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Recruitment of a phospholipase C/sphingomyelinase into non-lamellar lipid droplets during hydrolysis of lipid bilayers

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Abstract

When giant unilamellar vesicles (GUV) composed of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and cholesterol are treated with PlcHR₂, a phospholipase C/sphingomyelinase from *Pseudomonas aeruginosa*, the initial stages of lipid hydrolysis do not cause large changes in vesicle morphology (Ibarguren et al., 2011). However, when hydrolysis progresses confocal fluorescence microscopy reveals the formation of lipid aggregates, whose morphology is not compatible with that of bilayers. Smaller vesicles or droplets can also be seen inside the GUV. Our studies indicate that these aggregates or droplets are enriched in the non-lamellar lipid ceramide, an end-product of PlcHR₂ reaction. Moreover, the aggregates/droplets appear enriched in the hydrolytic enzyme PlcHR₂. At a final stage GUVs containing the enzyme-enriched droplets disintegrate and vanish from the microscope field. The observed non-lamellar enzyme-rich structures may be related to intermediates in the process of aggregation and fusion although the experimental design prevents vesicle free diffusion in the aqueous medium, thus actual aggregation or fusion cannot be observed.

Keywords

Phospholipase C/sphingomyelinase HR₂; *Pseudomonas aeruginosa*; non-lamellar phases; lipid aggregates

1. Introduction

PLCs and SMases are often secreted by bacterial pathogens in order to interact with host eukaryotic cells where they can affect multiple processes, either through direct alteration of host membrane function (e.g. lysis) or by the generation of potent signaling products (e.g. increased ceramides → apoptosis) in some specific cell types (e.g. endothelial) (Vasil et al., 2009). They are also expressed by eukaryotes, where they frequently provide key intracellular signaling functions (Goñi et al., 2012). However, significant morphological studies of the effects of PLCs/SMases on model or cell membranes are not yet widely available.

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Rapid formation of Cer-containing domains upon treating SM-containing GUV with a SMase has been already observed (Taniguchi et al., 2006). Some years later, morphological observations of supported lipid bilayers (SM:DOPC:Chol, 40:40:20 mol ratio) were described after addition of *Bacillus cereus* SMase (Chao et al., 2010). After the initial stages of enzyme activity, micrometer-sized three-dimensional domains were observed, that contained SM, Cer and SMase. The enzyme would act on these domains until SM was almost completely hydrolysed (Chao et al., 2010). Maggio and co-workers performed remarkable studies on the subject of SMase activity on lipid monolayers at an air-water interface (Fanani et al., 2010). The latter authors commented on the results by Chao et al. (Chao et al., 2010) and suggested that the three-dimensional bright spots corresponded to inverted phase-structures generated by the presence of Cer.

PlcHR₂ is a toxin secreted by *Pseudomonas aeruginosa*, which exhibits PLC and SMase activities (Stonehouse et al., 2002). The hydrolytic activity of PlcHR₂ on bilayers of varying lipid compositions was described previously (López et al., 2011; Montes et al., 2007). Ibarguren et al. studied the effects of the enzyme lipidic end-products Cer and DAG on enzyme (i.e. PlcHR₂) binding to bilayers and on its hydrolytic activity (Ibarguren et al., 2010). Then the same authors (Ibarguren et al., 2011), unaware of the studies previously published (Chao et al., 2010), described a number of morphological observations on the effect of PlcHR₂ on GUV, as seen by fluorescence confocal microscopy. The lipids and the PlcHR₂ were separately labeled with specific fluorescent markers. It was found that, although PlcHR₂ diffused rapidly throughout the observation chamber, detectable enzyme binding appeared to be a slow, cooperative process, occurring for several minutes. After the initial cluster of bound PlcHR₂ molecules was detected further binding and catalytic activity followed rapidly. At some late stage of enzyme activity, apparently three-dimensional structures or lipidic aggregates were formed (Ibarguren et al., 2011). The presence of such structures marked a new burst of enzyme activity, and the lysis of the giant vesicle.

The experiments in this paper are directed to increase our understanding of the latter stages of vesicle disintegration due to lipase activity. In particular the composition and structure of the observed lipid droplets, and their putative enrichment in enzyme have been explored using GUV treated with PlcHR₂. The use of vesicles offers the advantage, over that of supported bilayers, of providing conditions of lateral pressure and bending properties more akin to those prevailing in living cells.

2. Materials and Methods

2.1. Materials

Egg PC, egg PE and DAG were purchased from Lipid Products (South Nutfield, UK). Egg SM, egg Cer and Chol were from Avanti Polar Lipids (Alabaster, AL). 1,1-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl) sphingosyl phosphocholine (BODIPY FL C₁₂-sphingomyelin), Alexa Fluor 633 goat anti-mouse IgM and Alexa Fluor 633 protein labeling kit were from Invitrogen (Eugene, OR) (Ibarguren et al., 2011). PlcHR₂ was purified as previously described (Stonehouse et al., 2002). Monoclonal antibody to ceramide (MID 15B4) was from Grupo Taper (Enzo, New York, USA).

2.2. GUV preparation and fluorescence microscopy

GUVs were prepared using the electroformation method as previously described (Angelova et al., 1992), in a special chamber supplied by L. A. Bagatolli (Odense, Denmark) that allows direct visualization under the microscope. 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. 3 µl of the appropriate stocks were added on the surface of platinum electrodes and solvent

traces were removed by evacuating the chamber under high vacuum for at least 2 h. Then the platinum electrodes were covered with 400 μ l of 10 mM HEPES pH 7.4, previously heated to 60°C. The platinum wires were connected to an electric wave generator (TG330 function generator, Thurlby Thandar instruments, Huntington, U.K.) under AC field conditions (freq. 10 Hz, amplitude 1 V) for 2 hours at 60° C. After GUV formation, the chamber was placed on an inverted confocal fluorescence microscope (Nikon D-ECLIPSE C1, Nikon Inc., Melville, N.Y.). The excitation wavelength for DiI was 561 nm. The images were collected using band-pass filters of 593 ± 20 nm. When required, 2 μ l of PlcHR₂ labelled with Alexa Fluor 633, at 12 μ g/ml, were added to study its effect on the GUVs. In the latter case, an excitation light at 635 nm was used and images were collected using a long-pass filter of 650 nm. In the case of vesicles with Bodipy FL the excitation light was 488 nm and the collecting long-pass filter was 515 ± 15 nm. All these experiments were performed at 25–28° C. Image treatment and quantification was performed using the software EZ-C1 3.20 (Nikon Inc., Melville, N.Y.).

The primary and secondary antibodies were supplied simultaneously (dilutions: anti-ceramide 1:2 and Alexa Fluor 633 goat anti-mouse IgM 1:200) (Vielhaber et al., 2001). The excitation light for Alexa Fluor 633 was 635 nm and the emission was collected using a long-pass filter of 650 nm.

3. Results and Discussion

When GUV composed of SM:PC:PE:Chol (1:1:1:1 mol ratio) are treated with PlcHR₂ at 28° C and examined by confocal fluorescence microscopy the overall morphology of the vesicles changes little within the first 3–5 min after enzyme addition apart from the localized changes in fluidity described previously (Ibarguren et al., 2011). At a later stage of enzyme activity (Fig. 1) some kind of lipid aggregates, or droplets appear to form at, or near the bilayer, as seen by DiI staining. DiI is a general-purpose fluorescent lipid dye. The DiI-stained aggregates in Fig. 1 look clearly non-lamellar with an average apparent thickness about 4-fold that of the control bilayer (average of 25 measurements). This occurs at a stage when non-bilayer lipids (Cer, DAG, Chol) are accumulating while those that favour the lamellar structure (SM, PC) are being degraded. Essentially the same results are obtained when DAG and/or Cer are included in the GUV composition at the preparation stage, before enzyme addition (Fig. 1B, C). Mixtures, including a fraction of DAG and/or Cer, exhibit somewhat different responses to PlcHR₂ activity (Ibarguren et al., 2010; Ibarguren et al., 2011). Note that when 10 mol % DAG is present in the original composition, a condition that increases enzyme activity, aggregates become visible at an earlier stage and small vesicles appear to originate via fission from the large one (Fig. 1C). Fission and fusion probably share a very similar mechanism, and similar if not identical lipid intermediates. Thus DAG, that is known to facilitate fusion (Goñi and Alonso, 1999; Nieva et al., 1993) is, in this case, making fission possible as well (Fig. 1C). “Vectorial shedding” or pinching off of vesicles from plasma membrane cells upon addition of a bacterial sphingomyelinase has been previously observed (Zha et al., 1998). With regard to PlcHR₂, aggregate generation and fission of smaller vesicles are accompanied by a decrease in vesicle diameter, of up to 40 % (Fig. 2).

The final outcome of vesicle lipid hydrolysis by PlcHR₂ is the vesicle lytic burst. Vesicle lysis starts when at least 20% of the substrate (PC + SM) has been hydrolysed (enzyme activity data at 28° C, data not shown). Beyond the stage of non-lamellar aggregate formation, the vesicles start shrinking until they eventually disappear from the microscope field, often leaving behind remnants in the form of amorphous aggregates and/or smaller vesicles (Fig. 3).

The presence of high concentrations of Cer, a non-bilayer lipid that occurs as a result of SM hydrolysis by PlcHR₂, was confirmed in the aggregates by two independent procedures. The use of an anti-Cer antibody, with an Alexa 633-coupled secondary antibody (Fig. 4), reveals the presence of Cer-rich areas within the DiI-stained aggregates. Not all aggregates were stained by the anti-Cer antibody, but the areas that were stained by the antibody overlap consistently with the DiI aggregates. Ceramides are known to form macroscopically separated gel phases (Boulgaropoulos et al., 2011; Goñi and Alonso, 2009).

Cer-rich domains were also observed using a different method, which is based on the presence of Bodipy-labelled SM in the GUV (Fig. 5). Bodipy-SM is hydrolysed by PlcHR₂, thereby giving rise to Bodipy-Cer, whose fluorescence emission is readily distinguished from that of DiI. In the absence of PlcHR₂ a regular bilayer structure is observed in the GUV (Fig. 5, left-hand panel), however, ceramide, in the form of Bodipy-Cer, is enriched in distinct areas, which always overlap with some, but not all, the large lipid aggregates revealed by DiI. The absence of reliable and specific staining methods for DAG prevents us from performing a parallel study with the other lipidic end-products of PlcHR₂. The lack of a full overlapping between BODIPY-Cer and DiI-stained aggregates may be related to the capacity of ceramide to segregate laterally into Cer-rich domains in the presence of other lipids (Veiga et al., 1999; Carrer and Maggio, 1999; Goñi and Alonso, 2009). The morphology of the aggregates seen in Figs. 1, 4 and 5 are evocative of non-lamellar mixtures, because of their three-dimensional appearance and because their dimensions are clearly larger than the bilayer width.

Moreover, when PlcHR₂ derivatised with Alexa 633 is used in the hydrolysis assays, and at the time when lipid aggregates begin to form, within or adjacent to the bilayer (Fig. 1), PlcHR₂ is seen to exhibit a marked preference for these regions (Fig. 6). The data have been quantified to show that both DiI (lipid marker) and Alexa 633 (enzyme marker) are concentrated in the aggregates, but the relative enrichment in enzyme is higher than that of lipid (Table 1).

The aggregates in Figs. 1, 3, 4 and 6 may arise from the coalescence of much smaller domains, originally resulting from the localized enzyme action during the latency period observed in previous studies, with PlcHR₂ and other phospholipases (Basañez et al., 1996a; Ibarguren et al., 2010; Ibarguren et al., 2011). That these regions, enriched in non-lamellar lipids, have a particular affinity for PlcHR₂ would confirm the observations and predictions of Basañez et al. (Basañez et al., 1996a), who explored the burst of a PLC (i.e. *Bacillus cereus*) activity after the lag period. An increased affinity of non-lamellar regions (micro- or macrodomains) for the enzyme would also be in agreement with previous work (Ahyayauch et al., 2005) for the phosphatidylinositol-specific PLC from *Bacillus cereus*. The activity of several phospholipases is known to increase concomitantly with the formation of inverted hexagonal phases (Basañez et al., 1996b; Maggio, 1996). It should also be noted that membrane insertion of proteins, including enzymes other than lipases, can also be facilitated by non-bilayer forming lipids (Alonso et al., 2000; Martin et al., 2004; Ostolaza et al., 1993). Moreover, in the case of PlcHR₂, membrane insertion could follow interaction with specific cell receptors (e.g. integrins) (Vasil et al., 2009).

The aggregates observed in Fig. 1 may well share the non-lamellar structure of lipidic fusion intermediates or “stalks” (Basañez et al., 1998; Chernomordik and Kozlov, 2005; Chernomordik and Zimmerberg, 1995; Weinreb and Lentz, 2007; Yang and Huang, 2003). Furthermore, in the presence of adjacent, aggregated vesicles, either the droplets, or their precursors, would constitute fusion points. Indeed, our experimental design, which hinders the diffusion of GUV within the aqueous medium to facilitate image capturing, does not allow vesicle aggregation or fusion. Consequently, our data do not yet provide direct

evidence that the non-lamellar droplets can also be regions of vesicle-vesicle contact. In contrast, our images do reveal the possibility that, even in the absence of inter-vesicular contacts, structural intermediates for vesicle aggregation, fusion and/or fission, may be formed.

4. Conclusions

When lipid membranes are treated with SMase and/or PLC, the end-products respectively, Cer or DAG, have important effects on the bilayer architecture. Spectroscopic studies of large vesicle populations in cuvettes revealed that lipase activities can lead to vesicle aggregation, leakage, hemifusion and/or fusion. The newly accessible technique of confocal fluorescence microscopy of GUV allows the observation of enzyme-driven structural changes in single vesicles. In particular, the results in this study show that, at late stages in SMase/PLC attack on lipidic GUV, part of the lipid reorganizes into droplets or aggregates, enriched in the enzyme itself. These large droplets may be the result of the coalescence of much smaller nanodomains. We propose that the aggregates, or their smaller precursors, are the structural intermediates responsible for vesicle aggregation and fusion under conditions when large amounts of vesicles are allowed to interact.

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Abbreviations

GUV	giant unilamellar vesicles
PlcHR₂	phospholipase C/sphingomyelinase HR2
NMR	nuclear magnetic resonance
PLCs	phospholipases C
SMase	sphingomyelinase
Cer	ceramide
SM	sphingomyelin
Chol	cholesterol
DAG	diacylglycerol
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
PE	phosphatidylethanolamine
PC	phosphatidylcholine

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- Lipid bilayers in the form of GUV have been treated with a phospholipase C/sphingomyelinase.
- Enzyme action leads to formation of nonlamellar domains in the bilayers.
- These domains are enriched in ceramide and diacylglycerol.
- The nonlamellar domains are also enriched in the enzyme.

Fig. 1.

Generation of lipid aggregates at the advanced stages of PlcHR₂ action. Representative GUV images. DiI staining. Lipid composition: (A) Control (SM:PC:PE:Chol equimolar mixture). (B) + 10 mol % Cer. (C) + 10 mol % DAG. Times after enzyme addition are given in each frame. Bar = 10 μ m.

Fig. 2. Decrease in perimeter of PlcHR₂-treated vesicles. Data obtained from experiments as shown in Fig. 1. Full bars, perimeter before enzyme addition. Empty bars, perimeter at 4–5 min after enzyme addition. Average values of 6–8 vesicles.

Fig. 3.
Lysis of GUV at the last stages of PlcHR₂ action. Vesicle composition: SM:PC:PE:Chol equimolar mixture. DiI staining. Times after enzyme addition are indicated for each frame. Bar = 10 μ m.

Fig. 4. Detection of ceramide in the lipid aggregates. GUV are stained with DiI. Ceramide is revealed through the use of an anti-Cer antibody, and an Alexa 633-bound secondary antibody. (A) Control. (B) + 10 mol % Cer (C) + 10 mol % DAG. Images of two representative vesicles are shown. Bar = 10 μ m.

Fig. 5. Newly generated ceramide is concentrated in the lipid aggregates. (Left-hand side panel) GUV are stained with both DiI (pseudocolour yellow) and Bodipy-SM (pseudocolour green). (Center and right-hand side panels) Bright green domains are formed as a result of Bodipy-Cer becoming concentrated in some of the lipid droplets. Images of two representative vesicles are shown. Bar = 10 μ m.

Fig. 6. Imaging of PlcHR₂ binding to lipid aggregates in GUV's. Left-hand column, Control mixture. Right-hand column, + 10 mol % Cer. Lipids are stained with DiI (yellow). Enzyme is derivatised with Alexa 633 (pseudocolour blue). The bottom figures correspond to the merging of DiI + Alexa 633 images. Bar = 10 μ m.

Table 1
Preferential partition of PlcHR₂ into enzyme-generated lipid aggregates

Fluorescence intensities (DiI and Alexa 633, corresponding to lipid and enzyme, respectively) were measured at different points in the vesicle perimeter, either within the lipid aggregates or in the aggregate-free regions of the bilayer. Both dyes are concentrated in the aggregates, but the relative enrichment in enzyme (right-hand column) is higher than that of lipid. The Nikon software used allows the operator to ensure that no saturation of the fluorescence signal occurs. Average values of 15–20 points in 4–6 vesicles.

Relative intensity (DiI)		DiI-enrichment in aggregates (-fold)		Relative intensity (Alexa 633)		Alexa 633-enrichment in aggregates (-fold)		Alexa 633/DiI	
Bilayer	Aggregates			Bilayer	Aggregates				
Control ^a	25±3.2	100	4.0	10±4.2	100	10.0		2.5	
+10 mol % Cer	11±2.7	100	9.1	2.8±0.5	100	35.7		3.9	
+10 mol % DAG	12±3.2	100	8.5	1.2±0.9	100	83.3		9.8	

^aSM:PC:PE:Chol. equimolar ratio.