



End-products diacylglycerol and ceramide modulate membrane fusion induced by a phospholipase C/sphingomyelinase from *Pseudomonas aeruginosa*

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ABSTRACT

A phospholipase C/sphingomyelinase from *Pseudomonas aeruginosa* has been assayed on vesicles containing phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and cholesterol at equimolar ratios. The enzyme activity modifies the bilayer chemical composition giving rise to diacylglycerol (DAG) and ceramide (Cer). Assays of enzyme activity, enzyme-induced aggregation and fusion have been performed. Ultrastructural evidence of vesicle fusion at various stages of the process is presented, based on cryo-EM observations. The two enzyme lipidic end-products, DAG and Cer, have opposite effects on the bilayer physical properties; the former abolishes lateral phase separation, while the latter generates a new gel phase [Sot et al., FEBS Lett. 582, 3230–3236 (2008)]. Addition of either DAG, or Cer, or both to the liposome mixture causes an increase in enzyme binding to the bilayers and a decrease in lag time of hydrolysis. These two lipids also have different effects on the enzyme activity, DAG enhancing enzyme-induced vesicle aggregation and fusion, Cer inhibiting the hydrolytic activity. These effects are explained in terms of the different physical properties of the two lipids. DAG increases bilayers fluidity and decreases lateral separation of lipids, thus increasing enzyme activity and substrate accessibility to the enzyme. Cer has the opposite effect mainly because of its tendency to sequester sphingomyelin, an enzyme substrate, into rigid domains, presumably less accessible to the enzyme.

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1. Introduction

Membrane fusion is an essential event in a variety of cellular processes, e.g., neurotransmitter release, certain viral infections (influenza, HIV) or membrane biogenesis. The mechanism by which the two involved bilayers transiently break down their structures to become a single continuous bilayer is not fully understood yet. The most widely accepted model of membrane fusion involves the formation of a highly curved semitoroidal intermediate, “the stalk,” between the two membranes [1]. It is known that the presence of diacylglycerol in the membrane greatly facilitates membrane fusion [2–4].

Phospholipases are essential enzymes in maintaining membrane homeostasis and in the generation of metabolic signals. They are also powerful tools for bacteria to infect and eventually destroy eukaryotic

cells, helping in the degradation of the target cell membranes. Phospholipases C (PLC) cleave the phosphodiester bond between the glycerophospholipid DAG moiety and the phosphoryl groups. Most sphingomyelinases hydrolyze the equivalent bond between ceramide and phosphorylcholine in sphingomyelin (SM). From the point of view of their enzyme activity, phospholipases, and lipases in general, are rather unique among enzymes in that their substrates and most end-products do not occur free in solution but are found instead making part of cell membranes [5–8].

PlcHR₂ is a novel phospholipase C/sphingomyelinase from *Pseudomonas aeruginosa* [9] equally active on PC and on SM when these lipids are in the monomeric form, or in mixed micelles with detergents. In a previous paper [10] we found that, when acting on vesicles, the enzyme produces both DAG and Cer, which are in turn responsible for vesicle aggregation, and fusion. Aggregation occurs when DAG and/or Cer are formed in the membrane above a certain level. Aggregation is necessary but not sufficient for fusion to occur. DAG, an end-product of the enzyme, induces membrane fusion [2,11–13]. In the present work we have applied our previous knowledge to study the effects of the end-products DAG and Cer on PlcHR₂-induced membrane fusion assayed in LUV in suspension.

Abbreviations: Cer, egg ceramide; DAG, diacylglycerol; LUV, large unilamellar vesicles; NBD-PE, N-(7-nitrobenzen-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLC, phospholipase C; PlcHR₂, phospholipase C/sphingomyelinase from *Pseudomonas aeruginosa*; Rho-PE, rhodamine phosphatidylethanolamine; SM, sphingomyelin

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2. Materials and methods

2.1. Materials

PlcHR₂ was purified as previously described [9]. Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE) and egg diacylglycerol (DAG) were purchased from Lipid Products (South Nutfield, United Kingdom). Egg SM, egg Cer and cholesterol were from Avanti Polar Lipids (Alabaster, AL). NBD-PE and Rho-PE were supplied by Molecular Probes, Inc. (Eugene, OR).

2.2. LUV preparation

LUV of diameters 100–150 nm were prepared by the extrusion method [14] using Nuclepore filters 0.1 or 0.2 µm pore diameter at room temperature, in 25 mM HEPES, 100 mM NaCl, pH 7.2. Quantitative analysis of the lipid composition of our LUV preparations, as described by Ruiz-Argüello et al. [12], showed that it did not differ significantly from the initial lipid mixture. All experiments were performed at 37 °C. Lipid concentration was 0.3 mM, and PlcHR₂ was used at 0.5 µg/ml.

2.3. Cryo-electron microscopy

Samples were prepared under controlled conditions (37 °C, 99% humidity) in a Vitrobot (Eindhoven, Nederland). 3 µl of a suspension containing 10 mg phospholipid/ml were applied on a grid. After careful spreading of the drop, excess liquid was immediately blotted with filter paper. A 3-µl droplet creates a cylinder of about 0.5-mm thick on a grid and by blotting away excess liquid this cylinder is reduced to a layer of about 100 nm. After blotting, the sample was immediately plunged into liquid ethane held at its freezing point [15]. The specimen was transferred to a cryoholder of a microscope. After a thermal equilibration period (20 min), images are taken at 100 kV and low-dose conditions in a Philips CM 12 microscope (Philips, Eindhoven, the Netherlands).

2.4. PlcHR₂ binding to vesicles

The intrinsic fluorescence spectra of PlcHR₂ either alone or in the presence of vesicles were recorded in an Aminco Bowman Series 2 luminescence spectrometer equipped with a thermostated cell holder and a magnetic stirrer. Small aliquots of a concentrated LUV suspension were added to a fixed concentration of enzyme solution (3.5 µg/ml) in a cuvette with continuous stirring, exciting the samples at 295 nm and collecting emission between 300 and 420 nm. The slit widths were 5 nm for both excitation and emission. The intrinsic fluorescence of PlcHR₂ was titrated with increasing amounts of lipid, in the 0–60 µM concentration range, added at 1.5-min intervals.

Spectra were corrected for the light scattered by the LUVs of identical composition and concentration but in the absence of protein. The signal was also corrected using the soluble Trp analogue, NATA, which does not partition into membranes. The apparent mole fraction coefficients, $K_{x(\text{app})}$, were determined by fitting the experimental values to the hyperbolic function:

$$F/F_0 = 1 + \frac{(F_{\text{max}}/F_0 - 1)[L]}{K + [L]}$$

where [L] is the lipid concentration and K is the lipid concentration at which the bound enzyme fraction is 0.5. Therefore, $K_{x(\text{app})} = [W]/K$, where [W] is the molar concentration of water.

The fraction of PlcHR₂ bound to the membranes was estimated according to the equation

$$f_{\text{bound}} = \left(K_{x(\text{app})}[L] / [W] + K_{x(\text{app})}[L] \right) \times 100$$

2.5. Enzyme activity

PlcHR₂ activity was assayed by determining phosphorous contents [16]. The liposome concentration was 0.3 mM in all experiments. For optimal catalytic activity, the enzyme was assayed at 37 °C, in 25 mM HEPES, 100 mM NaCl, pH 7.2. Enzyme concentration was 0.5 µg/ml. Enzyme activity was assayed by determining water-soluble phosphorous. Aliquots (50 µl) were removed from the reaction mixture at regular intervals and extracted with 250 µl of a chloroform/methanol/hydrochloric acid mixture (66/33/1, v/v/v) and the aqueous phase was assayed for phosphorous content.

2.6. Aggregation and fluorescence measurements

Liposome aggregation was estimated as an increase in absorbance at 500 nm, measured in a Uvikon 922 (Kontron instruments, Groß-Zimmern, Germany). Vesicle-vesicle fusion was assayed as mixing of inner monolayer lipids by the resonance energy transfer method [17] modified by Montes et al. [10], using NBD-PE and Rho-PE. Vesicles containing 2% NBD-PE and 2% Rho-PE are briefly treated with sodium dithionite, a reagent that does not penetrate the membrane and that bleaches the fluorescence of the probes located in the outer monolayers. The resulting vesicles, containing fluorescent probes only in the inner monolayer composition were mixed with probe-free liposomes at 1:4 ratio. When vesicles fuse the inner monolayers of the fusing vesicles become into contact, lipid mixing ensues, and the subsequent probe dilution leads to decreased resonance energy transfers from NBD to rhodamine and increased NBD emission. NBD emission was followed at 530 nm (excitation wavelength at 465 nm) with a cut off filter at 515 nm. Zero percent mixing was established as the equilibrium fluorescence emission in the absence of enzyme. One hundred percent mixing was set after addition of 1 mM Triton X-100. These experiments were performed in an Aminco Bowman Series 2 luminescence spectrometer.

2.7. Statistics

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

3. Results and discussion

3.1. Cryo-electron microscopy. Membrane fusion

As shown by Montes et al. on the basis of spectroscopic techniques [18], the addition of PlcHR₂ to LUVs composed by PC/PE/SM/Ch (1:1:1:1) leads to extensive membrane fusion. Ultrastructural evidence for this phenomenon is presented here using cryo-electron micrographs taken at various times after enzyme addition (Fig. 1). At 0 min the size of the vesicles is 162 ± 27 nm. After 2 min the vesicle size does not change, probably because of the lag time of the enzyme activity [7]. At longer times (5 min) some very large vesicles are seen, together with others whose size has not changed. Finally, after 10 min huge vesicles and vesicle aggregates (1156 ± 285 nm) can be observed. Inside these aggregates some of the honeycomb structures, marked by arrows, arising from vesicles undergoing fusion events, are found. These honeycomb patterns had been observed in the fusion of

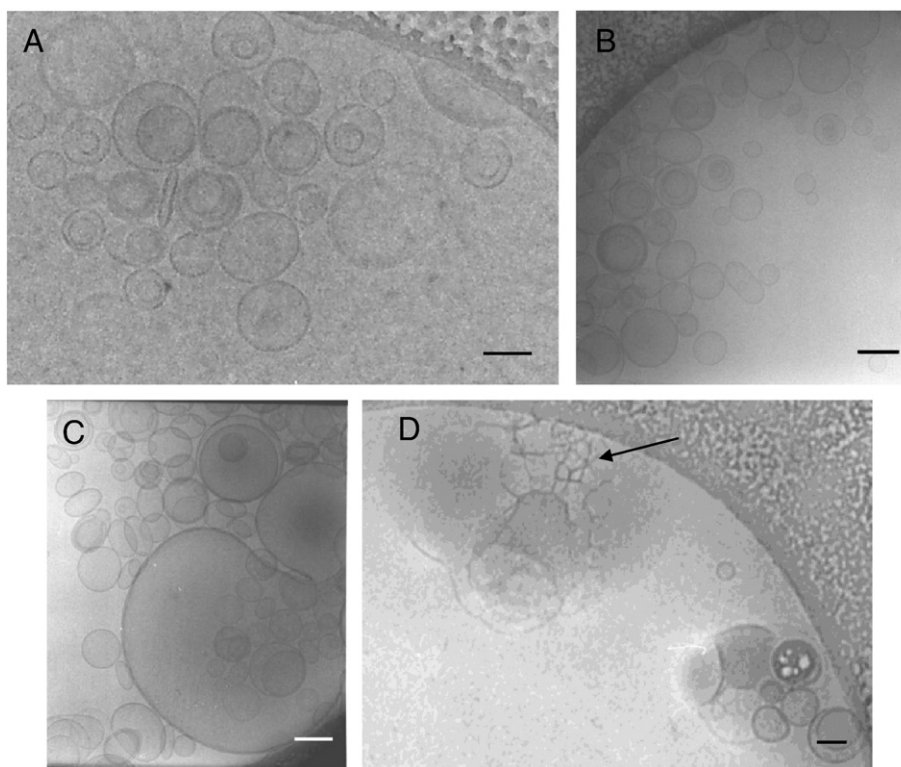


Fig. 1. Cryo-TEM micrographs of LUV of PC/PE/SM/Ch (1:1:1:1) incubated with PlcHR₂ at 37 °C for (A) 0 min, (B) 2 min, (C) 5 min and (D) 10 min. Arrow in (D) indicates the “honeycomb structures.”

LUV induced by phospholipase C from *Bacillus cereus* [4,19] using freeze-fracture and cryo-transmission electron microscopies.

3.2. Enzyme binding to vesicles

In order to estimate the enzyme affinity for the various bilayer compositions a fixed concentration of the enzyme PlcHR₂ (3.5 µg/ml) was titrated with increasing concentrations of lipid in the form of LUVs up to 60 µM. The increase in fluorescence was measured 1 min after enzyme addition at 37 °C. Plots of fluorescence intensity vs. lipid concentration are shown in Fig. 2A. The enzyme binds the lipid with much higher affinity when DAG and/or Cer are present in the bilayer (Table 1). The enzyme being catalytically active under these conditions, it was possible that the data in Fig. 2A were influenced by the presence of increasing amounts of DAG + Cer in the bilayer. In order to overcome this difficulty, the initial rates of binding (or maximum rates, when there was a lag time) were computed from time-resolved studies of the increase in enzyme intrinsic fluorescence secondary to membrane binding. The results (Fig. 2B and Table 1) confirm that both DAG and Cer, either alone or in combination, facilitate PlcHR₂ binding to the lipid bilayers.

In a previous work where the lag time of the PC-PLC from *B. cereus* [20] was studied, it was suggested that the enzyme worked at very low rates during the initial period. Then, after a given concentration of DAG had been reached in the bilayers, a kinetic transition occurred and maximum rates of hydrolysis were observed. The same paper described experiments showing that lag times became almost zero when DAG was present in the membrane before enzyme addition. The data in Fig. 2 indicate that DAG or Cer increase dramatically PlcHR₂ binding. With yet another enzyme, PI-PLC from *B. cereus*, Ahyauch et al. [21] observed that including DAG in bilayers increased enzyme binding. Thus, it may be a general phenomenon that adding the lipidic end-products of a lipase to a bilayer facilitates enzyme binding and decreases the lag times of enzyme activity. The mechanism by which addition of Cer or DAG enhances PlcHR₂ binding cannot be ascertained

at present. In fact, the possibility of various coexisting mechanisms cannot be ruled out. The data by Ahyauch et al. [21] suggest that, in general, increasing membrane fluidity, e.g., by adding DAG [22], facilitates enzyme binding; however, Cer gives rise to less fluid domains [22], yet it also enhances binding (Fig. 2). In the absence of direct evidence, the hypothesis can be proposed that the enzyme has an affinity for the interfaces between the Cer-rich and -poor domains. There are several examples of proteins that became preferentially inserted at rigid–fluid interfaces [20,23].

3.3. Enzyme activity. Vesicle aggregation and fusion

Montes et al. [18] have shown that PlcHR₂ activity induces aggregation and fusion of phospholipid vesicles (LUV) composed of PC/SM/PE/Ch (1:1:1:1, mol ratio). In the present paper, we describe the effects of including DAG and/or Cer in the initial composition. As shown in Fig. 2A, the maximum rates of hydrolysis decrease when Cer is present in the vesicle composition. Maximum rates rather than initial rates are given because, as discussed by Montes et al. [18], a latency period or lag time is observed with this enzyme, as with many other lipases. When the extent of hydrolysis after 5 min, an intermediate stage in the fusion process (Fig. 1C), is measured (Fig. 3B). Cer but not DAG, is clearly seen to act as an enzyme inhibitor. Our interpretation for the data in Fig. 3 is that, beyond their role as end-product inhibitors, Cer and DAG have opposite physical properties that influence enzyme activity. Cer separates into rigid SM-Cer domains [22,24], thus presumably a large fraction of the substrate SM is not available to the enzyme. Recent evidence [25,26] with *B. cereus* sphingomyelinase has shown that the enzyme acts in the fluid phase by producing oversaturation of ceramide which causes end-product inhibition of the enzyme activity and eventually segregation of ceramide-enriched domains.

On the contrary, DAG increases membrane fluidity, and this facilitates enzyme activity and compensates end-product inhibition. Ahyauch et al. [21] have shown for another PLC (PI-PLC from *B.*

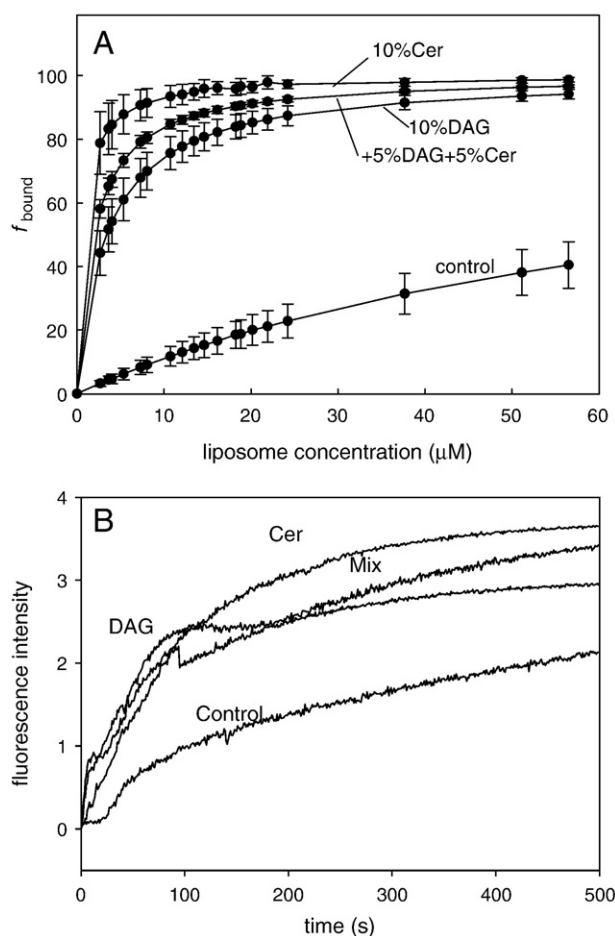


Fig. 2. PlcHR₂ binding to LUV of different lipid composition. (A) The enzyme has been titrated with increasing concentrations of lipids. See main text for experimental details. Control: basic lipid composition, consisting of an equimolar mixture of PC/PE/SM/Ch. Average values \pm S.D. ($n = 3$). (B) The enzyme binding depending of the time.

cereus) that the enzyme is activated by an increase in membrane fluidity. The results with vesicles containing initially 5% Cer + 5% DAG can be interpreted in the same way. DAG homogenizes or solubilizes Cer-enriched domains, thus the observed decrease in enzyme rate (Fig. 3A) is probably due to end-product inhibition. However, most of the lipid is in the fluid state, thus accessible to the enzyme, and this explains the small difference in the amount of lipid cleaved after 5 min between the “control” and the “5% DAG + 5% Cer” samples. Moreover, it should be noted, from the comparison of Figs. 2 and 3, that there is no correlation between enzyme binding and rates of hydrolytic activity.

Table 1

Effects of ceramide and/or diacylglycerol in bilayers originally composed of PC/SM/PE/Ch (1:1:1:1, mol ratio).

Composition	Enzyme binding ^a $K_{0.5}$ (μM)	Enzyme binding maximum rates ^b (s^{-1})	PlcHR ₂ aggregation lag time ^c (s)
Control	20 ± 2.5	3 ± 1	46 ± 0.5
+10% Cer	3.0 ± 0.325	8 ± 0.5	12 ± 0.5
+10% DAG	1.8 ± 0.01	6 ± 0.5	5 ± 0.2
+5% Cer + 5% DAG	0.72 ± 0.205	7 ± 1	8 ± 0.3

Data are average \pm S.D.

^a Data derived from the experiments in Fig. 2A ($n = 3$).

^b Data derived from the experiments in Fig. 2B ($n = 3$). Maximum rates are also the initial rates when lag time is ≈ 0 .

^c Data derived from the experiments in Fig. 4 ($n = 3$).

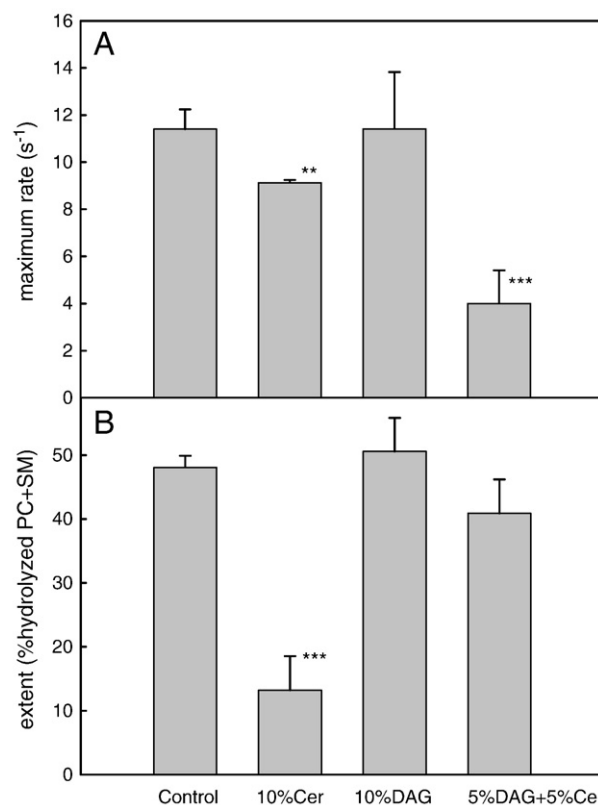


Fig. 3. Activity of PlcHR₂ for the four different lipid mixtures. (A) Maximum slopes. (B) Extent of the activity. Average values \pm S.D. ($n = 3$). The asterisk denotes the results of the Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

PC and SM hydrolysis by PlcHR₂ leads to vesicle aggregation. Lipid hydrolysis and vesicle aggregation progress in parallel with this [10] and other lipases [27,28]. Aggregation is assayed as an increase in light scattering by the vesicle suspension [18]; thus, light scattering measurements provide a convenient method to measure lag times. Time-courses of enzyme-induced LUV aggregation are shown in Fig. 4.

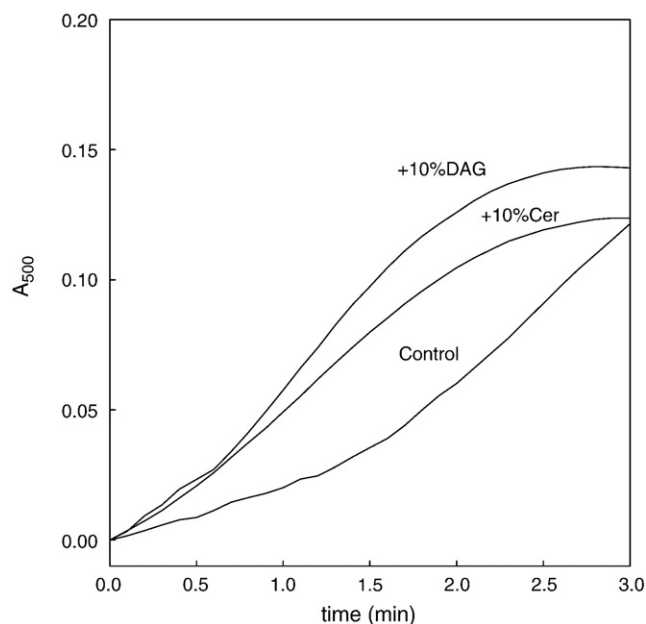


Fig. 4. Time-course PlcHR₂-induced LUV aggregation, for the three different lipid compositions. Aggregation is assayed as an increase in suspension turbidity (A_{500}). A latency period is clearly observed for the control vesicles.

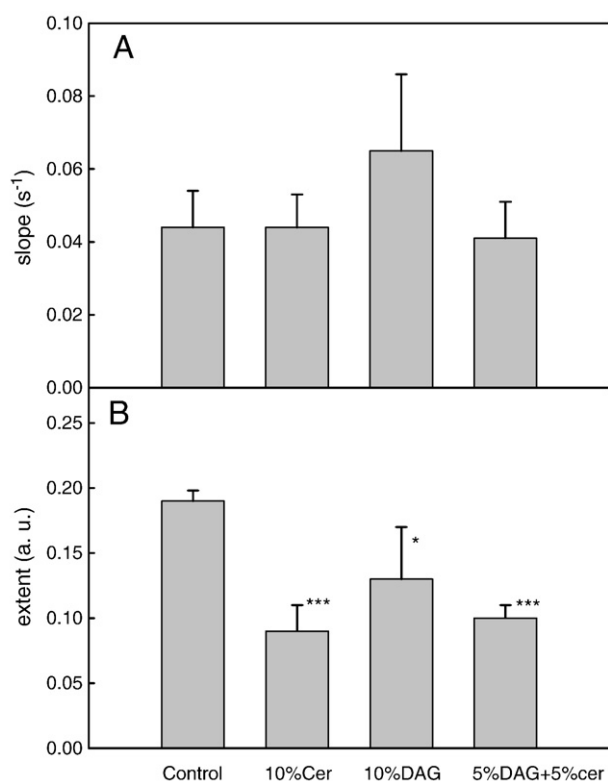


Fig. 5. PlcHR₂-induced LUV aggregation. (A) Maximum slopes. (B) Extent of aggregation (A₅₀₀) after 5 min, under apparent steady-state conditions. Average values \pm S.D. ($n = 3$ –4). The asterisk denotes the results of the Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

It is clear that both Cer and DAG reduce greatly the latency period after which maximum aggregation rates are observed. Aggregation lag times for the four populations of LUV under study are given in Table 1. There is a very good parallelism between enzyme affinity for the vesicles (data from experiments in Fig. 2) and lag times (data from experiments in Fig. 3). We conclude that PlcHR₂ behaves as shown previously for *Bacillus* PC-PLC [27]: the latency period is decreased when a small proportion of the end-product is present from the beginning of the enzyme assay. Moreover, the combined observations of Figs. 2 and 4 indicate that the transition from the slow to the fast regime of enzyme activity is accompanied (caused) by the increased binding of enzyme. Note also the lack of correlation between lag times (Table 1) and maximum enzyme activity (Fig. 3). A ceramide-increased binding of *B. cereus* sphingomyelinase and subsequent enhancement of the precatalytic steps (lag time shortening) was thoroughly described and kinetically analysed by Fanani et al. [29] working on lipid monolayers at the air–water interface.

The rates and extents of LUV aggregation induced by PlcHR₂ are shown in Fig. 5. The maximum rates (slopes) appear to be independent of lipid composition, the increase observed for the mixture containing DAG being statistically non-significant. The extents of aggregation after 5 min, when an apparent equilibrium has been reached, follow a somewhat similar pattern as the corresponding data for lipid hydrolysis (Fig. 3B), ceramide inhibition being the main result. A ceramide-dependent decrease in enzyme activity was also found by Fanani et al. [29] for *B. cereus* sphingomyelinase. The data in Fig. 5 confirm a trend that has been observed previously for other PLCs, namely that aggregation appears to be a direct effect of lipid cleavage.

As shown for other PLCs [12,28,30–32] enzyme-induced LUV aggregation may lead, under certain circumstances, to vesicle fusion. This is also the case for PlcHR₂, as shown by Montes et al. [18] and in Fig. 1. Among the various available fusion assays, the inner monolayer

lipid mixing assay is particularly specific for intervesicular fusion, as distinct from hemifusion [18,28]. Results for vesicles containing DAG and/or Cer are summarized in Fig. 6A. Only maximum slopes are given, because in this kind of assay the total extent of fusion cannot be reliably ascertained. As shown in Fig. 6A, 10% DAG increases significantly the fusion rate, while Cer, or DAG + Cer mixtures are not effective. The data in Fig. 6B show that the enhancing effect of DAG is dose-dependent. This confirms our previous observations [2,11,12,32–35] that DAG, but not Cer, is a potent fusogen.

Previous studies have pointed out the mutual modulation of phospholipase C and sphingomyelinases when these enzymes are added separately to a lipid bilayer [32,36]. In our case, we have both activity associated to a single protein, but the interplay of bilayer physical properties and enzyme activities is probably very similar. In our former study with PlcHR₂ [18] we described that the catalytic activity of the enzyme that cleaves PC and SM at about the same rates induced vesicle fusion in the absence of vesicular leakage. In the present contribution we have explored in further detail the effects of the enzyme end-products DAG and Cer. From the various assays of enzyme activity and enzyme-induced vesicle aggregation and fusion, we conclude that both DAG and Cer decrease the lag times of lipid hydrolysis, that Cer inhibits the PlcHR₂ hydrolytic activity, and that DAG increases fusion rates. The observed decrease in lag times confirms our previous observation that, in PLCs, the latency period is used to accumulate a certain amount of lipid end-product, and that the lag time can be substantially decreased if the corresponding end-product is already present in the bilayer at the time of enzyme addition [27]. Cer inhibition of the enzyme hydrolytic activity appears to be due mainly to lateral segregation of rigid SM–Cer domains [22],

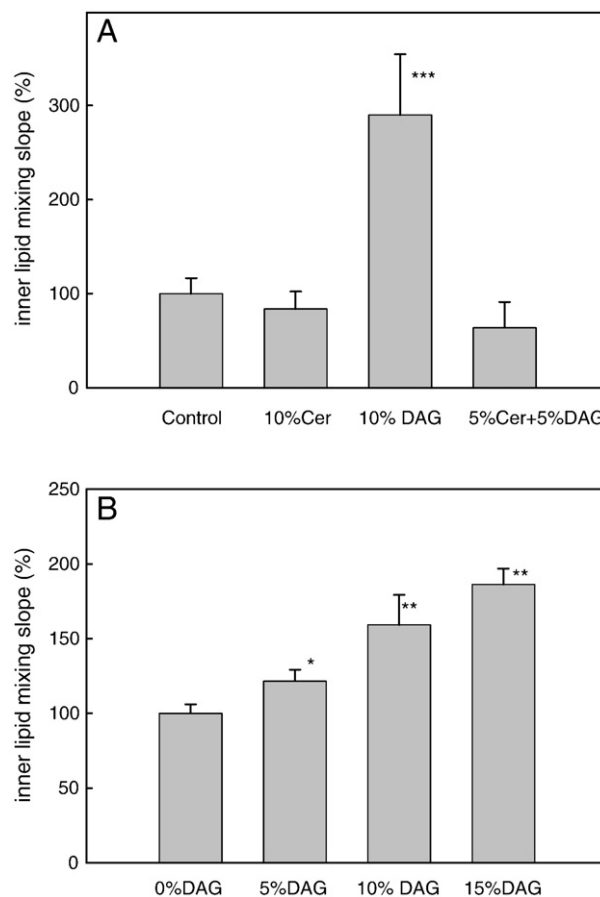


Fig. 6. PlcHR₂-induced intervesicular mixing of inner monolayer lipids. (A) Maximum slopes for the various lipid compositions. (B) Dose-response effect of DAG. Average values \pm S.D. ($n = 3$). The asterisk denotes the results of the Student's *t*-test. (*) $0.01 < P < 0.05$; (**) $0.001 < P < 0.01$; (***) $P < 0.001$.

thus to a decrease in accessible substrate. Moreover, activation of fusion by DAG is probably related to the ability of DAG to give rise to the inverted non-lamellar phases whose structure is reproduced in the fusion intermediate “stalk” [1,11,13].

Whether the mechanisms demonstrated in our model system would also be applicable to cell membranes remains an open question. The observed effects, including gel-phase separations, that require a relatively high concentration of enzyme end-products could indeed exist, although certainly limited in space and time to a particular region (nanodomain?) of the membrane and for a short time. However, the current technologies are still unable to detect these small, transient domains in an unambiguous way.

Finally, the observations reported herein that ceramide formation inhibits PlcHR₂ hydrolytic activity seems to be counter intuitive in the context of the recently reported biological activities [37] associated with this protein. That is, the cytotoxicity of PlcHR₂ appears to be mediated primarily by its sphingomyelinase activity rather than its PC-PLC activity. This is the consequence of the fact that PlcHR₂ induces a calcium signalling-mediated apoptosis in the endothelial cells, which strongly suggest that its cytotoxicity is mediated by the generation of ceramide, rather through the generation of DAG. On the other hand, this interpretation may be overly simplistic since PlcHR₂, besides being PC-PLC and a SMase, also has sphingomyelin synthase activity [38]. That is, it can remove the phosphorylcholine head group of PC and then transfer it to ceramide, resulting in a net synthesis of sphingomyelin. Accordingly, further studies of the biochemistry and biophysics as described in this report may help in dissecting the mechanisms by which very low concentrations of PlcHR₂ (i.e., picomolar) are selectively cytotoxic to endothelial cells and the mechanisms by which this protein causes the hemolysis of sheep erythrocytes, which have virtually no PC in their outer leaflet of their membranes, which in terms of their lipids are essentially composed only of sphingomyelin [39].

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