Imaging the early stages of phospholipase C/sphingomyelinase activity on vesicles containing coexisting ordered-disordered and gel-fluid domains

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Abstract The binding and early stages of activity of a phospholipase C/sphingomyelinase from Pseudomonas aeruginosa on giant unilamellar vesicles (GUV) have been monitored using fluorescence confocal microscopy. Both the lipids and the enzyme were labeled with specific fluorescent markers. GUV consisted of a mixture of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and cholesterol in equimolar ratios, to which 5–10 mol% of the enzyme end-product ceramide and/or diacylglycerol were occasionally added. Morphological examination of the GUV in the presence of enzyme reveals that, although the enzyme diffuses rapidly throughout the observation chamber, detectable enzyme binding appears to be a slow, random process, with new bound-enzyme-containing vesicles appearing for several minutes. Enzyme binding to the vesicles appears to be a cooperative process. After the initial cluster of bound enzyme is detected, further binding and catalytic activity follow rapidly. After the activity has started, the enzyme is not released by repeated washing, suggesting a “scooting” mechanism for the hydrolytic activity. The enzyme preferentially binds the more disordered domains, and, in most cases, the catalytic activity causes the disordering of the other domains. Simultaneously, peptide- or figure-eight-shaped vesicles containing two separate lipid domains become spherical. At a further stage of lipid hydrolysis, lipid aggregates are formed and vesicles disintegrate.—Ibarguren, M., D. J. López, L.-R. Montes, J. Sot, A. I. Vasil, M. L. Vasil, F. M. Goñi, and A. Alonso. Imaging the early stages of phospholipase C/sphingomyelinase activity on vesicles containing coexisting ordered-disordered and gel-fluid domains. J. Lipid Res. 2011. 52: 635–645.

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Phospholipases are essential enzymes in maintaining membrane homeostasis and in the generation of metabolic signals. They are also powerful tools that bacteria use to infect and eventually destroy eukaryotic cells by helping in the degradation of the target cell membranes. Phospholipase C (PLC) enzymes cleave the phosphodiester bond between the diacylglycerol moiety and the phosphoryl base (phosphorylcholine, phosphorylethanolamine, and others), characteristic of each phospholipid class. Most sphingomyelinases hydrolyze the equivalent bond between ceramide (Cer) and phosphorylcholine in sphingomyelin (SM). From the point of view of their enzyme activities, phospholipases and lipases in general are rather unique among enzymes in that their substrates and end products do not occur freely in solution but are instead found to make up part of the cell membrane.

Early work from our laboratory (1) had shown that phospholipid hydrolysis catalyzed by PLC from Bacillus cereus was able to induce aggregation and fusion of large unilamellar vesicles (LUV). That work was confirmed by other studies (2) and was later extended to other PLCs (3–7). More recently, Montes et al. (8) described the leakage-free membrane fusion induced by the hydrolytic activity of...
PlcHR<sub>2</sub> from <i>Pseudomonas aeruginosa</i>. This enzyme is the paradigm member of a novel PLC/phosphatase superfamily, first described in one of our studies (9).

A different line of research, also pertinent to the present work, has underlined the significance of lateral heterogeneity, i.e., the existence of domains in cell membranes (10). Studies of pure lipid vesicles (liposomes) have demonstrated that even with pure lipids or simple two-lipid mixtures, domain formation can occur. The coexistence of gel and fluid (11–13), gel and liquid-ordered (14), and fluid-ordered and fluid-disordered domains (15) has been shown by a variety of physical methods (16–18). Moreover, the advent of giant unilamellar vesicles (GUV) (19, 20) and the application of confocal fluorescence microscopy (21, 22) have allowed visualization of domains and domain coexistence in a variety of lipid membranes. In a recent study from one of our laboratories (23), fluorescence confocal microscopy and differential scanning calorimetry were used in combination to study the phase behavior of GUV bilayers composed of phosphatidylcholine, SM, phosphatidylethanolamine, and cholesterol (PC:SM:PE:Chol) in equimolar mixtures, in the presence or absence of 10 mol% egg Cer. In the absence of Cer, separate liquid-ordered and liquid-disordered domains were observed in the vesicles. In the presence of Cer, gel-like domains appeared within the liquid-ordered regions. The morphological and calorimetric data were interpreted in terms of cholesterol displacement by Cer from SM- and Chol-rich liquid-ordered regions. In contrast to Cer, when 10 mol% egg diacylglycerol (DAG) was added to the PC:SM:PE:Chol mixture, homogeneous vesicles consisting only of liquid-disordered bilayers were observed (23). This study confirmed and expanded several related observations from other laboratories (24–26).

The relationship between phospholipase activity and lipid phase behavior remains a field of active research. Several details regarding the kinetics, morphology, and mechanisms of formation of segregated domains by some phospholipases, including sphingomyelinase, have been elucidated using different model membrane systems and mixtures of lipids. The dependence of the activities of phospholipase A<sub>2</sub> (PLA<sub>2</sub>), PLC, and sphingomyelinase on the lipid physical state, showing that these enzymes preferably degrade the more fluid phase state, was demonstrated in early studies using lipid monolayers and bilayer vesicles (27–32). Our previous studies of PLC-promoted membrane fusion correlated enzyme activity and the generation of nonlamellar structures that would in turn be fusion intermediates (33). Further results from our laboratory (34) have related the activity of phosphatidylinositol-specific PLC to the degree of molecular order in membranes. Also, Bell and co-workers (35, 36) and Mouriizen and co-workers (37, 38) have explored the relationship between PLA<sub>2</sub> activity and the physical state of the membrane. Holopainen et al. (39) observed lipid microdomain formation that resulted from sphingomyelinase activity on fluid membranes of PC/SM. Contreras et al. (40) reported that bacterial sphingomyelinase activity varied with the physical state of the substrate, increasing in the order gel < liquid-ordered < liquid-disordered. Mechanisms for phospholipase action, including domain formation by sphingomyelinase, have been reported recently (41–43). Also, the dependence of sphingomyelinase activity on the membrane physical properties has been stressed in a recent report (44). GUVs have been used in connection with the study of lipases (45, 46). In particular, Holopainen et al. (47) described the vectorial budding of vesicles by asymmetric enzymatic formation of Cer in giant liposomes. Equally relevant in this context are the studies by Holopainen et al. (48) and López-Montero et al. (49), in which lipases were added in situ to single GUVs, and various effects, e.g., vesicle shrinking or generation of surface tension, were observed.

The present contribution is intended to provide a link between the bulk (in cuvette) studies of lipase activity on lipid bilayers in different physical states (27–44) and the morphological data obtained largely with GUV of homogenous lipid compositions (45–49). This work is based on our previous studies of phase separation in GUVs (23) as well as our experience with PlcHR<sub>2</sub> (8, 50). A detailed description of the binding and activity of PlcHR<sub>2</sub> on GUV exhibiting gel/fluid and fluid-ordered/fluid-disordered domain coexistence is offered, and mechanistic aspects of PlcHR<sub>2</sub> activity are discussed.

**MATERIALS AND METHODS**

**Materials**

PlcHR<sub>2</sub> was purified as previously described (9). Egg PC, egg PE, and egg DAG were purchased from Lipid Products (South Nutfield, UK). Egg SM, egg Cer, and Chol were from Avanti Polar Lipids (Alabaster, AL). The compound 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and an Alexa Fluor 633 protein labeling kit were from Invitrogen (Eugene, OR).

**Construction and purification of PlcHR<sub>2</sub> T178A**

The Thr178Ala mutant PlcH was identified based on an alanine scanning mutagenesis experiment to identify enzymatically deficient mutants. A 1,000 bp region of the plcH gene was mutagenized, and the pooled mutated DNA was used to replace the corresponding wild-type plcH gene in an <i>Escherichia coli</i> plasmid (51). Individual clones carrying the mutated plcH gene were then screened for PLC activity using the synthetic PLC substrate p-nitrophenyl phosphatidylcholine (NPPC) (9). Individual clones that expressed either low or no NPPC activity compared with one that had the wild-type plcH gene were fully sequenced to determine the location of the individual mutations. One of these clones that carried the mutation, in which Thr was changed to Ala at position 178, was chosen for further characterization. The plcH gene carrying the Thr178Ala mutation, along with the plcR gene (encoding a chaperone for PlcH secretion), was then cloned into an expression plasmid (pADD9268) with a T7 promoter and transformed into a <i>P. aeruginosa</i> strain (pADD1976), which carries a chromosomal isopropyl β-D-thiogalactopyranoside-inducible T7 polymerase driven by a lacUV5 promoter (9). This expression system allows the expression and secretion of mutant PlcHR proteins (e.g., Thr178Ala). This mutant was then purified from the culture supernatant as previously described for native PlcHR (9). However, because the Thr178Ala mutant has little or no detectable PLC activity, we tracked its purification using
monoclonal or affinity-purified antibodies against PlcH and PlcR (9). Circular dichroism spectroscopy and thermal denaturation studies revealed there were no significant differences between the structure of the wild-type PlcHR and that of the Thr178A mutant (data not shown).

It is noteworthy that Thr178 of PlcH corresponds to Ser175 in a PlcH ortholog (i.e., AcpA), which is expressed by Francisella tularensis (52). However, unlike PlcH, AcpA has phosphatase activity but not authentic PLC activity (51). The Ser175 of AcpA was identified in the active site of AcpA, which is only defined based on the crystal structure of AcpA in context of the interactions of 10 amino acids, including Ser175, with vanadate (a phosphate analog), which provides a model for the phosphate moiety of a phosphomonoester or phosphodiester bond of an AcpA or PLC substrate (52).

**GUV preparation and fluorescence microscopy**

GUVs were prepared using the electroformation method developed by Angelova et al. (19), using a specialized chamber supplied by L. A. Bagatolli (Odense, Denmark) that allows direct visualization under the microscope (53). Stock solutions of lipids (0.2 mg/ml total lipid containing 0.2 mol% DiI) were prepared in a chloroform-methanol (9:1, v/v) solution. Three milliliters of the appropriate stocks were added to the surface of Pt electrodes, and solvents traces were removed by evacuating the chamber under high vacuum for at least 2 h. Then, the Pt electrodes were covered with 400 µl of 10 mM HEPES (pH 7.4), previously heated to 60°C. The Pt wires were connected to an electric wave generator (TG330 function generator; Thurlby Thandar Instruments, Huntingdon, UK) under AC field conditions (frequency, 10 Hz; amplitude, 1 V) for 2 h at 60°C. After GUV formation, the chamber was placed on an inverted confocal fluorescence microscope (D-Eclipse C1 model; Nikon Inc., Melville, NY). The excitation wavelength for DiI was 561 nm. The images were collected using band-pass filters of 593 ± 20 nm. When required, 2 µl of PlcHR2, labeled with Alexa Fluor 633, at 12 µg/ml, was added to study its effect on the GUVs. In the latter case, an excitation wavelength at 635 nm was used, and images were collected using a long-pass filter of 650 nm. All these experiments were performed at 25–28°C. Image treatment and quantification were performed using EZ-C1 version 3.20 software (Nikon Inc., Melville, NY).

**LUV preparation**

LUV with diameters of 100–150 nm were prepared by the extrusion method using Nuclepore filters of 0.1 µm pore diameter at room temperature in a solution of 25 mM HEPES, 100 mM NaCl (pH 7.4). Quantitative analysis of the lipid composition of our LUV preparations, as described by Ruiz-Argüello et al. (54), showed that it did not differ significantly from the initial lipid mixture. All experiments were performed at 28°C. Lipid concentration was 0.3 mM, and PlcHR2 was used at 0.5 µg/ml.

**Protein labeling**

An Alexa Fluor 633 protein labeling kit from Molecular Probes, Inc. (Eugene, OR) was used. This kit labels primary amino groups. The concentration of labeled protein was 60 µg/ml, and each mole of protein contained 4 M dye. Alexa Fluor 633 dye-labeled proteins have fluorescence emission maxima of approximately 692 nm and 647 nm.

**Enzyme activity**

PlcHR2 activity was assayed by determining water-soluble phosphorus. Liposome concentration was 0.3 mM in all experiments. The enzyme was assayed at 28°C in a solution of 25 mM HEPES, 100 mM NaCl (pH 7.4). Enzyme concentration was 0.5 µg/ml. Aliquots (50 µl) were removed from the reaction mixture at regular intervals and extracted with 250 µl of a chloroform-methanol-hydrochloric acid mixture (200:100:1, v/v/v), and the aqueous phase was assayed for phosphorus.

**Statistics**

Unless otherwise indicated, data are average values of at least three independent measurements ± 1 standard deviation. Student’s t-test was used in order to assess the significance of observed differences.
RESULTS AND DISCUSSION

Enzyme activity on LUV

The hydrolytic activity of PlcHR2 was assayed on LUV composed basically of PC:SM:PE:Chol (1:1:1:1, molar ratio) at 28°C, the temperature at which confocal microscopy observations were performed. This mixture was used because it contains the main lipids found in mammalian plasma cell membranes and because it was well characterized from previous studies (8, 23). The basic lipid composition was varied, including either 10 mol% Cer, 10 mol% DAG, or 5 mol% Cer plus 5 mol% DAG, to test the effect of enzyme end products in the bilayer. The results can be seen in Table I. The initial rate of hydrolysis for the basic lipid mixture was $3.1 \pm 0.23$ nmol/min. Initial rates are only slightly, albeit significantly, influenced by lipid composition. The presence of egg DAG with and without Cer causes a small increase in enzyme rate, presumably because of the overall fluidifying effect of DAG and/or due to its tendency to favor negative membrane curvature (23, 33). At 5 min after enzyme addition, about 15% of the PC plus SM solution was hydrolyzed in all mixtures, except the one containing DAG plus Cer, in which 29% hydrolysis was found. At 10 min after PlcHR2 addition, the corresponding values were approximately 33% and 53%, respectively. The 5 and 10 min times were selected for quantitating the extent of lipid hydrolysis because they mark the end of two different periods in the evolution of vesicle morphology (see below).

Hydrolytically inactive T178A mutant

A PlcHR2 mutant was generated in which Thr178 was changed into an Ala residue. This mutation led to a complete loss of phospholipase activity (Fig. 1A). The PlcHR2 T178A mutant was also unable to induce vesicle aggregation or release intravesicular aqueous contents (55), while the native enzyme did exhibit those properties (8, 50). However, the T178A mutant’s ability to bind LUV composed of PC:SM:PE:Chol (1:1:1:1, molar ratio) was undistinguishable from that of the wild type (Fig. 1B), according to measurements in which the intrinsic enzyme fluorescence was titrated with increasing concentrations of lipid.

Imaging PlcHR2 binding to GUV

The use of GUV allows the direct observation of vesicle and domain morphology and the enzyme effects thereupon. PlcHR2 has been added to preformed GUVs under confocal fluorescence microscopy, and the subsequent morphological changes have been observed and quantitated. Because of the experimental limitations derived from the vesicle thermal motion, the continuous observation of a single vesicle for a period of minutes, as required in this experiment, was only possible at or below 28°C. Thus, GUV experiments were performed at 26–28°C. Lipids from a natural origin (egg) have been used for GUV preparation, despite the additional complexity that this brings to the system, including the possibilities of interdigitation and fluid–fluid immiscibility (56). However, our choice of lipids was guided by our previous work with

Fig. 2. Images show PlcHR2 binding to GUVs. The enzyme is derivatized with Alexa 633 (pseudocolor blue). Vesicles newly stained with Alexa 633 are shown at different times after enzyme addition. A–C: The gradual binding of enzyme to the same set of vesicles is shown over 50 s (bar = 10 μm). D: A summary of the number of newly stained vesicles is shown as a function of time in 10 videos (10 independent experiments for each lipid composition). Mixture (Mix) = 5 mol% DAG plus 5 mol% Cer. E–J: A gallery of newly stained vesicles is shown at the time they were first detected.

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could be complementary to the one based on supported “membrane islands,” as proposed by Simonsen et al. (41).

PlcHR2 was labeled with Alexa 633 in order to visualize its binding and localization in the vesicle bilayer. Control experiments had shown that Alexa derivatization had not altered the enzyme-specific activity. Adding the Alexa-stained enzyme to the GUV chamber causes the instantaneous appearance of a uniform bright background against which the vesicles are seen as dark objects (Fig. 2A). This shows that the enzyme diffuses freely and uniformly in the medium, under our experimental conditions, in a very short time (<10 s) and that it does not penetrate into GUV. Afterward, the accumulation of bright spots on the vesicle surface indicates enzyme absorbance/binding. Interestingly, enzyme binding does not occur with all vesicles simultaneously but is, rather, a random process that extends over minutes. Figure 2B and C shows two stages separated by 30 and 50 s of enzyme binding to the vesicles in the frame seen in Fig. 2A. Note that the initial enzyme binding does not occur uniformly along the vesicle surface. Rather, the enzyme appears to be located initially in small areas (Fig. 2E–J) from which binding propagates along larger fractions of the GUV surface.

When a large number of vesicles are observed, in independent experiments, the phenomenon can be shown in a quantitative way, as shown in Fig. 2D, which summarizes the results of about 10 videos (i.e., 10 independent experiments) for each lipid composition. In all cases, the number of new vesicles containing enzyme clusters increases linearly and slowly for several minutes. Control vesicles, i.e., those containing PC:SM:PE:Chol (in equimolar proportions) become stained at a somewhat higher rate than those containing DAG and/or Cer in addition. Note that...
the data in Fig. 2D correspond to a count of the vesicles that appeared to the observer as newly enzyme bound. Presumably a sizable amount of enzyme molecules must adhere/bind to the GUV bilayer to be detected. It may be that enzyme binding occurs initially in a uniform way and that only after localized cooperative phenomena are the enzyme-enriched membrane regions observed that allow us to detect bound enzyme-containing vesicles. The reason why newly stained vesicles appear over a long time interval is presently unknown. Speculatively, enzyme binding would be favored by thermal instabilities occurring at random in the membranes. According to the data shown in Fig. 2D, control vesicles bind the enzymes faster than those containing DAG or DAG plus Cer. As described in our previous work (23), macroscopic phase separation (liquid-ordered and liquid-disordered) was observed in control GUV but not in those containing DAG or DAG plus Cer. Lipid phase separation may occur, at the phase boundary, favorable conditions for instabilities and enzyme binding. Also, as shown in Fig. 2D, GUV containing Cer are seen to be slow enzyme binders. In this case, the Cer-rich rigid domains described previously (23) may display low affinity for enzyme binding. De Tullio et al. (42), in studies using lipid monolayers containing SM and Cer, have observed that sphingomyelinase is preferably located in the SM-rich, liquid-expanded phase, rather than in the Cer-enriched, liquid-condensed areas. Interestingly, those authors do not find an enrichment in enzyme at the phase boundaries.

The results in Fig. 2 support the hypothesis of a cooperative enzyme binding to the bilayer, which would give rise to enzyme-enriched regions detectable under the microscope. Interestingly, Basañez et al. (61), on the basis of cuvette kinetic measurements of PLC on LUV, suggested that the end of the lag period of this enzyme would be marked by the cooperative binding of enzyme molecules to DAG-enriched spots. The data in Fig. 2 appear to confirm this proposal, thus clarifying a mechanistic aspect that may be common at least to several PLC enzymes, namely the cooperative binding to discrete membrane spots at the start of the “burst” of lipase activity. This lag-burst behavior was observed for PLC in GUV by Holopainen et al. (48).

Once a newly stained vesicle is spotted, the increase in membrane-bound Alexa 633 can be quantitated. The results (Fig. 3) show that once started, enzyme binding progresses linearly at least for the first 20 s. Reliable measurements at longer times are precluded by the secondary effects caused by enzyme products, DAG and Cer, and concomitant degradation of PC and SM, which alter the bilayer structure in various ways. Remarkably, GUV containing Cer, which were the slowest to show enzyme binding (Fig. 2D), displayed the highest rate of binding once the first enzyme cluster had been detected (Fig. 3B). This is in agreement with the observation that the formation or presence of Cer in lipid monolayers causes increased enzyme adsorption and faster precatalytic activation (but reduced enzyme activity) in independent, surface-regulated processes (62). A comparison of the data in Figs. 2 and 3 suggests that the initial and subsequent steps of phospholipase binding are different phenomena. For vesicles containing 10% additional DAG (Fig. 3C), enzyme binding cannot be reliably measured beyond 15 s due to fast formation of lipid aggregates, presumably nonlamellar, in the bilayer.

**PlcHR 2 preferentially binds the more disordered domains**

With respect to the distribution of PlcHR 2 among the various domains, the enzyme has a preference for the more disordered domains (Fig. 4), i.e., those into which DiI partitions preferentially (23). The preferential partition of DiI into the more disordered domains cannot be taken for granted (63). In our study, control experiments using Laurdan staining and two-photon confocal microscopy demonstrated that, indeed, DiI partitions preferentially into the liquid-disordered domains (see supplementary material).

**Fig. 5.** Effect of repeated washing on the binding of PlcHR 2 to GUV is shown. The persistence of bound enzyme after several washing steps is compatible with a processive or “scooting mode” kinetic mechanism of the enzyme.

**Fig. 6.** Binding of the inactive T178A mutant of PlcHR 2 to GUVs is shown. The mutant enzyme is derivatized with Alexa 633 (pseudocolor blue), and the more fluid domains in the membrane are stained with DiI (pseudocolor orange). Experimental conditions are as shown in Fig. 3. Images were taken at 0, 480, and 960 s after mutant enzyme addition.
The results (Fig. 5) indicate that PlcHR2 binding to GUV bilayers is irreversible in the time scale of minutes. This would be compatible with the enzyme operating in the processive or “scooting mode” (65). It is interesting in this respect that Code et al. (66) have observed formation of amyloid-type fiber upon interfacial activation of PLA2. This can be related to the aggregation of PLC molecules in defined patches, as proposed by Basañez et al. (61), as the required step before the burst in enzyme activity.

GUV binding of the catalytically inactive PlcHR2 T178A mutant

In order to measure enzyme binding to GUV in the absence of perturbations due to the catalytic activity of PlcHR2, the catalytically inactive T178A mutant was used. Alexa 633 labeling was performed as with the wild type. As shown in Fig. 1B, the mutant binds GUV in much the same way as the wild type, except that the structural changes caused by the wild-type enzyme in the bilayer (see below) are not observed with the mutant, even after long incubation times. Extensive merging of the Alexa 633 (enzyme) and DiI (disordered lipid) are observed (Fig. 6, bottom images), confirming the enzyme’s tendency to preferentially bind domains in the liquid-disordered state.

Effect of enzyme concentration

Enzyme concentration in the observation chamber was estimated to be about 12 μg/ml under our conditions. This value is higher than most concentrations used for PLC or sphingomyelinase assays in cuvette or in the trough of a Langmuir balance. However, it is not clear that our conditions are comparable to those used in the bulk studies, as the small thickness of our chamber (<200 μm) and the presence of the giant vesicles must decrease the enzyme diffusion due to a massive effect of unstirred layers. Generally, our observations can be considered a slow-motion version of the in vivo situation. Several experiments were performed at approximately 6 and 3 μg/ml enzyme. The same results were observed, except that reactions took longer to occur (see supplementary Fig. 1).

Irreversible binding of PlcHR2 to GUV bilayers

The irreversibility of PlcHR2 binding to GUV bilayers was tested by incubating the vesicles with Alexa-stained enzyme for 5 min and then flushing the chamber thoroughly with buffer until the background fluorescence, corresponding to free enzyme had disappeared. The vesicle fluorescence was photographed, and the washing and photographing cycle was repeated twice more. The results (Fig. 5) indicate that PlcHR2 binding to GUV bilayers is irreversible in the time scale of minutes. This would be compatible with the enzyme operating in the processive or “scooting mode” (65). It is interesting in this respect that Code et al. (66) have observed formation of amyloid-type fiber upon interfacial activation of PLA2. This can be related to the aggregation of PLC molecules in defined patches, as proposed by Basañez et al. (61), as the required step before the burst in enzyme activity.

Fig. 7. Evolution of the bright (more-fluid) and dark (less-fluid) domains in GUVs is shown as a function of enzyme activity. Times after enzyme addition are given for each frame: (A) control; (B) with 10 mol% Cer; (C) with 5 mol% Cer plus 5 mol% DAG. Equatorial planes are shown. Assays were performed at 28°C. Bar = 10 μm.

In order to measure enzyme binding to GUV in the absence of perturbations due to the catalytic activity of PlcHR2, the catalytically inactive T178A mutant was used. Alexa 633 colocalizes with DiI in all cases. The ratios of enzyme (Alexa 633) in the more-disordered region to enzyme in the less-disordered region per unit length are 2.9 ± 1.0 (control mixture) or 6.7 ± 3.9 (with 10% Cer), as estimated from experiments similar to those shown in Fig. 4. Note that rigorous calculation of this parameter would require quantitation of bright and dark areas, rather than perimeters. Only recently has a procedure allowing area measurements been published (64). However, our values can still be considered useful approximations. Also, Alexa 633 is known to exhibit a degree of self-quenching so that the actual ratios may be somewhat higher than estimated.

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2011/01/20/jlr.M012591.DC1.html
shown in Fig. 7. The plotted values would parallel the progressive increase in size of the fluid-disordered domains. The overall intensity of DiI fluorescence emission per vesicle was also quantitated and found to be constant over time in the time scale of the experiments shown in Fig. 7 (data not shown). This implies that the overall vesicle structure is maintained under these conditions, when 15%–30% of PC plus SM vesicles have been hydrolyzed. The invariant overall intensity of DiI fluorescence also shows that bilayer order influences probe partition but not probe fluorescence yield.

A similar morphology is observed for enzyme-treated vesicles containing 10 mol% Cer, i.e. the bright domains increase at the expense of the dark ones (Fig. 7B). However, Cer at these concentrations is known to give rise to gel domains in bilayers (59, 60, 67–69), and Sot et al. (23) have shown that under the conditions of the present study, the dark domains seen in Fig. 7B at time 0 consist partly of liquid-ordered and partly of gel phases. Thus, in this case, a gel phase and a fluid-ordered phase are being converted into a fluid-disordered phase. Quantitative data are also very similar (Fig. 8B). Cer-containing vesicles are sometimes found to have a figure-8 (or peanut) shape (23), in which the dark domain occupies one, usually the largest, of the spherical segments. With enzyme activity, the vesicles become spherical, as shown quantitatively in Fig. 9. This is probably related to the morphological effects of Cer generation in GUV, as described by Devaux and co-workers (49, 70).

An interesting phenomenon is observed with vesicles containing 5 mol% Cer plus 5 mol% DAG. These vesicles are nearly homogeneous in appearance, with the occasional small dark domain (Fig. 2C). However, the enzyme activity in these vesicles gives rise to the formation of dark, gel-like or ordered domains (Figs. 7C, 8C). Rigid domain formation appears to be the result of a subtle equilibrium between the Cer tendency to lateral separation and the DAG activity toward lateral homogenization of the bilayer, both of which are modulated by the remaining lipids, PC, SM, PE, and Chol. The enzyme activity alters the compositional equilibrium by decreasing PC and SM and converting them into DAG and Cer, thus also modifying the lateral distribution of lipids in the bilayer. Fanani and Maggio (31) described the mutual modulation of sphingomyelinase and PLA2 activities when both enzymes were added to a mixture of the corresponding substrates. Considering that PlcHR2 has both sphingomyelinase and PLC activity, a similar phenomenon may be taking place here, only with one enzyme and two sets of substrates/products.

CONCLUSIONS

A system containing a complex, laterally heterogeneous lipid bilayer and an enzyme that both modifies and is influenced by the bilayer properties constitutes an attractive, although complicated, object of study. Enzyme addition generates a nonequilibrium situation in the membrane. Still, the combination of biochemical and morphological

**Fig. 8.** Quantification of the data in vesicles was carried out as shown in Fig. 5. A: control; (B) with 10 mol% Cer; (C) with 5 mol% Cer plus 5 mol% DAG. C: Open circles indicate bright portions of the perimeters; closed circles indicate dark portions of the perimeters. Data are average values of 6–8 vesicles ± SD. Total (100%) time is 300 ± 153 s (A), 214 ± 114 s (B), and 208 ± 83 s (C).
techniques provides a wealth of information for the lipid-protein interactions involved. For *P. aeruginosa* PlcHR₄ acting on bilayers composed basically of PC, SM, PE, and Chol at equimolar ratios, the following main conclusions may be reached. *a* Measurable enzyme binding does not occur simultaneously on all the vesicles in a preparation, even when enzyme is readily available to all of them. Rather, the phenomenon extends over a period of minutes, during which new enzyme-containing vesicles are gradually detected. Enzyme binding is initially localized to small areas from which it progresses to larger fractions of vesicle surface. *b* The enzyme preferentially binds fluid-disordered domains over fluid-ordered or gel domains. *c* Enzyme activity will, in most cases, fluidify or disorder the less fluid, more-ordered domains. *d* The data are compatible with a mechanism in which enzyme binding to the bilayer is a cooperative phenomenon, and hydrolysis occurs in the “scooting mode.”

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**REFERENCES**


