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Leakage-free membrane fusion induced by the hydrolytic activity of $PlcHR_2$, a novel phospholipase C/sphingomyelinase from *Pseudomonas aeruginosa*

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Abstract

$PlcHR_2$ is the paradigm member of a novel phospholipase C/phosphatase superfamily, with members in a variety of bacterial species. This paper describes the phospholipase C and sphingomyelinase activities of $PlcHR_2$ when the substrate is in the form of large unilamellar vesicles, and the subsequent effects of lipid hydrolysis on vesicle and bilayer stability, including vesicle fusion. $PlcHR_2$ cleaves phosphatidylcholine and sphingomyelin at equal rates, but is inactive on phospholipids that lack choline head groups. Calcium in the millimolar range does not modify in any significant way the hydrolytic activity of $PlcHR_2$ on choline-containing phospholipids. The catalytic activity of the enzyme induces vesicle fusion, as demonstrated by the concomitant observation of intervesicular total lipid mixing, inner monolayer-lipid mixing, and aqueous contents mixing. No release of vesicular contents is detected under these conditions. The presence of phosphatidylserine in the vesicle composition does not modify significantly $PlcHR_2$-induced liposome aggregation, as long as Ca^{2+} is present, but completely abolishes fusion, even in the presence of the cation. Each of the various enzyme-induced phenomena have their characteristic latency periods, that increase in the order lipid hydrolysis < vesicle aggregation < total lipid mixing < inner lipid mixing < contents mixing. Concomitant measurements of the threshold diacylglyceride + ceramide concentrations in the bilayer show that late events, e.g. lipid mixing, require a higher concentration of $PlcHR_2$ products than early ones, e.g. aggregation. When the above results are examined in the context of the membrane effects of other phospholipid phosphocholine hydrolases it can be concluded that aggregation is necessary, but not sufficient for membrane fusion to occur, that diacylglycerol is far more fusogenic than ceramide, and that vesicle membrane permeabilization occurs independently from vesicle fusion.

Keywords: PlcHR2; Bacterial pathogenesis; Membrane fusion; Membrane leakage; Phospholipase C; Sphingomyelinase; Bacterial toxin; Pseudomonas

1. Introduction

Phospholipid phosphocholine hydrolases include the phosphatidylcholine-preferring phospholipases C (PC-PLCs) and the sphingomyelinases (SMases). PC-PLCs hydrolyse phosphatidylcholine (PC) yielding diacylglycerol (DAG) and phosphocholine. SMases hydrolyse sphingomyelin (SM) yielding ceramide (Cer) and phosphocholine. Both phospholipases C (PLCs) and SMases have been associated with a growing number of critical signal transduction mechanisms in eukaryotic cells, mainly through their hydrophobic end-products DAG and Cer [for reviews see [1–4]]. In eubacteria, PLCs and SMases appear to be mainly exocellular enzymes with degradative roles, often involved in pathogenic processes as virulence factors, thus being part of the complex world of "microbial toxins" [1,2,5–7]. Some bacterial and eukaryotic PLCs and SMases are related from several points of view. SMase from *Bacillus cereus* is structurally related to a mammalian neutral SMase [8]. From a functional point of view, the PC-PLC gene of *B. cereus* can be expressed in fibroblasts leading to the oncogenic transformation of these cells [9].

Abbreviations: ANTS, 8-Aminonaphthalene-1,3,6-trisulfonic acid; Cer, ceramide; Ch, cholesterol; 2D-IR, two-dimensional infrared; DAG, diacylglycerol; DPX, *p*-xylenebis(pyridinium bromide); LUV, large unilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC-PLC, phosphatidylcholine-specific phospholipase C; PLC, phospholipase C; SM, sphingomyelin; SMase, Sphingomyelinase

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Bacterial phosphocholine hydrolases have been found in both Gram-positive and Gram-negative bacteria. Best studied in Gram-positive bacteria are the PC-PLC and the SMase of *B. cereus* [1,2,5,6,10], the PLC/SMase from *Listeria monocytogenes* [7,11], and the PLC (α-toxin) from *Clostridium perfringens* [12]. Among Gram-negative bacteria, two PC-PLC from the opportunistic plant and human pathogen *Pseudomonas aeruginosa* have been characterized, a haemolytic (PlcH) and a non-haemolytic (PlcN) isoforms [13]. PlcH has been shown to be a virulence factor in a wide variety of organisms, mammalians, insects, yeast and plants [14–17]. Previous studies from one of our laboratories (Vasil and co-workers) have characterized in detail $PlcHR_2$ from *P. aeruginosa* as a heterodimeric complex, formed by PlcH, the subunit containing the active centre, and $PlcR_2$, a chaperone that modulates the enzyme activity [18] and is required for the secretion of PlcH. $PlcHR_2$ (95.5 kDa) is the paradigm member of a novel PLC/phosphatase superfamily, with members in various prokaryotic species including *Mycobacterium tuberculosis*, *Bordetella* spp., *Francisella tularensis* and *Burkholderia* spp. Homologs were also recently identified in fungi (*Aspergillus fumigatus*) and plants (*Arabidopsis*). $PlcHR_2$ is equally active on PC and on SM when these lipids are in the monomeric form, or in mixed micelles with detergents. It has been further discovered that PlcH has a SM synthase (transferase) activity [19], thus making PlcH a multifunctional enzyme.

Alonso and co-workers [20–23] first described that PLC activity of the *B. cereus* enzyme had important structural effects on the bilayer that supported both the substrate (PC) and hydrophobic end-product (DAG) of the reaction. Namely PLC activity induced aggregation and fusion of large unilamellar vesicles (LUV) without loss of internal aqueous contents. These results were later confirmed by Luk et al. [24] using a similar system. PLC-induced changes in membrane architecture have been attributed to the formation of transient, non-lamellar lipidic intermediates formed in the presence of DAG [25, 26; see review in 10]. The structurally related SMase from *B. cereus* did not cause membrane fusion, but instead gave rise to extensive release of aqueous contents [27]. Moreover, the joint activity of *B. cereus* PLC and SMase did cause membrane fusion under conditions where none of the enzymes were fusogenic by themselves [8]. This, together with the observation that the product of *plcB* from *L. monocytogenes* has both PLC and SMase activities [28,29] prompted the exploration, by the Goldfine and Alonso groups, of the structural effects of the *Listeria* PLC/SMase. When acting on LUV, the enzyme was found to cause vesicle aggregation, intervesicular mixing of lipids, and mixing of aqueous contents, with very low leakage of vesicular contents, i.e., vesicle fusion [11]. The observation of membrane fusion induced by the *Listeria* enzyme suggests a mechanism for the cell-to-cell propagation of the bacterium, which requires disruption of a double-membrane vacuole.

In the present work, we have applied our previous knowledge to study the effects of the founding member ($PlcHR_2$) of a completely distinct class of PLCs on LUV of defined compositions. The enzyme complex induces vesicle fusion without concomitant release of vesicular aqueous contents ("leakage"). However, fusion is abolished in the presence of the negatively-charged phosphatidylserine.

2. Materials and methods

2.1. Materials

$PlcHR_2$ was purified as previously described [18]. Egg PC, egg PE and PS were purchased from Lipid Products (South Nutfield, UK). Egg SM and cholesterol were from Avanti Polar Lipids (Alabaster, AL). 1-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS), *p*-xylenebis(pyridinium bromide) (DPX), octadecylrhodamine B chloride (R_{18}), NBD-PE and Rho-PE were supplied by Molecular Probes, Inc. (Eugene, OR). Ch was from Sigma.

2.2. Preparation of liposomes

Large unilamellar vesicles (LUV) of diameters 100–150 nm or 400–450 nm were prepared by the extrusion method [30] using Nuclepore filters either 0.1 μm or 0.4 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. [27], 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 $PlcHR_2$ was used at 0.5 μg/ml.

2.3. Enzyme activity

$PlcHR_2$ activity was assayed by determining phosphorus contents [31] in the aqueous phase of a chloroform:methanol:water (2:1:0.6) extraction mixture to which aliquots of the reaction mixture were added at different times. In order to calculate the percentage of individual lipids hydrolysed by $PlcHR_2$, aliquots were extracted at different times with chloroform:methanol:HCl (66:33:1). Each organic phase was concentrated and separated on thin layer chromatography Silica Gel 60 plates, with chloroform:methanol:acetic acid: H_2O (15:9:2.4:0.6). Plates were then stained with 5% sulphuric acid and after charring at 120 °C for about 10 min, the density of the spots was measured in a Bio-Rad GS800 Calibrated Densitometer, using the Quantity One program, also from Bio-Rad.

2.4. Aggregation and fluorescence measurements

Liposome aggregation was estimated as an increase in absorbance at 500 nm, measured in a Uvikon 922 (Kontron). Fusion was assayed as mixing of aqueous contents, using the ANTS:DPX fluorescent probe system described by Ellens et al. [32]. Vesicle efflux was also measured with the ANTS:DPX system. Calibration of these procedures has been described in detail previously [21]. Lipid mixing was assayed either by the octadecylrhodamine B method [33,11] or by the resonance energy transfer method described previously [34] using NBD-PE and Rho-PE. Vesicles containing 0.6% NBD-PE and 0.6% Rho-PE in their bilayer composition were mixed with probe-free liposomes at a 1:4 ratio. NBD emission was followed at 530 nm (excitation wavelength at 465 nm) with a cutoff 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. Fusion and efflux measurements were performed in an Aminco Bowman Series 2 luminescence spectrometer.

2.5. $PlcHR_2$ binding to vesicles

To measure $PlcHR_2$ binding to membranes, liposomes of diameters 400–450 nm were used. 100 μl samples containing 16 μg/ml protein and 1 mM lipid in 25 mM HEPES, 100 mM NaCl, pH 7.2, were incubated at 37 °C for 5 min, transferred to a Millipore Ultrafree-MC filtration unit (cut-off diameter 100 nm) and centrifuged at 400×*g* for 30 min. Then the filter was rinsed with 50 μl buffer and recentrifuged for a further 20 min until all except 5–10 μl had passed through the filter. Unbound protein in the flow-through fraction was concentrated in a Microcon YM-10 down to 5–10 μl. Both fractions, filtrate and retentate, were quantified in a 10% SDS-PAGE gel stained with a Bio-Rad Silver Stain Plus kit.

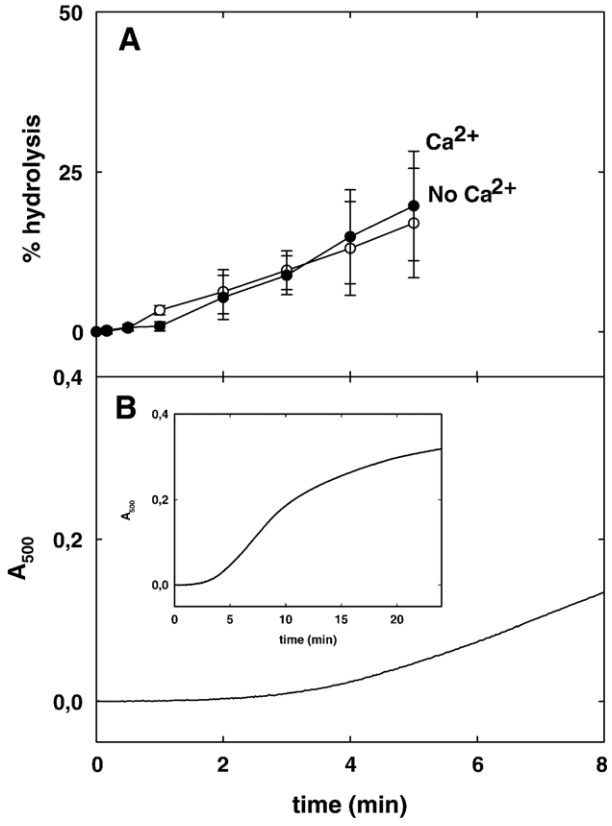

Fig. 1. Effects of $PlcHR_2$ on LUV composed of SM:PC:PE:Ch (1:1:1:1 mol ratio) (A) Phospholipid hydrolysis. 100%=SM+PC (0.15 mM). Assays in the absence (○) or in the presence (●) of 5 mM Ca^{2+}. (B) Time course of vesicle aggregation, measured as increase in turbidity (A_{500}). Enzyme added at time 0. Inset: same process, at a longer time scale. Average values±S.D. (n=3).

2.6. Statistics

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

3. Results

3.1. Phosphocholine hydrolase activity

In order to complement previous assays in which the substrates were mainly in the form of phospholipid monomers, or detergent–phospholipid mixed micelles, $PlcHR_2$ activity has been assayed on LUV with different bilayer compositions, and in the presence or absence of various cations. In this assay water-soluble phosphate (from free phosphorylcholine) is determined at different times after enzyme addition. Typical results are shown in Fig. 1A, for bilayers composed of SM:PC:PE:Ch (1:1:1:1 mol ratio), in the presence and absence of 5 mM Ca^{2+}. Enzyme addition (at time 0) leads to a latency period of 0.5–0.8 min, then hydrolysis proceeds linearly for at least 5 min, during which 30–40% of the substrate (SM+PC) is cleaved. Numerical values of the enzyme activity under various conditions are summarized in Table 1. Four lipid compositions were tested, of which SM:PC:PE:Ch (1:1:1:1) and PC:PE:Ch (2:1:1) have been extensively used in studies with phosphocholine hydrolases [see, e.g., 11, 21, 35]; the PC:PE:Ch:SM:ganglioside mixture is meant to represent the composition of the red blood cell membrane outer leaflet, and the PS:PC:PE:Ch composition is used to study the effect of the PS net negative charge on the enzyme.

In the absence of added cations bilayer composition appears to have little effect on $PlcHR_2$ activity (Table 1), except when PS is present. In the latter case the activity is decreased to about one third of the values in the absence of PS. Ca^{2+}, Mg^{2+}, or EGTA at millimolar concentrations do not appear either to affect phosphohydrolase activity, except that 5 mM Ca^{2+} reverts the inhibition caused by PS in the PS:PC:PE:Ch mixture. In the latter case the enzyme activity reaches the same values observed for bilayers with no net electrical charge. 1 mM Ni^{2+}, that had been found to inhibit $PlcHR_2$ with the substrate in monomeric form [18], also inhibited phosphohydrolase activity on bilayers (Table 1).

Table 1
Phosphocholine hydrolase activity of $PlcHR_2$ on LUV and $PlcHR_2$-induced LUV aggregation

Lipid composition (mol ratio)	Cations/EGTA [a]	Activity (nmol/min)	Aggregation lag (min)	Aggregation slope (min^{-1})	Aggregation range (A.U.) [b]
SM:PC:PE:Ch (1:1:1:1:1)	None	12±3.3	1.4±0.14	0.051±0.0087	0.27±0.045
	1 mM EGTA	14±0.2	1.9±0.25	0.081±0.0059	0.31±0.0088
	5 mM Ca^{2+}	14±3.7	0.86±0.021	0.12±0.0066	0.36±0.0013
	1 mM Mg^{2+}	12±2.2	1.4±0.18	0.061±0.0003	0.30±0.11
	1 mM Ni^{2+}	3.3±1.5 *	n.d. [c]	0.0048±0.0013	0.018±0.013
PC:PE:Ch (2:1:1)	None	16±1.0	0.89±0.55	0.050±0.0076	0.57±0.020
	5 mM Ca^{2+}	17±1.6	0.85±0.24	0.11±0.050	0.33±0.011
PC:PE:Ch:SM:ganglioside (12:3:17:10:1)	None	11±1.6	1.3±0.20	0.0077±0.0010	0.18±0.0035
	5 mM Ca^{2+}	12±2.8	0.76±0.42	0.0084±0.0010	0.18±0.0046
PS:PC:PE:Ch (1:1:1:1)	None	3.4±0.7	n.d. [c]	0.0009±0.0002	0.036±0.0007
	5 mM Ca^{2+}	15±1.0 **	1.0±0.014	0.16±0.014	0.57±0.023

Effect of lipid composition and of cations. Average values±S.D. (n=3).
[a] These are additions to the basic assay buffer consisting of 25 mM HEPES, 100 mM NaCl, pH 7.2.
[b] Absorbance units at 500 nm.
[c] n.d.=not detected.
* Significantly different with respect to the control experiment, in which no cations or EGTA had been added, p=0.031.
** Significantly different with respect to the control experiment, in which no cations or EGTA had been added, p=0.0006.

The effect of Ca^{2+} was further explored in assays in which SM:PC:PE:Ch (1:1:1:1) LUV were treated with the enzyme in the presence and absence of 5 mM Ca^{2+}. At various time intervals between 0 and 10 min aliquots of the reaction mixture were withdrawn and the individual lipid components quantitated after thin-layer chromatographic separation. It was found that (i) PC and SM were degraded at similar rates, (ii) PE was not hydrolysed, and (iii) Ca^{2+} did not influence the degradation rates of the individual phospholipids, PC and SM (data not shown). These results are in agreement with similar studies performed by Stonehouse et al. [18] in which the substrate was usually in micellar or monomeric form. In view of the data in Table 1 all further experiments in this paper were performed, unless otherwise stated, on SM:PC:PE:Ch (1:1:1:1, mol ratio) LUV, in the absence of Ca^{2+}.

3.2. Vesicle aggregation

$PlcHR_2$-induced vesicle aggregation was studied as an increase in vesicle suspension turbidity (ΔA_{500}). Representative experiments are shown in Fig. 1B. Aggregation causes an increase in turbidity. It starts only when a certain fraction of lipid (5–10 mol%) has been hydrolysed. The lag times, maximum rates and extents of aggregation are included in Table 1 for the various lipid compositions and buffers. In all cases a good parallelism is observed between enzyme activity and vesicle aggregation. In general, rates are more meaningful than amplitudes (or extents) in these experiments, because maxima are often the result of several competing phenomena, thus more difficult to analyze. No aggregation was detected, even after several hours at 37 °C, in the absence of the enzyme.

3.3. Release of vesicle aqueous contents

When LUV containing entrapped water-soluble fluorescent probes lose the integrity of their permeability barriers, the probes diffuse rapidly into the outer aqueous medium and this often leads to fluorescence changes that can be readily detected. In our case, LUV were loaded with the fluorescence emitter/fluorescence quencher couple ANTS/DPX. Upon release from the LUV, the complex dissociates and ANTS fluorescence increases. Such an increase is clearly observed with enzymes such as PI-PLC from *B. cereus* [36], but not with $PlcHR_2$ (Fig. 2A).

3.4. Vesicle–vesicle fusion

Demonstration of vesicle–vesicle fusion usually requires the observation of both intervesicular lipid mixing and intervesicular mixing of aqueous contents. Lipid mixing in SM:PC:PE:Ch (1:1:1:1) vesicles was observed as a consequence of $PlcHR_2$ activity by following fluorescence resonance energy transfer (FRET) between donor and acceptor fluorescent probes, respectively NBD-PE and rhodamine-PE (Fig. 2B). As mentioned for vesicle aggregation, observation of a maximum does not mean that lipid mixing can be reverted but rather that, beyond the initial stages, changes in fluorescence due to lipid mixing become very complex [37]. Intervesicular mixing of aqueous contents was monitored with the ANTS/DPX method. This procedure can be applied when, as in the present case, there is only a limited amount of vesicle leakage, otherwise the observations can be seriously altered. The results are shown in

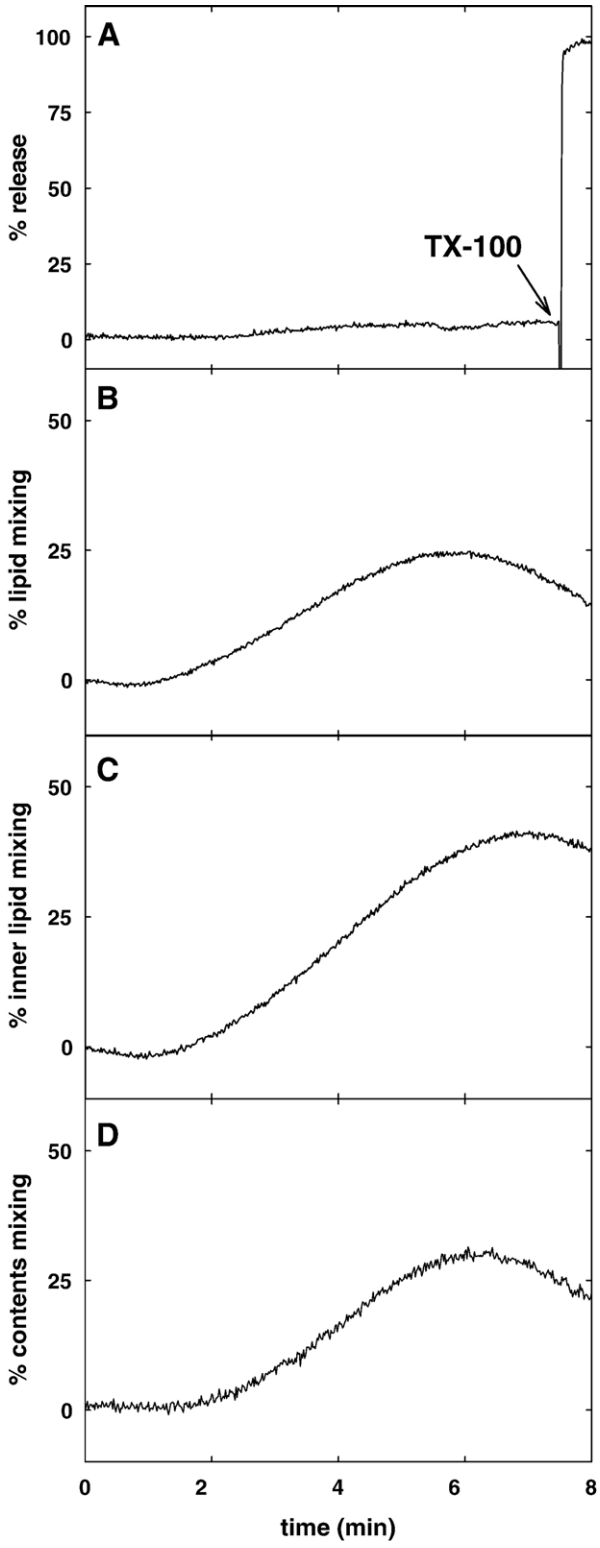

Fig. 2. Intervesicular lipid and contents mixing induced by $PlcHR_2$. (A) Lack of release of aqueous contents. (B) Lipid mixing. (C) Inner monolayer lipid mixing. (D) Aqueous contents mixing.

Table 2
Rates and extents of different phenomena caused by $PlcHR_2$ activity on LUV

Composition	Phenomena	Slope [a]	Amplitude [a]
SM:PC:PE:Ch (1:1:1:1)			
	Aggregation	0.051±0.0087	0.27±0.045
	Total lipid mixing	0.084±0.019	28±2.1
	Inner monolayer lipid mixing	0.050±0.016	19±2.1
	Contents mixing	0.13±0.016	32±5.2
PS:PC:PE:Ch (1:1:1:1)			
	Aggregation	0.001±0.0002 *	0.036±0.0007 *
PS:PC:PE:Ch (1:1:1:1)+5 mM Ca^{2+}			
	Aggregation	0.16±0.014 *	0.57±0.023 *
PS:PC:PE:Ch (1:1:1:1)+5 mM Ca^{2+} (No enzyme)			
	Aggregation	0.016±0.013 *	0.18±0.026 *

Average values±S.D. (n=3).
[a] Aggregation units are absorbance units (amplitude) and absorbance units×min^{-1} (slope). Lipid and content mixing units are % change in fluorescence (amplitude) and fluorescence units×min^{-1} (slope).
* Significantly different with respect to the SM:PC:PE:Ch composition, p<0.02.

Fig. 2D and Table 2. Both lipid and contents mixing reach a maximum after ≈6 min, when ≈30% of the vesicles appear to have undergone fusion events. The combined observations of vesicle aggregation, lipid and contents mixing strongly support the idea that $PlcHR_2$ induces vesicle fusion.

Intervesicular lipid mixing alone does not prove vesicle fusion, because lipid mixing can also be observed in the phenomenon called "hemifusion" [38] or "close apposition" [39], in which the outer monolayers of two adjoining vesicle membranes mix their lipids, but no fusion pore is formed. To overcome this problem, a technique can be applied [11] that allows the direct observation of mixing of inner monolayer lipids. Naturally, this occurs only when a fusion pore is opened, thus detection of intervesicular mixing of inner monolayer lipids is diagnostic for vesicle–vesicle fusion. The procedure is also based on FRET between two vesicle populations, but in this case the fluorescence probes in the outer monolayer have been chemically bleached so that only information arising from the inner monolayer is being recorded. The results in Fig. 2C show that $PlcHR_2$ does induce inner monolayer mixing. The rates and, less accurately, the extents of inner monolayer lipid mixing are roughly one half of those corresponding to total lipid mixing, as expected. Note that the ordinate scale in Fig. 2B corresponds to 100% *total* lipid mixing, while that in Fig. 2C corresponds to 100% *inner* lipid mixing, the inner monolayer containing about one half of the total lipids. The experiments in Fig. 2 indicate that $PlcHR_2$ induces vesicle fusion in the absence of vesicle leakage.

3.5. Enzyme binding to LUV

Enzyme binding to SM:PC:PE:Ch vesicles was estimated by incubating LUV with enzyme and then separating the bound from unbound enzyme by ultrafiltration. Vesicles and enzyme were used at concentrations similar to those in the other experiments described in this paper. In the absence of added Ca^{2+}, the proportion of bound enzyme is 23±11.3% (average±S.D., n=3), while in the presence of 5 mM Ca^{2+} the corresponding figure is 35±4.7%. Assuming the numbers of lipids per vesicle given by Cullis et al. [40], this would correspond approximately to 1 enzyme per 26200 lipids or 100 enzyme molecules/vesicle (no Ca^{2+}) and 1 enzyme per 17000 lipids or 150 enzyme molecules/vesicle (5 mM Ca^{2+}) respectively. The rather similar proportions of bound enzyme are in agreement with the parallel rates of hydrolysis and fusion in the presence and absence of the cation.

3.6. Negatively charged bilayers

The possible influence of negatively charged lipids in the bilayers was tested by preparing LUV of a composition PS:PC:PE:Ch (1:1:1:1, mol ratio). As mentioned above, this composition supports only a low hydrolase activity in the absence of Ca^{2+}, although addition of this cation restores full activity (Table 1). In fact, in the absence of Ca^{2+}, there was no detectable vesicle aggregation, nor fusion, nor contents release (Fig. 3 and data not shown). Ca^{2+} in the absence of enzyme promoted some degree of aggregation (Fig. 3A) in agreement with data from other laboratories [37].

Ca^{2+} did promote extensive enzyme-induced vesicle aggregation (Fig. 3 and Table 2) but no lipid or contents mixing (data not shown). The slope and extent of negatively charged-vesicle aggregation in the presence of Ca^{2+} appear to be the sum of two processes, enzymatic and non-enzymatic Ca^{2+}-induced aggregation (see Table 2). When the non-enzymatic contribution to PS-containing vesicle aggregation is subtracted, the slope and range (respectively 0.14 A.U.×min^{-1}, and 0.40 A.U.) are very similar to those found for the zwitterionic bilayer. Lag times were the same under both conditions (see below). All this seems to indicate that enzyme-promoted vesicle aggregation is not influenced by the bilayer net negative charge.

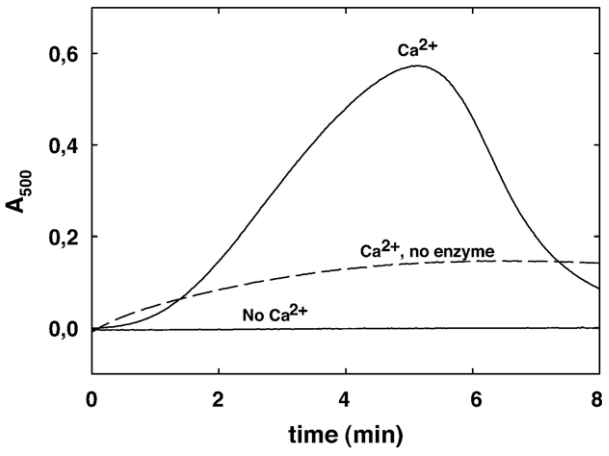

Fig. 3. $PlcHR_2$-induced aggregation and permeabilization of LUV composed of PS:PC:PE:Ch (1:1:1:1, mol ratio). Time course of vesicle aggregation (A_{500}). Experiments in the presence or absence of Ca^{2+}, as indicated near each curve. Discontinuous line: Ca^{2+}-induced aggregation, in the absence of enzyme.

4. Discussion

This paper reports on the effects of $PlcHR_2$ on LUV bilayers, particularly vesicle fusion in the absence of leakage. Ca^{2+} does not affect the hydrolytic activity of $PlcHR_2$, nor the enzyme-induced vesicle–vesicle fusion of electrically neutral vesicles. The presence of the negatively-charged PS abolishes fusion, even if aggregation of PS-containing vesicles may occur in the presence of Ca^{2+}. The observation of a time progression in the different events leading to membrane fusion may deserve some discussion. Moreover, a comparison of data collected with a variety of phospholipases C and/or sphingomyelinases sheds additional light onto these processes.

4.1. Latency periods and the sequence of events

Lipases are commonly found to exhibit latency periods, sometimes very lengthy [41], and $PlcHR_2$ is no exception to the rule (Fig. 1A). Furthermore, a detailed examination of the various structural effects of the enzyme activity (Figs. 1 and 2) reveals that the corresponding lag times increase progressively, giving rise to an apparent sequence of events, that may in turn be informative on the overall process of the structural changes caused by $PlcHR_2$. These lag times are represented in Fig. 4. Lag times increase in the order: phosphocholine hydrolysis < vesicle aggregation < lipid mixing < inner lipid mixing < contents mixing. This is exactly the sequence of events leading to vesicle or cell fusion, as proposed by a variety of authors [42–44].

Thus it appears as if, once a given stage is completed, the system becomes ready for performing the next step. The whole process is driven by the hydrolytic activity of the enzyme, and DAG, or ceramide, or both, have been deemed responsible for the various steps [10,22,26]. In general, longer lag times mean higher concentrations of DAG/ceramide in the bilayers. It is illustrative in this respect to observe the data collected in Table 3 for a variety of fusogenic phospholipases C or sphingomyelinases. DAG/ceramide concentrations required for fusion (lipid mixing) are invariably higher than those found at the onset of aggregation. Thus the progression of latency times and the measurements of fusogenic lipid (DAG and/or ceramide) at the onset of the various stages, concur in underlining the role of DAG and/or ceramide as the agents responsible for the structural changes observed in membranes in which these lipids are being generated.

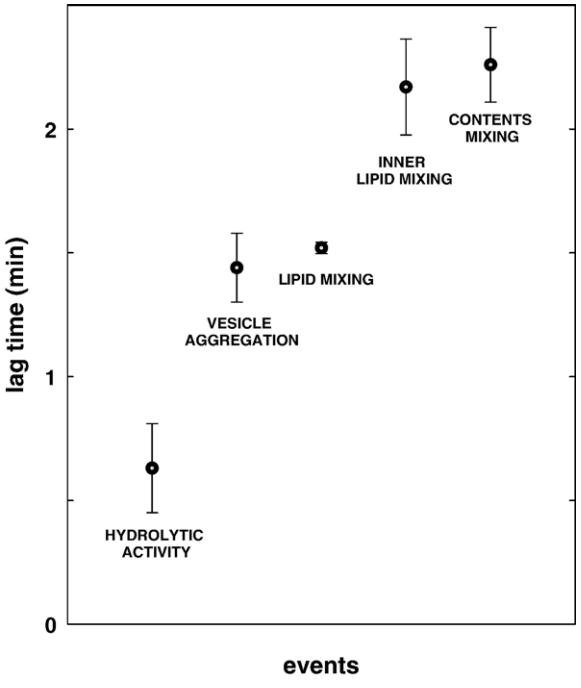

Fig. 4. Latency periods of the various events caused by $PlcHR_2$ activity on LUV composed of PC:SM:PE:Ch (1:1:1:1, mol ratio). Lag times measured after enzyme addition, in the absence (●) or in the presence (■) of 5 mM Ca^{2+}. Average values ± S.D. (n = 3).

Table 3
A comparison of several phospholipid phosphohydrolases and their effects on lipid bilayers

Enzyme	Bilayer composition (mol ratio)	[a]mol% DAG/Cer at the onset of		
		Aggregation	Fusion	Reference
B. cereus PC-PLC	PC:PE:Ch (2:1:1)	1.8	4.0	21
B. cereus SMase	SM:PE:Ch (2:1:1)	2.8	n.o.[b]	27
B. cereus PI-PLC	PI:PE:Ch (1:2:1)	3.3	4.4	36
L. monocytogenes PLC/SMase	PC:SM:PE:Ch (1:1:1:1)	3.3	16.3	11
P. aeruginosa $PlcHR_2$	PC:SM:PE:Ch (1:1:1:1)	4.7	7.0	This work

[a] The percent DAG (or ceramide, or DAG + ceramide, as required for each enzyme) in the vesicles at the onset of the indicated events was either experimentally measured, for $PlcHR_2$ data, or calculated from the published data in the remaining cases. In this context, fusion was assayed as intervesicular lipid mixing.
[b] n.o. = not observed.

The data in Fig. 4 may also be relevant to the current thinking about the mechanism of triggered native membrane fusion [45]. Although the model process described in this paper is clearly slower than its native counterpart, the relative temporal differences between the various parameters analyzed may have their correlates in the native fusion pathway, e.g. the onset of lipid mixing corresponds probably to the hemifusion stage, while the start of the inner lipid mixing process would indicate the opening of the fusion pore. Moreover, the fact that specific lipids, mainly diacylglycerol [1,21] but also ceramide, PE or Ch [1,35], are promoting several of the above stages in model systems provides strong support to the hypothesis that these or similar lipids are important in eukaryotic membrane fusion. This hypothesis has not been given much attention in the context of cell fusion, in spite of the extensive evidence that lipids inducing negative monolayer curvature, such as the above, are also involved in promoting non-lamellar structures as occurring in the fusion intermediate(s) [10,42,44]. Of course this would not exclude the role of fusion proteins, not only in modifying lipid architecture, but also in modulating, either positively or negatively, the rate of fusion pore opening and closing [45].

4.2. The effects of electrostatic charge

In the experiments reported in this paper, the two main reagents with a net electrostatic charge are PS and Ca^{2+}. PS-

containing vesicles are negatively charged, and sustain only a limited enzyme activity, thus they are not prone to aggregation (Fig. 3A). However Ca^{2+} is enough to neutralize this effect, so that even PS-containing LUV display extensive aggregation as a consequence of $PlcHR_2$ activity. This is in agreement with our previous data on phospholipase-induced aggregation, according to which aggregation would be the result of contacts between diacylglyceride-(or ceramide-) enriched domains [25]. Diacylglyceride or ceramide are produced at the same rates with or without PS in the presence of Ca^{2+} (Table 1), thus buffering of the PS negative charges by Ca^{2+} leads to comparable rates of $PlcHR_2$-induced aggregation with or without PS (Table 2). The situation is different for vesicle fusion, the latter phenomenon has not been observed in the presence of PS. This can also be explained in the light of our previous studies. Fusion appears to occur through the formation of a transient non-lamellar intermediate (the "stalk") [42,44], whose structure resembles that of the inverted cubic phases [26]. Because of its negative charge and surrounding water molecules, PS is unlikely to allow formation of inverted phases, thus no fusion occurs in PS-containing bilayers. Ca^{2+} will counteract the negative charge, but will not decrease the hydration sphere. Previous studies from our laboratory have shown the inhibitory effect on fusion of lipids with large, hydrated polar headgroups [41,23].

4.3. A comparison with other phosphocholine hydrolases

The present data can be inscribed in a series of studies in which vesicle aggregation, leakage and fusion induced by a variety of phosphocholine phospholipases have been observed. The currently available data are summarized in Table 3. Examination of this table leads to a number of conclusions that may have general validity regarding structural effects of phospholipases C and sphingomyelinases on membranous systems, as follows:

(i) Aggregation occurs every time diacylglycerol and/or ceramide are formed in the membrane above a certain level. Aggregation is necessary but not sufficient for fusion to occur. Negatively charged bilayers impair aggregation: extensive phosphatidylinositol (PI) hydrolysis is required by PI-PLC in order to observe aggregation of PI:PE:Ch vesicles [36]; Ca^{2+} is required to compensate the negative charge of PS (this work).

(ii) Fusion is induced by diacylglycerol-producing enzymes, or by mixtures of diacylglycerol and ceramide, but not by sphingomyelinases alone. The reason by which ceramide alone is hardly capable of inducing fusion was discussed by Ruiz-Argüello et al. [35]. Note however that, under certain conditions *B. cereus* sphingomyelinase appears to induce some degree of fusion [20]. Fusion is inhibited by PS (this paper) and by other lipids possessing a highly hydrated headgroup [41].

(iii) Leakage (or vesicle membrane permeabilization) [46] appears to be unrelated to fusion, since both leakage in the absence of fusion [27] and conversely fusion in the absence of leakage [11,21], this work] have been observed.

(iv) Ca^{2+} does not appear to play an essential role in either aggregation, leakage or fusion induced by phosphocholine phospholipid hydrolases, since all three phenomena have been often observed in the absence of this cation.

4.4. A final remark

It is important to note in the context of these studies that, while $PlcHR_2$ is able to hydrolyse PC and SM in vesicles and is cytolytic for human erythrocytes, its pathogenic potential is not merely associated with its ability to remove the head group from phospholipids in a vesicular structure. For example, although all eukaryotic cells contain PC and SM in the outer leaflet of their membranes, $PlcHR_2$ is not uniformly cytotoxic to all eukaryotic cell types. That is, we have found that while $PlcHR_2$ is only very weakly cytotoxic to epithelial cells (e.g. HeLa, A549), it is extremely cytotoxic (picomolar concentrations) to endothelial cells (Stonehouse, Vasil and Vasil unpublished observations). This specificity with regard to this cytotoxicity is unlike that seen with any other known bacterial PLC, and it may contribute to the virulence of *P. aeruginosa* particularly during septic infections, where this pathogen is known to be associated with thrombotic vascular lesions. The results reported herein, on the enzyme ability to induce large changes in membrane architecture and stability, may begin to shed some light on how this complex protein is able to interact with the membranes of vascular endothelial cells and ultimately induce their demise.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2007.04.024.

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