Macroscopic Consequences of the Action of Phospholipase C on Giant Unilamellar Liposomes

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ABSTRACT Macroscopic consequences of the formation of diacylglycerol by phospholipase C (PC-PLC) in giant 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) unilamellar vesicles (GUVs, diameter 10–100 μm) were studied by phase contrast and fluorescence microscopy. PC-PLC caused a series of fast stepwise shrinkages of fluid SOPC GUVs, continuing until the vesicle disappeared beyond the optical resolution of the microscope. The presence of *N*-palmitoyl-sphingomyelin (mole fraction $X = 0.25$) in the GUVs did not affect the outcome of the PC-PLC reaction. In addition to hydrolysis, PC-PLC induced adhesion of vicinal vesicles. When multilamellar SOPC vesicles were used only a minor decrease in their diameter was evident suggesting that PC-PLC can exert its hydrolytic activity only in the outer monolayer. A series of stepwise shrinkages was observed also for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) GUVs above their main phase transition temperature, T_m, i.e., when the bilayer is in the liquid crystalline state. However, this process was not observed for DMPC GUVs in the gel state, below T_m. These results are supported by the enhanced activity of PC-PLC upon exceeding T_m of DMPC large unilamellar vesicles (diameter ~0.1 μm) used as a substrate. Studies on SOPC monolayers revealed that PC-PLC can exert its hydrolytic activity only at surface pressures below ~30 mN/m. Accordingly, the lack of changes in the gel state DMPC GUVs could be explained by the equilibrium lateral pressure in these vesicles exceeding this critical value.

INTRODUCTION

Phospholipases represent a group of hydrolytic enzymes, which are intimately involved in different cellular signaling cascades and modify the structure of phospholipids in a number of specific ways (Roberts, 1996). More specifically, phospholipase A_1 and A_2 cleave the acyl chains at *sn*-1 and *sn*-2 positions, respectively, and phospholipase D (PLD) modifies the polar headgroup to yield phosphatidic acid, while phospholipase C (PLC) produces diacylglycerol. Phospholipases further involve enzymes with different substrate specificities, such as those acting on phosphatidylinositol (PI-PLC), sphingomyelin (SMase), and phosphatidylcholine (PC-PLC). The signaling molecules derived from the reactions catalyzed by phospholipases include arachidonic acid, lysophosphatidylcholine (lysoPC), diacylglycerol, ceramide, phosphatidic acid (PA), and lysophosphatidic acid (lysoPA, for a recent review see Gõni and Alonso, 1999). These lipids have been recognized to represent second messengers and some of them have been shown to represent high-affinity ligands for specific effectors, such as the specific G-protein coupled receptor described for lysoPA (e.g., Moolenaar, 1995).

While ceramide is involved in apoptosis the closely similar lipid, diacylglycerol generated by PC-PLC has been implicated as a messenger for cell growth (reviewed in Kinnunen, 1996). Similarly to PLA_2, PC-PLC exhibits interfacial activation, a lag time before a sudden burst in its activity (Hønger et al., 1997; Ruiz-Argüello et al., 1998a), sensitivity to lipid lateral packing (Rao, 1992; Rao and Sundaram, 1993), and increase in activity at the main phase transition of the substrate phospholipid (Vandenbranden et al., 1985; Gabriel et al., 1987). Increasing contents of diacylglycerol in phospholipid substrate enhance the activity of both PLA_2 and PC-PLC (Dawson et al., 1984). Diacylglycerol is a specific activator for protein kinase C (Zidovetski and Lester, 1992). Recently, Alonso and Gõni and their coworkers have shown that PC-PLC activity is not directly influenced by the formation of non-lamellar structures (Ruiz-Argüello et al., 1996) and suggested the tendency to form non-lamellar phases (Kinnunen, 1996) or release of the packing stress to activate this enzyme. Using cryo-transmission electron microscopy the same group showed that PC-PLC activation is linked to vesicle aggregation. This is followed by the formation of a closely packed "honeycomb structure" which eventually leads to the emergence of larger spherical vesicles (Basáñez et al., 1997). Under conditions where SMase or PC-PLC cannot produce major structural changes in the vesicles, the concerted action of these two lipases induces vesicle fusion. The above suggests "cross-talk" of the ceramide and diacylglycerol involving signal transduction pathways (Ruiz-Argüello et al., 1998b).

Liposomes have been extensively used as models for cell membranes for the past 20 years. Initially, Bangham and coworkers showed that lipids spontaneously form closed structures when dispersed into an aqueous medium (Bang-

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ham et. al., 1965). However, these structures are multilayered and inability to control their size and the number of the concentric lipid bilayers represent a significant drawback. Exposure to ultrasonication allows obtaining small unilamellar vesicles (Ø < 30 nm) with a relatively narrow size distribution. The small size and high curvature, however, limits their use. Extrusion through small pores yields large unilamellar vesicles (Ø 50–200 nm). However, also these vesicles are too small for studies on their morphology by light microscopy. To this end, topological changes in lipid membranes have gained only minor attention, perhaps due to the very lack of proper model membranes. Giant unilamellar vesicles (GUVs, Ø > 10 μm) can be formed by an AC electric field (Angelova and Dimitrov, 1986; Luisi and Walde, 2000). It is becoming evident that the vesicle size and curvature plays significant roles in the outcome of, for example, enzymatic reactions taking place on the lipid membrane (Hubner et al., 1998). For instance, when large unilamellar vesicles are treated with sphingomyelinase only aggregation and occasional fusion is observed (Ruiz-Argüello et al., 1996; Basáñez et al., 1997), whereas when the size of the vesicles is ~100–1000 times larger vectorial budding of small vesicles takes place (Holopainen et al., 2000a).

This study continues our efforts to characterize the morphological consequences due to specific modifications of the lipid composition of giant vesicles by different phospholipases. Here we show that the formation of diacylglycerol by PC-specific phospholipase C in fluid phosphatidylcholine giant unilamellar vesicles results in a series of consequent stepwise shrinkages of the liposome. The above is not observed for multilamellar vesicles nor for membranes in the gel state below the main transition temperature.

MATERIALS AND METHODS

Materials

1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) was from Avanti Polar Lipids (Alabaster, AL), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) from Sigma, and C16:0-sphingomyelin from Northern Lipids Inc. (Vancouver, British Columbia, Canada). The concentrations of BODIPY-sphingomyelin and 4-(4-(dihexadecylamino)styryl)-*N*-methylpyridinium iodide (DiA) (Molecular Probes, Eugene, OR) were determined spectrophotometrically using 91,000 cm^{-1} at 505 nm, and 52,000 cm^{-1} at 491 nm, respectively, as their molar extinction coefficients. Concentrations of the other lipids were determined gravimetrically using a high precision electrobalance (Cahn, Cerritos, CA). The purity of the lipids was checked by thin-layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) developed with chloroform/methanol/water (65:25:4, v/v/v). Examination of the plates after iodine staining or, when appropriate by fluorescence illumination revealed no impurities. PC-PLC (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* (Grade I, 4000 U/ml, specific activity 2000 units/mg protein) was from Boehringer Mannheim. The purity of the enzyme was analyzed by SDS polyacrylamide gel electrophoresis and revealed no impurities. Pro analysis grade solvents were from Merck and other chemicals from standard sources.

Liposome preparation

Lipid stock solutions (10, 10, and 2.5 mM for SOPC, DMPC, and C16:0-sphingomyelin, respectively) were mixed in chloroform to obtain the desired compositions, with either DiA or *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)sphingosyl phosphocholine (Bdp-SM) included as a fluorescent probe. Three different lipid solutions were made: 1) SOPC:DiA (molar ratio 1.0:0.005), 2) SOPC:*N*-palmitoylsphingomyelin (C16:0-SM):Bdp-SM (molar ratio 0.75:0.20:0.05), and 3) DMPC. The solvent was evaporated under a stream of nitrogen and subsequent evacuation under reduced pressure for at least 12 h. The dry residue was dissolved in diethylether:methanol (9:1, v/v) to yield a final total lipid concentration of 1 mM. Approximately 1 μl of this solution was applied onto the surface of the platinum electrodes (Angelova and Dimitrov, 1986) that were then dried with a stream of nitrogen and evacuation in vacuum for at least 1 h. A glass chamber with the attached electrodes and with a quartz window bottom was placed on a homemade temperature-controlled stage (supported by a Polystat cc3, Huber, Offenburg, Germany) of a Zeiss Axiovert 135 inverted fluorescence microscope (see instrument setup in Wick et al., 1996). An 0.2 V AC voltage (f = 4–10 Hz) was applied before adding 1.3 ml of 0.5 mM Hepes, pH 7.4 buffer. During the first minute of hydration the voltage was raised to 1–2 V. After 2 h the AC field was turned off and giant liposomes were observed through a phase contrast objective (Zeiss Achroplane LD 40X/0.60). For fluorescence microscopy excitation and emission wavelengths were selected with filters transmitting in the range 485 nm and >520 nm, respectively. Images were viewed with a Peltier-cooled CCD B/W camera (C5985–10, Hamamatsu, Japan) connected to a computer.

Microinjection of PC-PLC

Micropipettes with inner tip diameters of 0.5–1 μm (Schnorf et al., 1994) were made from borosilicate capillaries (1.2 mm outer diameter) by a microprocessor-controlled vertical puller (PC-10, Narishige, Tokyo, Japan). PC-PLC was dissolved in 9 mM $CaCl_2$, 1.8 mM $MgCl_2$ aqueous solution to yield a final enzyme concentration of 0.8–8 U/ml (0.4–4 μg/ml). The micropipette was filled with the enzyme solution using Eppendorf automatic pipettes. The micropipette was positioned with a Narishige MMN-1 and fine positioning was performed with hydraulic MMO-202N micromanipulator (Narishige, Tokyo, Japan). Subsequently, small aliquots (a few picoliters) of the enzyme were applied onto the outer surface of individual giant vesicles with a pneumatic microinjector (Transjector 5246, Eppendorf, Hinz, Germany). For easier handling only vesicles attached to the electrode surface were used. All experiments were repeated at least 5 times with excellent reproducibility.

Hydrolysis of monolayers

Action of PC-PLC on SOPC monolayers residing on an air/buffer interface was observed by monitoring decrease in surface pressure (π). Teflon-coated circular wells (subphase volume 1.2 ml, diameter 1 cm, Multiwell plate, Kibron Inc., Helsinki, Finland) were used and π was measured using a metal alloy wire attached to a microbalance (KBN 602, Kibron Inc.). Data were collected and analyzed using dedicated software (DeltaGraph) provided by the instrument manufacturer. SOPC (dissolved in $CHCl_3$) was applied on the air/buffer (0.5 mM Hepes, 10 mM $CaCl_2$, pH 7.4) interface using a Hamilton syringe. As the desired π was reached, the lipid film was allowed to equilibrate for 10 min before adding the enzyme into the subphase (final concentration 0.08 U/ml). Subsequent changes in π were monitored for 30 min. The reactions were conducted at ambient temperature (~22°C), with continuous magnetic stirring of the wells.

Liposome preparation and thin-layer chromatography

Appropriate amounts of DMPC stock solutions were mixed in chloroform and then evaporated to dryness under a stream of nitrogen. Traces of solvent were subsequently removed by evacuating under reduced pressure for at least 12 h . The lipid residues were hydrated at 50°C in 0.5 mM Hepes, 10 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4 buffer to yield a final lipid concentration of 2 mM. The lipids were maintained at this temperature for 30 min before irradiation for two min in a bath type ultrasonicator (NEY Ultrasonik 104H, Yucaipa, CA). The resulting dispersions were subsequently processed to large unilamellar vesicles (LUVs) by extrusion through one Millipore (Bedford, MA) 0.1 μm pore size polycarbonate filters using a Liposofast-Pneumatic (Avestin, Ottawa, Canada). A semiquantitative assay for the activity of PC-PLC acting on DMPC LUVs was performed, essentially as described previously for SMase (Holopainen et al., 2000b). In brief, DMPC LUVs in 0.5 mM Hepes, 10 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4 buffer were maintained for 15 min at various temperatures (10–30°C) at a final lipid concentration of 0.3 mM. Subsequently, the enzyme reactions were started at the desired temperatures by the addition of PC-PLC (5 U/ml). After 5 min the reactions were stopped by adding 1 ml of chloroform:methanol (4:1, v/v). From the lower organic phase 800 μl was separated and concentrated by evaporating the solvents where after 50 μl of chloroform was added to dissolve the lipids. Dimyristoylglycerol (DMG) was separated from the reaction mixture on TLC plates using hexane/ethyl-ether/acetic acid (80:20:1, v/v/v) as a solvent system. The plates were scanned and digitized with an Epson Perfection photo 1200 (Nagano, Japan) connected to Pentium PC and the amount of DMG was evaluated densitometrically by dedicated software (Aida/2D densitometry, version 2.00; Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany). All experiments were repeated at least twice.

RESULTS

Giant liposomes composed of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and fluorescent dye DiA ($X_{DiA} = 0.005$) readily formed in an AC field and were visible by phase contrast microscope (Fig. 1 *A*). Only vesicles that appeared quasi spherical and therefore were under minor membrane tension were selected. Following the addition of PC-PLC (0.8 U/ml, 0.4 μg/ml) close to the outer surface of the vesicle a lag time ranging from tens of seconds to several minutes was observed (Fig. 1 *B*). We did not attempt to characterize the nature or origin of the lag period at this stage. At the end of the lag, the vesicle surface began to undulate, followed by a sudden (within <1 s) shrinkage of the vesicle (Fig. 1 *C*). The latter was followed by another lag period and subsequent rapid shrinkage of the vesicle. This sequence of events repeated until the vesicle disappeared beyond the resolution of the optical microscope (Fig. 1, *D–H*). Changes in vesicle diameter *vs*. time are shown in Fig. 2 *A* and reveal the intermittent lag/shrinkage behavior. To emphasize this lag/shrinkage process the last 50 s, illustrating the stepwise disappearance of the SOPC GUV is depicted in Fig. 2 *B*. The above morphological consequences of PC-PLC reaction remained identical when a more concentrated (10X; 8 U/ml, 4 μg/ml) PC-PLC solution was used, with no apparent differences in the lag time (data not shown). This indicates that the rate of formation of 1-stearoyl-2-oleoyl-*sn*-glycerol (SOG) is not the rate-limiting factor for the above sequence of morphological transitions.

The observed lag/shrinkage behavior could be due to impurities, such as proteins, within the enzyme solution. It has, however, been previously shown that lysozyme (Wick et al., 1996) applied to the outer surface of GUVs composed of zwitterionic lipids does not induce morphological changes in the vesicle. In line with the above cytochrome c applied onto SOPC GUVs produces no morphological changes (J. M. Holopainen and P. K. J. Kinnunen, unpublished data). Finally to rule out the possibility that impurities, such as detergents, within the enzyme solution would be responsible for the observed lag-shrinkage behavior heat-inactivated PC-PLC was used. In brief, PC-PLC was incubated at 90°C for 2 h , which decreased enzyme activity by ~90% (data not shown). The heat-treated enzyme was microinjected onto SOPC GUVs. The inactivated enzyme did not cause any changes into SOPC GUVs (data not shown). In summary, these results provide strong support to the idea that the lag/shrinkage behavior is caused by the action of PC-PLC.

No vesiculation (inward or outward) was observed by phase contrast microscopy in the experiments described above. Accordingly, to trace the fate of the lipids lost from the surface in vesicle shrinkage we used fluorescence microscopy (data not shown). Uniform distribution of the fluorescent lipid, DiA, in the membrane was initially evident at the resolution of the optical microscope. Notably, we did use rather low probe concentration ($X = 0.005$) and did not want to increase this so as to limit the membrane perturbing effect of the dye. The intermittent lag period and stepwise shrinkages were evident also in fluorescence images, until the vesicle disappeared beyond the resolution of the microscope. However, there was no evidence for vesiculation or detachment of the lipids from the vesicles. The optics of our system would allow to observe vesicles with diameters of >1 μm. Accordingly, vesicles smaller than this would not be detected and therefore it is possible that <1 μm vesicles could be released from the GUV. In contrast, the intensity increased at the sites of attachment of the vesicle to the platinum wire, in an area with contacts to other vesicles and lipid residue on the platinum electrode (Fig. 3). The reason for the decrease in size of fluid vesicle could be the accumulation in the area of the vesicle-vesicle contact sites on the platinum wire of newly formed diacylglycerol either as isotropic droplets (Mountford and Wright, 1988) or possibly as topically enriched inverted cubic phase (Gõni et al., 1998) containing a number of contacting membranes. Third possibility would be the formation of submicroscopic vesicles. Importantly, all cases lead to the minimization of exposure of diacylglycerol to the aqueous phase.

Our previous study on the external action of SMase on GUVs demonstrated an endocytosis-like vesiculation of the reaction product, ceramide into the interior of the vesicle

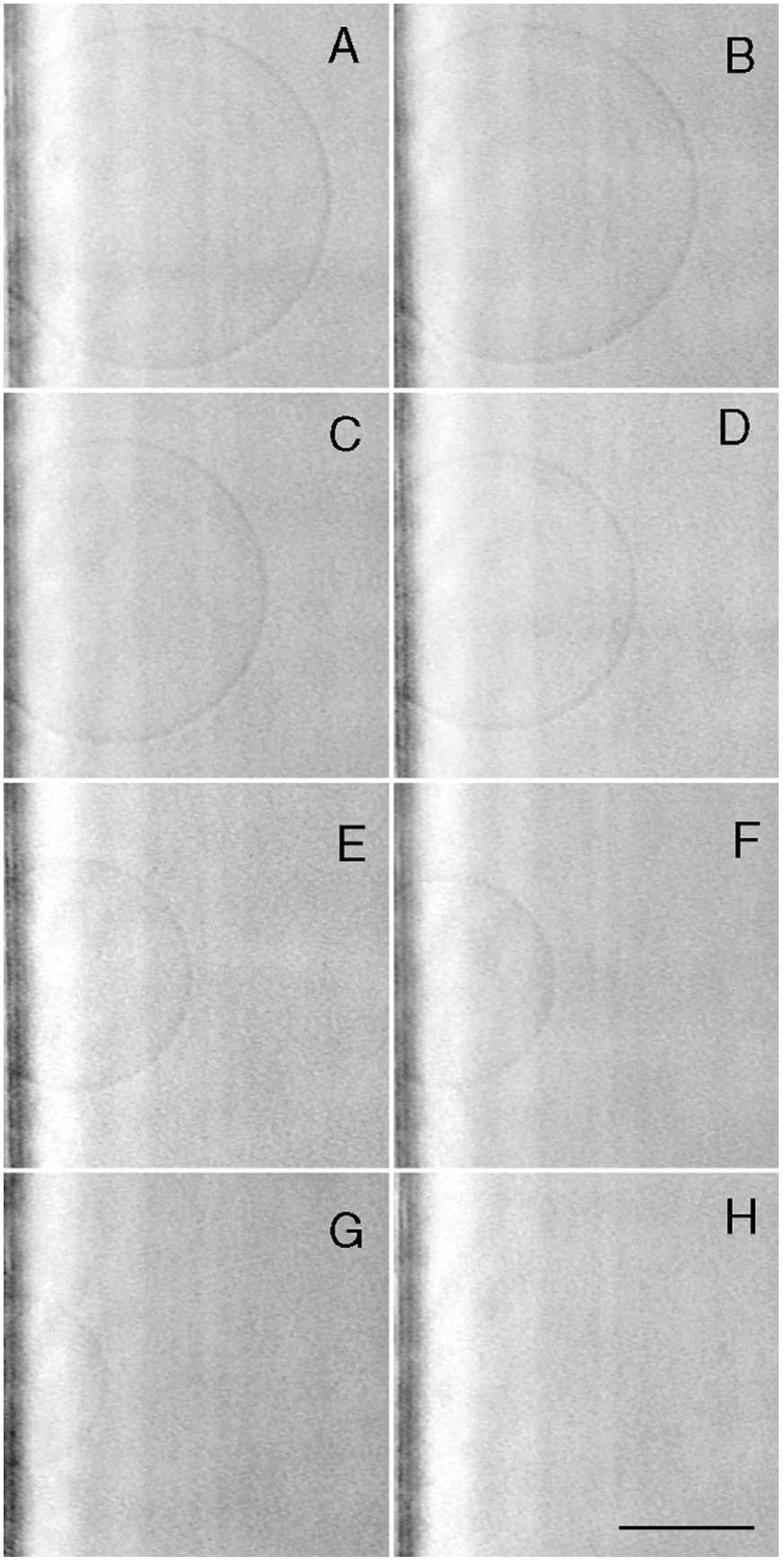

FIGURE 1 Transformations of a single SOPC/DiA (molar ratio 1:0.005) giant vesicle, induced by the addition of *B. cereus* phospholipase C (PC-PLC) in the vicinity of the vesicle. Still phase contrast images were taken at before enzyme administration (*A*) and after 6 min 18 s (*B*), 6 min 30 s (*C*), 6 min 35 s (*D*), 6 min 49 s (*E*), 6 min 53 s (*F*), 7 min 3 s (*G*), and 7 min 26 s (*H*). The length of the scale bar in *D* corresponds to 20 μm.

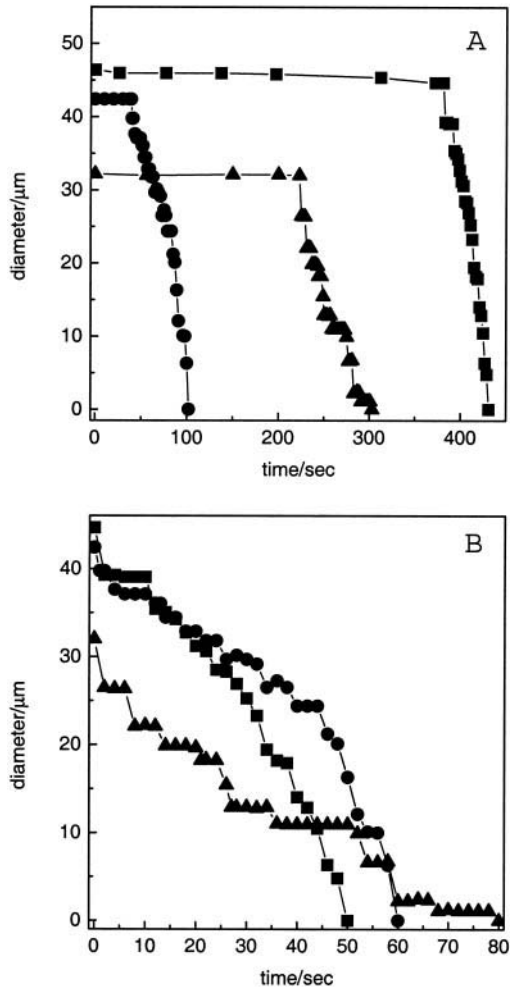

FIGURE 2 (*A*) Time course for the decrease of three SOPC GUV diameter following application of *B. cereus* phospholipase C to the vicinity of the membrane. (*B*) The last 50 s of the enzymatic digestion of the above GUVs shown in *A*.

(Holopainen et al., 2000a). In order to investigate the possibility that the difference in vesicle morphology caused by SMase and PC-PLC resulted from the presence of sphingomyelin, we used similar GUVs as in our earlier investigation, composed of SOPC, *N*-palmitoyl-sphingomyelin (C16:0-SM), and Bdp-SM as a fluorescent tracer (molar ratio 0.75:0.20:0.05, respectively). Upon the addition of PC-PLC to the vicinity of the outer surface of these GUVs, a stepwise shrinkage was again observed similarly to pure SOPC vesicles, thus ruling out the possibility that the vesiculation observed for SMase in our previous study was related to the vesicle composition (data not shown). Finally, we attempted to produce GUVs with high C16:0-SM contents. These were, unfortunately, significantly smaller compared to vesicles with no or low contents of SM and made it impossible to pursue sound experiments. A likely reason for this is the high main transition temperature for C16:0-SM (~41°C) hampering the electroformation of GUVs.

PC-PLC applied close to the surface of two or more initially adjacent vesicles can also cause their adherence (Fig. 4). Subsequently, the vesicles form a honeycomb structure with a flattened adhering surface. Slowly (within a few minutes) the adherence is then diminished while the vesicles remain in contact. Adhesion of GUVs in the buffer without PC-PLC was not observed, thus ruling out the possibility that the divalent cations (i.e., Ca^{2+}) added along with the enzyme would cause vesicle adhesion. Occasionally, yet only rarely, vesicle fusion was observed in the experiments after PC-PLC addition (data not shown). However, no obvious correlation with vesicle preparation, size, or enzyme amount could be established. The lack of vesicle shrinkage for two adhering GUVs could be due to the formation of the contact site due to the increased hydrophobicity while vesicle fusion may still not be energetically feasible. This is followed by the transfer and enrichment of diacylglycerols to the lipid residues on the platinum wire and subsequent detachment of the two vesicles allowing for the lack of vesicle shrinkage. Unfortunately, we are lacking a method to investigate this possibility.

In order to distinguish between unilamellar and multilamellar vesicles the emission intensity of the included fluorescent lipid tracer, DiA, was used. Up to approximately four bilayers the emission intensity of the GUV membrane increases as a multiple of the amount of bilayers present (Akashi et al., 1996). This allowed choosing vesicles with either one or multiple bilayers. The appearance of multilamellar GUVs in phase contrast microscope is somewhat smoother and occasionally also the different layers of the vesicle could be distinguished (data not shown). After the enzyme was applied on multilamellar GUVs only a minor decrease (<3%) in the vesicle diameter was observed in 60 min. After this time, the vesicle diameter remained practically unaltered. Notably, the series of sequential stepwise shrinkages such as described above for the unilamellar vesicles was not observed.

We then proceeded to study how the phase state of the lipid bilayer affects the PC-PLC induced changes in the topology of GUVs. For this purpose we used GUVs composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DMPC (Fig. 5 *A*). As the AC field method does not yield vesicles at temperatures below the main phase transition temperature (T_m), the vesicles were first formed at ~28°C, i.e., above T_m of DMPC at 24°C. Subsequently, the temperature was lowered to 13–16°C (see Materials and Methods for details) and the enzyme was applied externally to the vicinity of the outer surface of the vesicle. At $T < T_m$ the vesicles were not perfectly spherical (Fig. 5 *A*). After enzyme application the vesicles remained essentially unaltered (59.1 ± 0.05 μm) suggesting lack of hydrolysis of DMPC by PC-PLC even after prolonged incubation times (up to 100 min). When 10-fold more concentrated PC-PLC (8 U/ml; 4 μg/ml) solution was used the topological changes in the GUV were still absent (data not shown). Yet, fluid

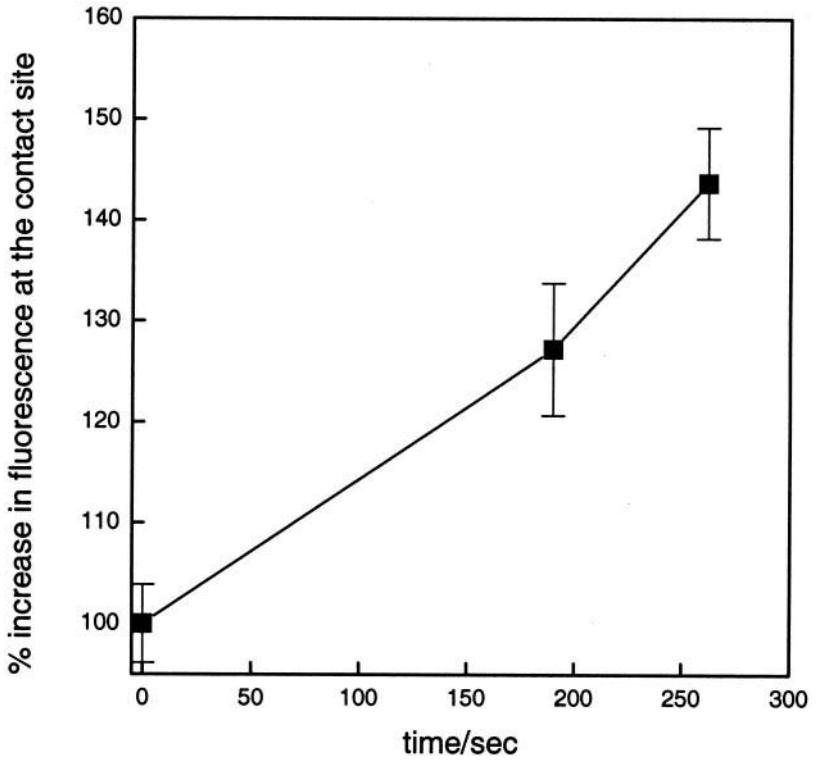

FIGURE 3 The increase in the emission intensity of DiA at the contact site of the vesicle to the platinum wire.

DMPC GUVs ($T > T_m$) behaved essentially similarly to the unsaturated SOPC vesicles, exhibiting a lag/shrinkage process following exposure to PC-PLC (data not shown).

Information on the hydrolysis rate and amount of product in gel state DMPC GUVs due to PC-PLC action would be of interest. Due to technical limitations this, however, is not feasible, as follows. First of all, a large population of GUVs with a reasonably small size distribution is difficult to obtain. Secondly, recovery of these GUVs without breaking them is not possible. Finally, the total amount of lipids in the GUV formation chamber is ~2–3 nmol, of which the amount of DAG is very small, thus preventing reliable quantitation. We attempted to quantify the progress of the PC-PLC reaction using a modification of the method used by Dorovska-Taran et al. (1996). In brief, headgroup labeled phosphatidylethanolamine (DPPF) was included into the GUVs which were then subjected to enzyme action. Upon cleavage of the phospholipid headgroup by PC-PLC the emission should decrease as the fluorescent probe is released into the aqueous phase. However, despite several trials we could not produce reliable data. This could result from small movements of the GUV in the focal plane, thus impeding quantitation in the decrease in emission intensity. Accordingly, we used large unilamellar vesicles (LUVs) to study the effect of the phase state on the action of PC-PLC. The enzyme exhibits a limited rate of hydrolysis with gel state DMPC as the substrate and shows a pronounced increase in activity at approximately the main phase transition temperature, whereas little changes in enzyme activity are evident upon further increase in temperature (Fig. 6). Although PC-PLC shows considerable activity toward DMPC LUVs at $T < T_m$, GUVs seem to be resistant to PC-PLC. This is likely to reflect the curvature difference between LUVs and GUVs which might play a role in producing packing defects and thus explaining the difference in the hydrolysis rates. The lack of lag/shrinkage behavior in gel state DMPC GUVs does not rule out that a small, critical amount of DMG could be formed in the gel state GUVs required for the observed phenomena. Alonso and Gõni and their coworkers, however, found that relatively high critical concentrations ~10 mol % were required for the sudden burst in the activity of PC-PLC (Basáñez et al., 1996). Taken together, it seems plausible to suggest that PC-PLC show no or only very little enzymatic activity toward gel state DMPC GUVs.

The activity of phospholipase A_2 toward large unilamellar vesicles has been demonstrated to depend on the lipid lateral packing (Lehtonen and Kinnunen, 1995). It has also been shown that the equilibrium lateral pressure for vesicles decreases upon the transition into the liquid crystalline state (Blume, 1979; Fulford and Peel, 1980; Konttila et al., 1988). For a phosphatidylcholine analogue, 1-palmitoyl-2-[10-(pyren-1-yl)-10-ketodecanoyl]-*sn*-glycero-3-phosphocholine, this decrement has been estimated to be ~22

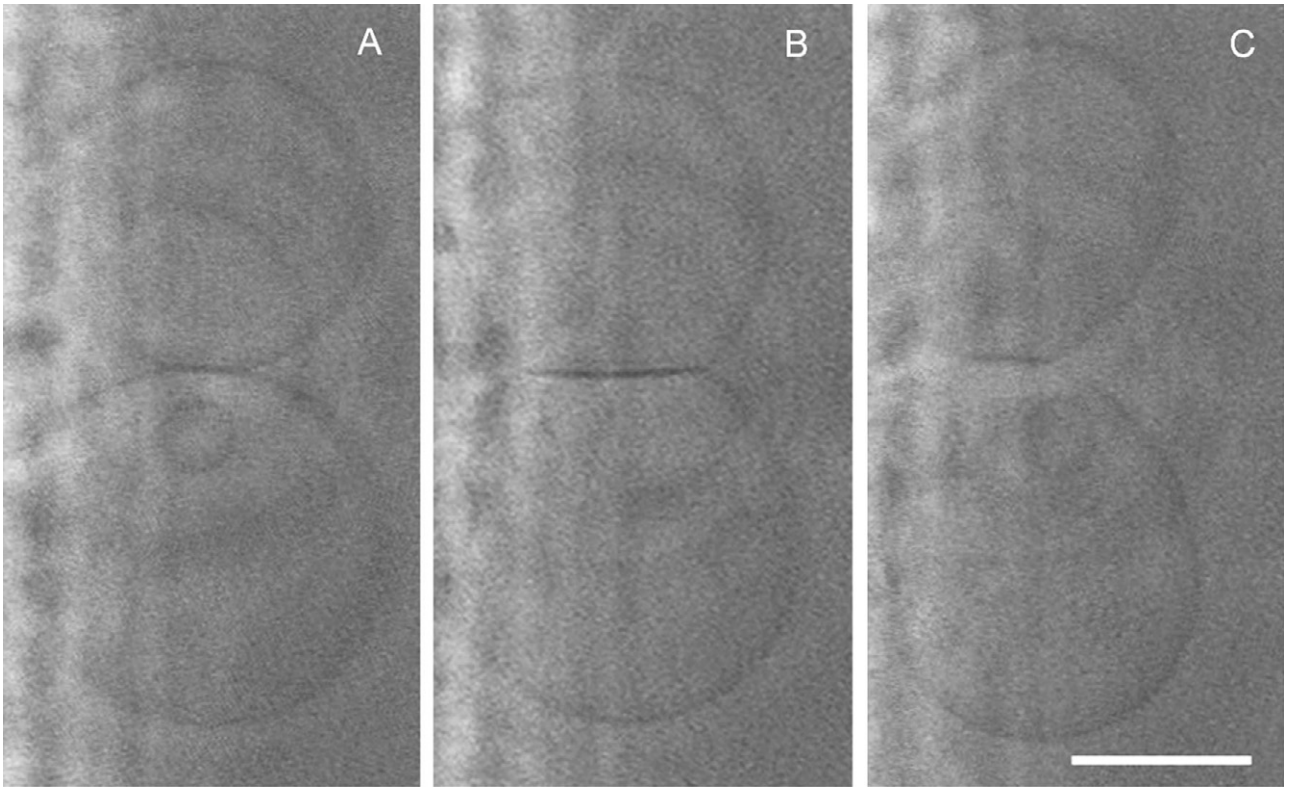

FIGURE 4 PC-PLC (*B. cereus*) causes the adherence of adjacent fluid phase SOPC giant vesicles. Still phase contrast images were taken before enzyme administration (*A*) and after 1 min 11 s (*B*) and 2 min 9 s (*C*). A small vesicle is visible inside the GUV (*A*). The length of the scale bar in *D* corresponds to 20 μm.

mN/m, from 39 to 17 mN/m (Konttila et al., 1988). For dipalmitoylphosphatidylcholine (DPPC) it has been estimated that this decrease would be somewhat larger, ~40 mN/m, from 65 to 25 mN/m (Fulford and Peel, 1980). For DMPC the decrease has been suggested to be ~20 mN/m (Blume, 1979). We explored the mechanistic basis for the above lack of PC-PLC activity toward gel state DMPC vesicles. Accordingly, we assessed the dependence of the activity of PC-PLC on the lipid lateral packing by measuring the decrease in surface pressure ($\Delta\pi$) caused by PC-PLC added into the subphase underneath a SOPC monolayer residing at the air/water interface at initial surface pressures (π_0) of 13 to 44 mN/m (Fig. 7 *A*). Below 30 mN/m the enzyme readily hydrolyzed the SOPC monolayer causing a rapid decrease in π. However, when $\pi_0 > 32$ mN/m the enzymatic reaction became very slow, with only minor decrement in π being evident. To emphasize dependence of the enzymatic reaction on π_0 these data are shown as the percent difference in the initial value for π_0 and final pressure π (20 min after enzyme addition) divided by the initial pressure ($(\pi_0 - \pi/\pi_0)$*100) vs. π_0, which revealed a sigmoidal behavior (Fig. 7 *B*). With increasing π_0 a major decrease in the relative surface pressure was evident until at $\pi_0 \sim 28$ mN/m, thus indicating that above this lateral pressure the enzymatic reaction was strongly attenuated, in accordance with previous studies (Daniele et al., 1996). These measurements with monolayers thus suggest that the lack of PC-PLC activity on DMPC GUV at $T < T_m$ could be due to the value of π in these vesicles exceeding 30 mN/m, in accordance with previous studies (Blume, 1979; Fulford and Peel, 1980; Konttila et al., 1988). Yet, although lipid monolayers are widely used as model membranes it should be emphasized that they cannot be considered to represent an exact equivalent to bilayers due to the very lack of the adjacent lipid monolayer (Brockman, 1999). The enzymatic reaction in monolayers may thus differ from that with a lipid bilayer as a substrate and the above conclusions should be considered as tentative only.

DISCUSSION

We have previously shown that SM specific PLC (SMase) cleaving the phosphocholine headgroup of sphingomyelin to yield ceramide induces vectorial budding of smaller vesicles from the surface of the GUV (Holopainen et al., 2000a). The experiments presented here addressed the effects of the closely related reaction catalyzed by PC-PLC removing the phosphocholine headgroup to yield diacylglycerol. Compared to the reaction catalyzed by SMase very

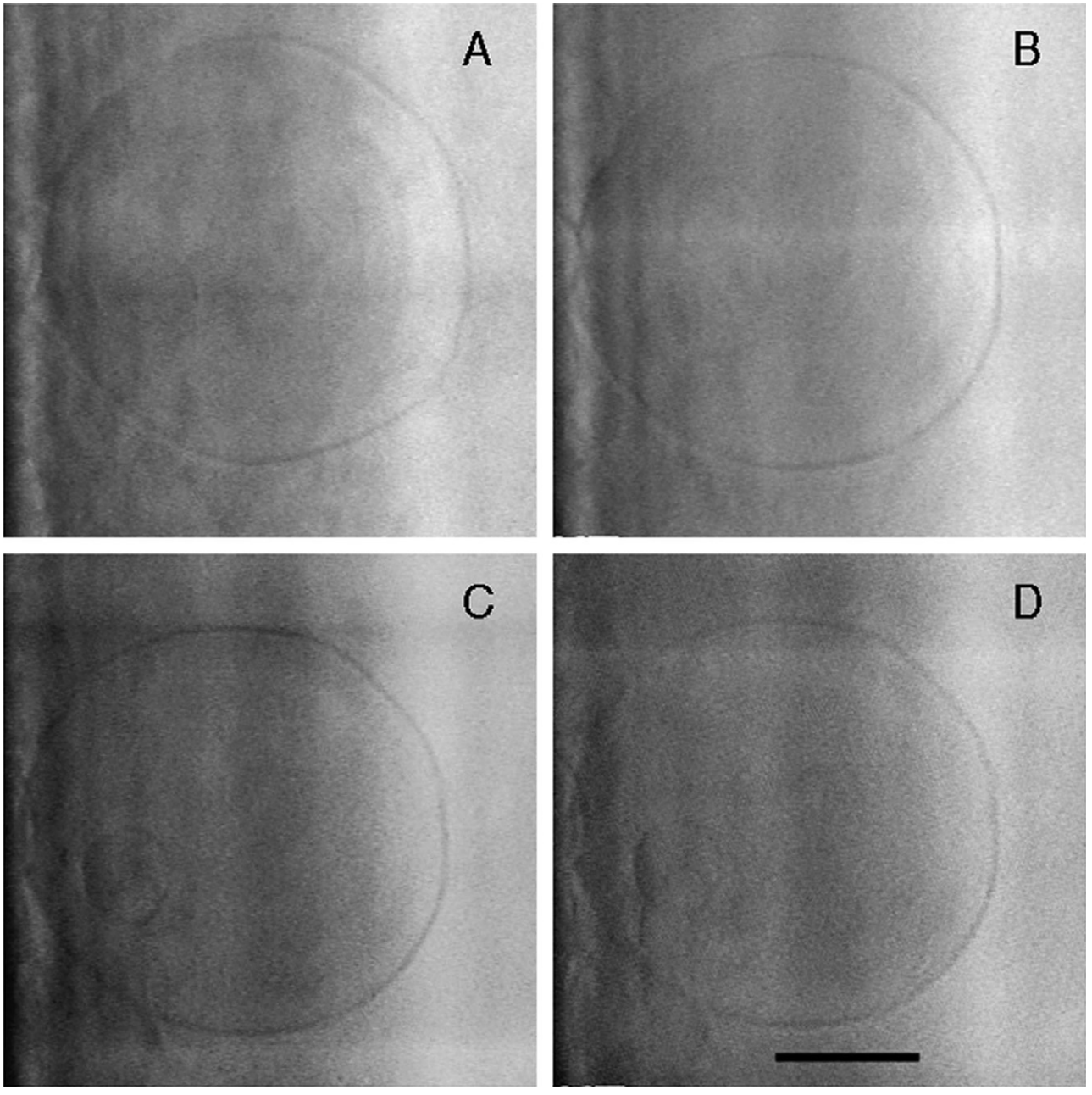

FIGURE 5 PC-PLC from *B. cereus* was applied to the vicinity of the outer surface of a single gel phase DMPC vesicle. Still phase contrast images were taken before enzyme administration (*A*) and after 15 min (*B*), 30 min (*C*), and 60 min (*D*). The length of the scale bar in *D* corresponds to 20 μm.

different morphological consequences were observed for the latter enzyme. In brief, the hydrolysis of SOPC GUVs by PC-PLC is characterized by sequential stepwise decrements in the vesicle diameter as a series of intermittent lags and shrinkages. A similar type of shrinkage/lag was also observed for saturated DMPC GUVs when in the liquid crystalline state, above the main phase transition temperature at 24°C. Instead, the above process was not observed for gel state liposomes, thus suggesting that in this state they cannot be hydrolyzed by PC-PLC. A probable reason for the apparent lack of activity of PC-PLC toward the DMPC vesicles at $T < T_m$ is the high lateral packing density of DMPC below the main transition and accordingly, for the enzyme it is more difficult to penetrate (even shallowly) into the lipid membrane. On the other hand, when in the fluid state ($T > T_m$) the lipid packing allows more efficient penetration to the membrane. This allows for optimal catalytic activity (Gabriel et al., 1987) and the observed lag-shrink behavior. This mechanism would be compatible with the critical surface pressure of ~30 mN/m observed for

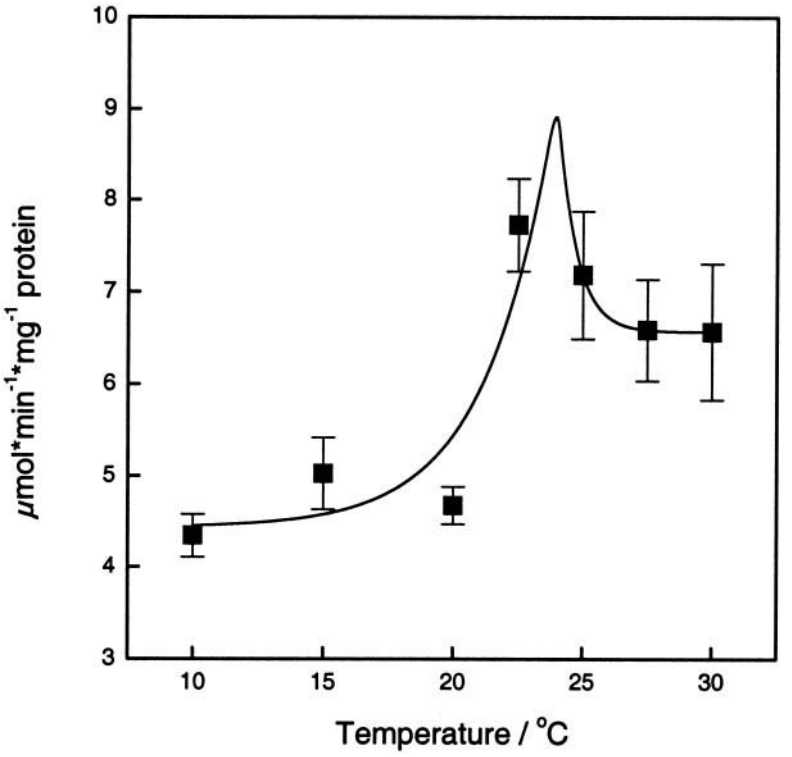

FIGURE 6 Temperature dependence of PC-PLC activity on DMPC large unilamellar vesicles. The reactions were started by the addition of 5 U/ml *B. cereus* PC-PLC and were allowed to proceed for 5 min in 0.5 mM Hepes, 10 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4. The formed dimyristoylglycerol was separated on a TLC plate developed with hexane/ethyl-ether/acetic acid (80:20:1, v/v/v) and subsequently quantitated densitometrically. The solid line represents a guide to the eye.

PC-PLC when using monolayers as a substrate (Fig. 7). Furthermore, the above shrinkage/lag behavior is not observed for multilamellar giant vesicles which only undergo a minor decrease in the vesicle diameter.

Phospholipases have been used as probes for membrane sidedness. Accordingly, PLA_2 can degrade 80% of the outer monolayer lipids without degrading those present in the inner monolayer (Sundler et al., 1978). In contrast, phospholipase C and D were not restricted in their action on the outer monolayer only. Yet, these authors used sonicated small unilamellar vesicles as the substrate. Comparison of these and our results therefore suggests that PC-PLC can act only on the outer monolayer of the GUV as demonstrated by the lack of the above morphological changes when using multilamellar giant vesicles as the substrate.

The dependence of the catalytic activity of PC-PLC on the physical state of the substrate has received significantly less attention compared to PLA_2, for instance. However, it was shown that similarly to PLA_2 the activity of PC-PLC is increased at the main phase transition temperature (Fig. 6; Vandenbranden et al., 1985) suggesting activation of these enzymes by the lateral heterogeneity of the membrane in the gel-fluid two phase region (Hønger et al., 1997).

Following the addition of PC-PLC to the vicinity of the GUV membrane surface the hydrolysis of phosphatidylcholine in the outer surface results in the production of diacylglycerol. The area per molecule of *sn*-1-stearoyl-2-oleoylglycerol (SOG) should be significantly smaller compared to that of SOPC. For the latter this is $\sim$62 Å^2 at $\pi = 30$ mN/m (H. L. Brockman, personal communication), unfortunately, for the former, exact A/molec is not available. Accordingly, as the volume of vesicle remains unaltered the conversion of SOPC to SOG by PC-PLC causes an increase in the membrane tension. Due to the formation of SOG the average area per molecule decreases compared to neat SOPC vesicles, leading to an increase in membrane tension. When tension reaches a critical value a transient pore is opened to release the water from the interior of the GUV and to restore the energy minimum. Accordingly, if a certain critical tension is needed to open the pore then the relative decrement in the vesicle surface area should be the same in all consecutive shrinkages. We analyzed the vesicle diameters for the decrement in different vesicles and the results show that the diameter decreases by 9.8 $\pm$ 1.4% in the consecutive shrinkages. This finding lends support to the idea that a constant mole fraction of SOG is needed to cause the stepwise shrinkages. Subsequently, due to the simultaneously progressing hydrolytic reaction, the membrane tension again increases, following the closing of the pore. This cycle, increase in membrane tension, opening of a transient pore, water efflux, and closing of the pore continues and is driven by the generation of SOG by PC-PLC. It should, however, be stressed here that evidence for the increased tension/pore/water efflux cycle is scarce. Unfortunately, we are lacking a suitable method to assess experimentally the above putative process. Phosphatidylcholine/diacylglycerol mixtures have been extensively studied. Diacylglycerol has a limited solubility at $\sim X = 0.40$ in lamellar phosphatidyl-

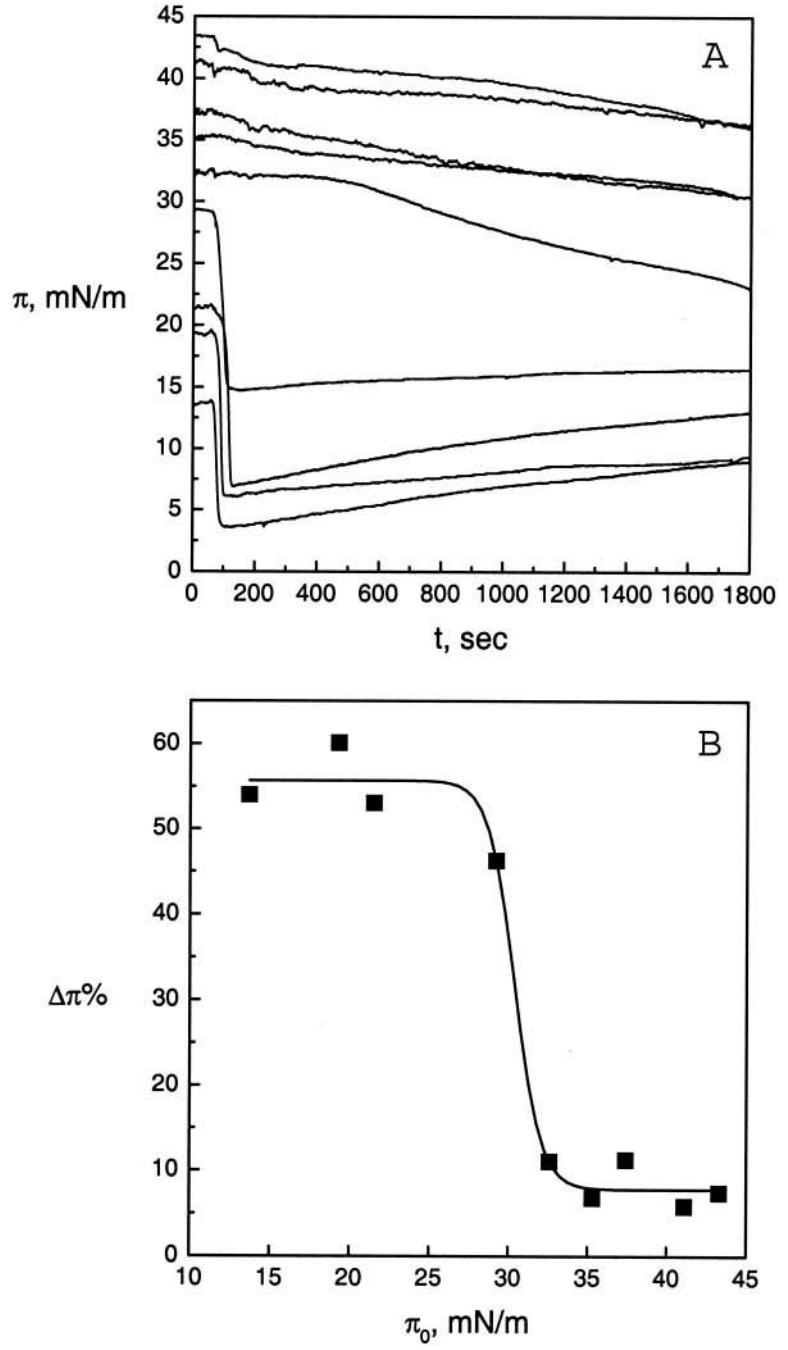

FIGURE 7 (*A*) Changes in the surface pressure following the injection of *B. cereus* phospholipase C into the subphase. (*B*) Relative changes in the pressure following the application of *B. cereus* PC-PLC. Data taken from *A*.

choline (Small, 1986; Cunningham et al., 1989). Exceeding this mole fraction an isotropic phase should form, probably separating as a fluid phase within the membrane. Our fluorescence microscopy data support the view that upon hydrolysis the newly formed SOG is accumulated in the area of the vesicle-vesicle contact sites on the platinum wire.

It is relevant to note that the in vitro conditions used here are very different from those in vivo. Allan et al. (1978) showed that when red blood cells were treated with PC-PLC diacylglycerol is produced only in the outer leaflet of the plasma membrane. Within one minute, however, a significant amount of diacylglycerol is found in the inner leaflet of the plasma membrane and is further rapidly converted into phosphatidic acid by diacylglycerol kinase. Cells remained intact with the hydrolysis of cells being less than 5%. Transbilayer diffusion of a fluorescent derivative of diacylglycerol is rapid, with a halftime of ~70 ms (Bai and Pagano, 1997). Accordingly, it is possible that the *sn*-1-stearoyl-2-oleoyl-glycerol (SOG) produced from SOPC transfers into the inner leaflet by flip-flop. In contrast, the flip-flop rate of ceramide is much slower (Bai and Pagano, 1997) and thus strain due to negative spontaneous curvature

of the outer leaflet would be caused upon the action of SMase, leading to the formation of small vesicles (Holopainen et al., 2000a; Kinnunen et al., 2000). Interestingly, if endogenous phosphatidylinositol-PLC is activated by Ca^{2+} vectorial budding of vesicles from the outer leaflet of the red blood cell is observed (Allan et al., 1976; Allan and Michell 1977). This suggests that also diacylglycerols could induce vesiculation when the processing of this lipid into phosphatidic acid is relatively slow. Yet, the peculiar acyl chain composition of phosphatidylinositol could also be important (Hodgkin et al., 1998).

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