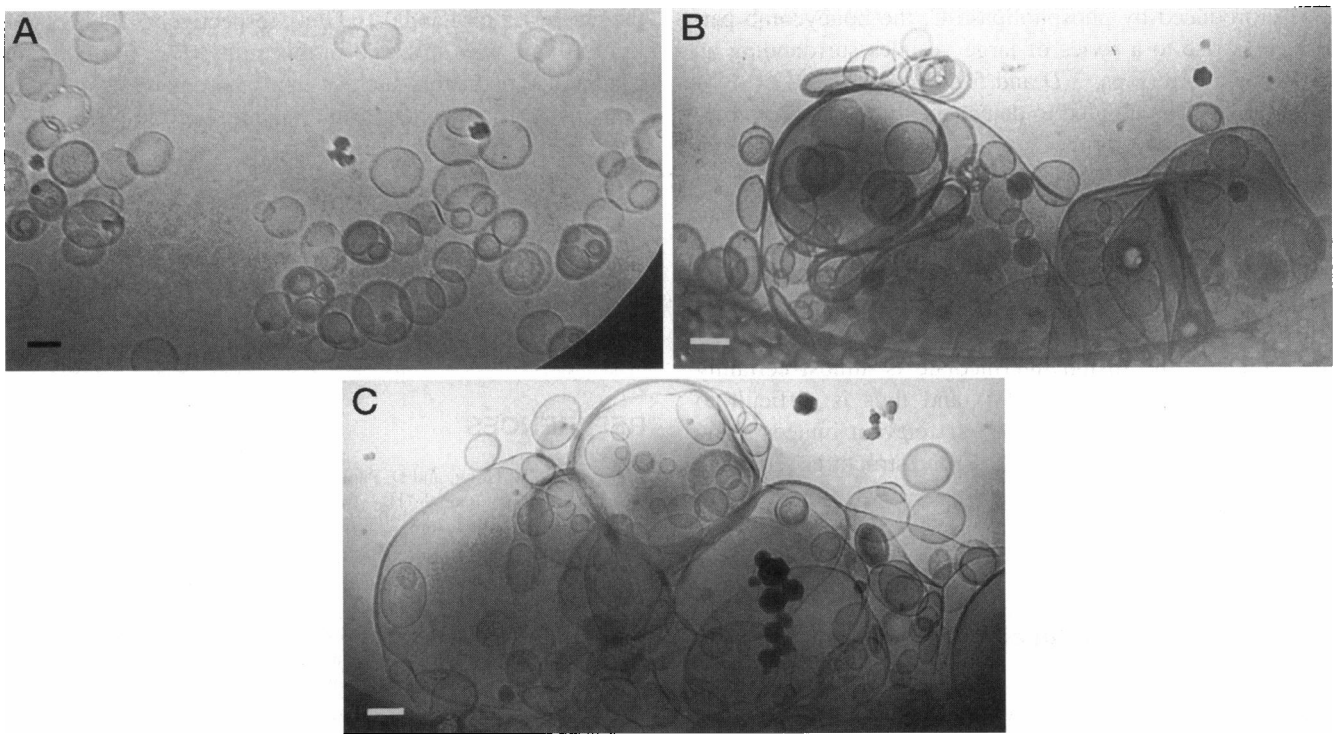

FIGURE 7 Cryo-TEM micrographs of large unilamellar vesicles of SM:PE:Ch (50:25:25) at various times after sphingomyelinase addition. (*A*) 0 min; (*B*) 3.5 min; (*C*) 6 min. Experimental conditions as in Fig. 6. Bar: 100 nm.

DISCUSSION

The main aim of this paper was to obtain structural information on the phospholipase C effects on LUV using cryo-TEM, to complement previous freeze-fracture studies (Burger et al., 1991), and to compare the above with the effects of sphingomyelinase on similar large vesicles.

Phospholipase C-promoted fusion and nonlamellar lipid phases

Lamellar to nonlamellar phase transitions in lipids have been repeatedly related to model membrane fusion. The relation has been confirmed by, among other techniques, electron microscopy (Frederik et al., 1991; Siegel et al., 1989b, 1994; Verkleij, 1984). Images of lipidic channels that would communicate different membranous spaces (fusion pores, or ILA in the terminology of Siegel et al., 1989) were obtained under conditions that favor formation of isotropic cubic phases. Because of the similarity between these structures and the so-called lipidic particles observed in various membrane fusion processes (Verkleij, 1984), a common fusion mechanism was proposed. The mechanism is based on the formation of a high-curvature lipidic intermediate (the "stalk") that would eventually give rise to the fusion pore (Chernomordik et al., 1995b,c; Siegel, 1993). According to this model, the progressive accumulation of the pores, and their disposition in an ordered pattern, could lead to the formation of structures reminiscent of inverted cubic phases.

Various studies have shown that DG is a potent destabilizer of the lamellar phase (Cheng and Hui, 1986; Das and Rand, 1986; De Boeck and Zidovetzki, 1989; Epand, 1985; Orädd et al., 1995; Siegel et al., 1989b; Takahashi et al., 1996). Also in LUVs of PC:PE:Ch (50:25:25), DG has been shown to promote the formation of the bicontinuous cubic phase Q^{224} in the proportions at which liposome fusion occurs (Nieva et al., 1995). Thus the putative relation between fusion and nonlamellar phases appears to apply to our system. More recently Takahashi et al. (1996) have shown the formation of bicontinuous and discontinuous cubic phases in mixtures of dipalmitoylphosphatidylcholine, dipalmitoyl-phosphatidylglycerol, and diacylglycerol. The present study supports the hypothesis of a fusion intermediate containing structural elements, the local geometry of which is reminiscent of the inverted cubic phases, because cryo-TEM shows similar images for liposome fusion induced by phospholipase C (e.g., Figs. 3 *B* and 5 *C*) and for the lamellar-cubic transition induced by DG (Fig. 1 *B*). In both cases the lipids organize themselves as compact aggregates, formed by polygonal compartments of varying sizes (the "honeycomb" structure). One important characteristic of such a structure is that it is far from equilibrium; when the DG proportion is constant it evolves toward a cubic phase (Fig. 1 *C*), whereas when DG is being contin-

uously produced by phospholipase C, the honeycomb pattern gives rise to a series of large vesicles surrounding an amorphous core (Figs. 3 *D* and 5 *D*).

Our studies have failed to detect specific structures compatible with the idea of the "stalk." This should not be taken as proof against their existence, however, because most of the observed images are complex, resulting from the projection of various planes, and the limit of resolution of cryo-TEM images is rather high (~3 nm) (see Materials and Methods). Furthermore, in our system DG is being continuously produced, so we never observe equilibrium structures. Finally, the fusion intermediate is almost certainly short-lived (Siegel et al., 1994) and thus is difficult to observe. Nevertheless, some of our relevant images show putative fusion pores (e.g., Fig. 3 *B*) and structures related to the ones found by Siegel et al. (1989b, 1994) in a different system.

Sphingomyelinase-induced leakage and vesicle growth

When added to SM:PE:Ch (50:25:25) LUV, sphingomyelinase produces an increase in suspension turbidity indicative of vesicle aggregation and/or growth. A similar effect is produced by phospholipase C on PC:PE:Ch (50:25:25) LUV. In all other respects, the two systems show very different behavior (Figs. 2 and 6): phospholipase C induces extensive fusion (mixing of aqueous contents) in the absence of leakage, whereas sphingomyelinase gives rise to leakage and an increase in size of at least part of the vesicles. The latter is accompanied by ~30% lipid mixing, suggesting that this many vesicles have participated in the vesicle growth process. The reasons for the different behavior of the PC- and the SM-based systems have been discussed elsewhere (Ruiz-Argüello et al., 1996) and related to the bilayer stabilizing properties of SM:Ch, as compared to PC:Ch, and to the reduced destabilizing properties of ceramides as compared to DG.

The cryo-TEM pictures of the SM:PE:Ch system clearly reveal that the increase in size of the vesicles occurs via a mechanism different from that observed for the PC-containing liposomes. The complete spillage of vesicle contents and the lack of detectable intermediates (honeycomb or other) suggest that, in this case, vesicle growth could occur via an "opening and reassembling" mechanism similar to the one proposed for the liposome fusion induced by surfactants (Alonso et al., 1981, 1982), which was later confirmed by Edwards et al. (1989). Only a fraction of the vesicles (~30% under our conditions, as deduced from lipid mixing measurements) would participate in the growth process, for reasons that remain unexplored at this moment.

It should be noted that in our previous studies with sphingomyelinase (Ruiz-Argüello et al., 1996), no lipid mixing was detected under any of the conditions tested. In that case lipid concentration was 0.3 mM, and enzyme concentration was 1.6 U/ml, whereas in the present study they were 1.5 mM and 0.16 U/ml, respectively. The fact that lipid mixing was observed in this study (Fig. 6) marks the importance of factors such as lipid and enzyme concentration, and perhaps others in the outcome of the process.

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