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Unexpected wide substrate specificity of *C. perfringens* α-toxin phospholipase C

Patricia Urbina [a], M. Isabel Collado [b], Alicia Alonso [c], Félix M. Goñi [c], Marietta Flores-Díaz [d,e], Alberto Alape-Girón [d,e,f], Jean-Marie Ruysschaert [a], Marc F. Lensink [g,*]

[a] Centre de Biologie Structurale et de Bioinformatique, Laboratoire de Structure et Fonction des Membranes Biologiques, Faculté des Sciences, Université Libre de Bruxelles, CP 206/2, B-1050 Brussels, Belgium
[b] SGIKER, Facultad de Ciencia y Tecnología, Universidad del País Vasco, 48940, Leioa, Spain
[c] Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain
[d] Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, Costa Rica
[e] Departamento de Bioquímica, Facultad de Medicina, Universidad de Costa Rica, Costa Rica
[f] Centro de Investigaciones en Estructuras Microscópicas (CIEMic), Universidad de Costa Rica, San José 2060, Costa Rica
[g] Biological Nanosystems, Interdisciplinary Research Institute, University of Sciences and Technology Lille1, USR3078 CNRS, 50 Avenue Halley, F-59658 Villeneuve d'Ascq, France

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ABSTRACT

Clostridium perfringens phospholipase C (CpPLC), also called α-toxin, is the main virulence factor for gas gangrene in humans. The lipase activity serves the bacterium to generate lipid signals in the host eukaryotic cell, and ultimately to degrade the host cell membranes. Several previous reports indicated that CpPLC was specific for phosphatidylcholine and sphingomyelin. Molecular docking studies described in this paper predict favorable interactions of the CpPLC active site with other phospholipids, e.g. phosphatidylethanolamine, phosphatidylinositol and, to a lesser extent, phosphatidylglycerol. On the basis of these predictions, we have performed experimental studies showing α-toxin to degrade all the phospholipids mentioned above. The molecular docking data also provide an explanation for the observed lower activity of CpPCL on sphingomyelin as compared to the glycerophospholipids.

1. Introduction

The protein α-toxin (CpPLC) is the key virulence determinant of *Clostridium perfringens* gas gangrene. α-Toxin is a zinc metallophospholipase C [1]. It belongs to a group of related bacterial phospholipases C (*Bacillus cereus* PC-PLC or BcPLC, *Clostridium bifermentans* PLC or CbPLC, *Listeria monocytogenes* PLC-B, and *Clostridium novyi* γ-toxin among others) which contain essential zinc ions and are reversibly inactivated by EDTA or o-phenanthroline [2–7]. From the structural point of view the crystal structure reveals a 370-residue, two-domain protein with the N-terminal domain composed of α-helices and the C-terminal domain consisting mainly of β-sheet [8]. These domains are joined by a flexible linker that favors interactions between the adjacent faces of the domains. Functionally, α-toxin may have at least two modes of action. At high concentrations it degrades eukaryotic cell membranes helping in the spread and growth of the bacterium. At low doses it causes limited phospholipid hydrolysis, which in turn activates DAG- and ceramide-mediated signal transduction pathways, leading to the uncontrolled production of several intracellular mediators [9].

Some of the bacterial phospholipases C (PLC) (sometimes referred to as PC-specific phospholipases C) are active toward PC and show a lower activity toward other phospholipids [2,10–12]. Activity of other phospholipases C (termed PI-specific phospholipases C) is optimal on

Abbreviations: Ala and A, alanine; Asp and D, aspartic acid; BcPLC, *Bacillus cereus* phospholipase C; BSA, bovine serum albumin; CbPLC, *Clostridium bifermentans* phospholipase C; $CDCl_3$-d, deuterated chloroform; Chol, cholesterol; CpPLC, *Clostridium perfringens* phospholipase C; C-terminal, carboxyl-terminal, DAG, diacylglycerol; DHPC, (3)-3,4-di-n-hexanoyloxybutyl-1-phosphocholine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; D5PC, dipentanoylphosphatidylcholine; D5PE, dipentanoylethanolamine; D5PG, dipentanoylglycerol; D5PI, dipentanoylinositol; D5PS, dipentanoylserine; EDTA-Cs, cesium ethylenediamine-tetra-acetate; EF hand, helix-loop-helix structural domain; Glu and E, glutamic acid; G, glycine; GTP, guanosine triphosphate; His and H, histidine; I, isoleucine; K, lysine; LGA, Lamarckian genetic algorithm; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; N, asparagine; N-domain, amino-terminal domain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; P-group, phosphate-group; Phe and F, phenilalanine; PI, phosphatidylinositol; PLC-B, *Listeria monocytogenes* phospholipase C; PlcH, *Pseudomonas aeruginosa* phospholipase C; $PlcHR_2$, *Pseudomonas aeruginosa* hetrodimeric complex composed of a phospholipase C and a chaperone protein; P, proline; PS, phosphatidylserine; Rac1, Ras-related C3 botulinum toxin substrate 1; RMSD, root mean square deviation; S, serine; SM, sphingomyelin; TIM barrel, triosephosphateisomerase barrel; Trp and W, tryptophane; Tyr and Y, tyrosine; Zn, zinc; 5SM, pentanoyl sphingomyelin; 5C, five carbon; ^{31}P-NMR, 31-phosphorus nuclear magnetic resonance

* Corresponding author. Tel.: +33 3 62 53 17 33; fax: +33 3 62 53 17 01.
E-mail address: marc.lensink@iri.univ-lille1.fr (M.F. Lensink).

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phosphatidylinositol (PI) [13] and some of the phospholipases C use sphingomyelin as a substrate [14].

Martin and coworkers provided some new insights into the molecular basis for substrate specificity in *B. cereus* PLC (BcPLC). They identified several residues responsible for substrate specificity using mutagenic, kinetic, and crystallographic experiments [15,16]. On the basis of site-directed mutagenesis of α-toxin and the structure–function relationship of BcPLC, it was confirmed that a highly conserved motif, consisting of three zinc atoms that coordinate residues including His, Glu, Asp and Trp, located in similar positions in several members of the bacterial PLC family, is essential for the catalytic activity [17]. Apart from Glu to Asp exchange, from BcPLC to CpPLC respectively, the residues involved in the active site are conserved [18]. The conserved motif in α-toxin is present in or near the active site cleft of the N-domain and is essential for the hydrolytic activity [8]. Despite the active domain of CpPLC showing 29% sequence identity with BcPLC, their hydrolytic activity has been reported to be quite different. While BcPLC is almost non-specific and hydrolyses phospholipids in the order of preference: phosphatidylcholine (PC) >phosphatidylethanolamine (PE) >phosphatidylserine (PS) [15,16], CpPLC is described to be specific for sphingomyelin (SM) and PC. According to these studies α-toxin can bind and disrupt artificial membranes (liposomes) composed of PC or SM but not of PS, PE or phosphatidylglycerol (PG) [4,19].

Molecular docking is an important tool for computer-aided drug design. This *in silico* technique can be used to calculate the three-dimensional structure of a protein–ligand complex starting from the individual structures of the constituent macromolecules. The crystal structure of CpPLC with a lipid ligand is not known, but the structure of BcPLC with a non-hydrolysable phospholipid has been deposited in the protein data bank (PDB Accession No. 1P6D). Moreover there are a number of important similarities with the non-specific BcPLC, namely the CpPLC overall fold similarity (160 ca. atoms can be aligned with a root-mean-square (r.m.s.) deviation of 1.49 Å), the 29% sequence identity they share, and the presence in both PLCs of the highly conserved motif mentioned above, located in a similar position in the bacterial PLC family and essential for the catalytic activity [17]. Thus BcPLC can be considered as a good template for docking experiments in CpPLC (Fig. 1). Our results show that, in addition to PC and SM, other phospholipids e.g. PE, PG and PI are also CpPCL ligands. Subsequent experimental results confirmed that these phospholipids are indeed CpPLC substrates.

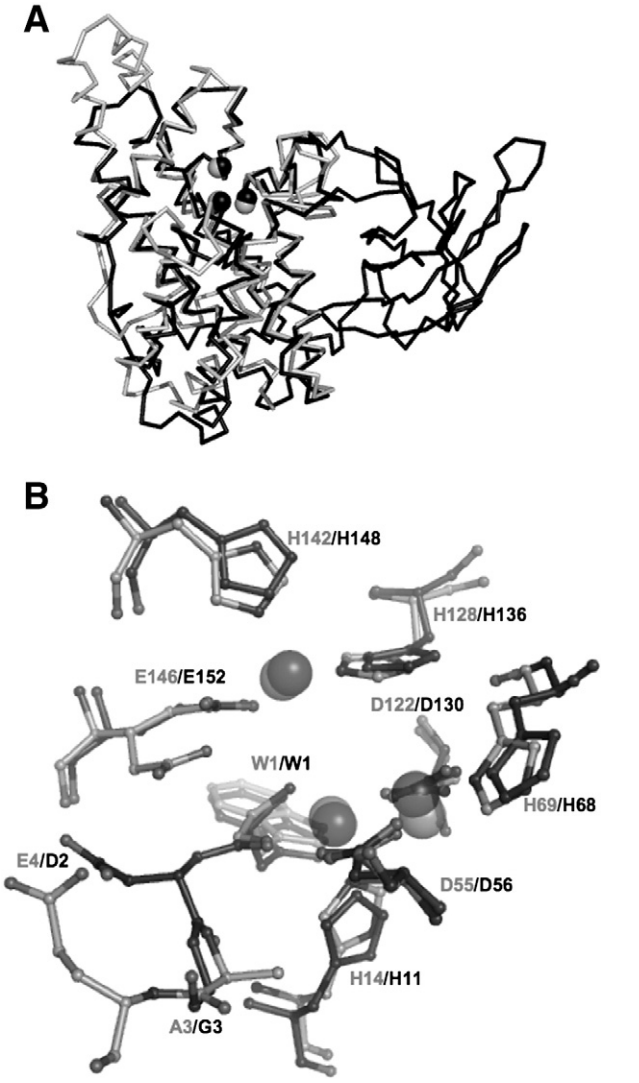

Fig. 1. Stereo picture of *Bacillus cereus* phosphatidylcholine-preferring phospholipase C (gray) and α-toxin (black). (A) Protein whole structure superposition. Spheres represent zinc ions in the active site. (B) Protein active site superposition. Solely aminoacid residues involved in the zinc coordination and phospholipid headgroup interaction were taken into account.

2. Materials and methods

2.1. Materials

Wild type recombinant *C. perfringens* α-toxin from strain 8–6 expressed in *Escherichia coli* was purified as described in Alape-Girón et al. (2000) [20]. Tris ultrapure (Tris) was purchased from Apollo Scientific, NaCl from Fluka and $CaCl_2$ and $ZnSO_4$ from Prolabo. Fatty acid-free bovine serum albumin (BSA) was from Sigma. Egg-yolk phosphatidylethanolamine (PE) was Grade 1 from Lipid Products, egg sphingomyelin (SM), cholesterol (Chol), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylserine (DOPS) and liver phosphatidylinositol (PI) were supplied by Avanti Polar Lipids (Alabaster, AL). The nature of the fatty acids from egg SM, egg PE or liver PI is the following: egg SM (86% 16:0, 6% 18:0, 3% 22:0, 3% 24:1, 2% unknown), liver PI (46% 18:0, 8% 18:1, 6% 18:2, 13% 20:3, 17% 20:4, 10% unknown) and egg PE (22% 16:0, 37.4% 18:0, 29.4% 18:1, 11.2% 18:2).

Chloroform, methanol, hexane, sulfuric acid, cesium ethylenediaminetetra-acetate (EDTA-Cs) and silicagel plates (20×20 cm), were purchased from Merck. Acetic acid and 37% hydrochloric acid were from Carlo Erba. Deuterated chloroform ($CDCl_3$-d) was purchased from Wilmad LabGlass.

2.2. Methods

2.2.1. Docking computations

The automated docking tool chosen for this purpose was AutoDock. This program allows automated docking of flexible ligands to proteins. It is very fast and provides high quality predictions of ligand conformations. The program is based on a Lamarckian genetic algorithm (LGA). Basically this program determines total interaction energies between random pairs of a ligand and selected portions of a protein to determine docking poses [21,22]. 3D structures used for docking experiments were:

- Template- BcPLC (PDB ID 1P6D).
- Target- CpPLC (PDB ID 1CA1). We removed all water molecules from the crystal structure, including those located in the binding pocket.
- Ligands- PC, PE, PG, PI, PS and SM. We used the natural isomer of the phospholipid (the R form) as the BcPLC-preferred isomer [23]. Since we were only interested in the protein-phospholipid headgroup interaction, 5-carbon acyl chains were used: D5PC, D5PE, D5PG, D5PI, D5PS and 5SM (pentanoyl sphingomyelin).

Ligand preparation: non-polar hydrogens were added to the ligand structures, following the program requirements [24]. Bonds of both 5C-acyl chains were turned into non-rotable bonds in order to reduce computing time and focus our study on the lipid polar head group.

Protein preparation: In order to ensure the overall stereochemical quality of the alpha-toxin and to show its local residue-by-residue reliability, the structure was evaluated by PROCHECK. 91% of the residues in the crystal structure of CpPLC were found in the most favored regions of the Ramachandran plot. Those residues in either generously allowed or disallowed regions, respectively K80/N82/W84/Y88/S89/P295/N297 and F78/S79/S83/I90, were found to be located away from the active site (Fig. S1).

Docking results and validation: Autodock predicts free energies of binding in kcal/mol. We adopted the rigid-docking protocol. Autodock generates a large number of duplicate poses within a RMSD of 2 Å. Distances from the BcPLC crystal structure in complex with a non-hydrolysable phospholipid (3)-3,4-di-n-hexanoyloxybutyl-1-phosphocholine (PDB ID 1P6D), were used as reference (Fig. S2A). It was found that the docked ligands were fairly superimposed with the reference for the best-scored conformations. We considered the best ranked to predict the binding mode of the ligand to the crystal structure. Figures were generated with Pymol [25] and ADT [24].

2.2.2. Model membrane preparation

The appropriate lipids were mixed in chloroform:methanol (2:1, v/v) and the solvents evaporated thoroughly. Multilamellar vesicles (MLVs) were prepared by hydration of the lipid film with buffer and intensive vortexing. The suspension was frozen in liquid nitrogen and thawed at 37 °C 10 times. Symmetric unilamellar vesicles (LUVs) were prepared by the extrusion method described by Nieva et al. (1989) [26]. Liposomes to be used in phospholipase and sphingomyelinase activity assays were routinely prepared in buffer A (10 mM Tris, 0.9% w/v NaCl, 3 mM $CaCl_2$, 0.005 mM $ZnSO_4$, pH 7.5). For enzyme dilution buffer A′ was used (10 mM Tris, 0.9% w/v NaCl, 3 mM $CaCl_2$, 0.005 mM $ZnSO_4$, 0.1% w/v BSA, pH 7.5). For the DOPS-containing liposome preparation, buffer B was used (10 mM Tris–HCl, 0.9% w/v NaCl, 0.005 mM ZnSO4) to reach a 3 mM calcium concentration, prior to adding the protein.

2.2.3. CpPLC activity measurements

α-Toxin hydrolytic activity on different potential lipid substrates was assayed by incubating 0.3 mM substrate (LUV) with enzyme in 1 ml buffer A [27,28]. 100 µl aliquots of the reaction mixture were removed at pre-fixed intervals, and added to 500 µl of a chloroform: methanol:HCl mixture (66:33:1 vol ratio). The chloroform phase was removed, evaporated and resuspended in 20 µl pure chloroform. The sample was loaded onto a silicagel plate and run in a glass cuvette with either chloroform:hexane:methanol:acetic acid (50:30:10:5 vol ratio) for separation of DOPC, SM, DOPG and DOPE, or chloroform: methanol:acetic acid:water (60:50:1:4 vol ratio) for separation of DOPC, SM, DOPS and liver PI. Two different staining methods were used, for preparative and analytical purposes respectively. For the analytical stain the plate was dried and treated with sulfuric acid (5% v/v) and heated for 10 min at 110 °C. The spots were quantified with a Bio-Rad G800 densitometer and the data processed with Quantity One (Bio-Rad, U.K.). For the preparative stain the plates were dried and stained with iodine salts. The spots were scraped off and the lipid extracted with a chloroform:methanol (2:1) solution.

Quantitative 31-phosphorus NMR (^{31}P-NMR) was used to confirm the differential hydrolysis of various substrates without separation of reaction products. 5 mM LUV in buffer A was mixed with the protein, previously diluted in buffer A′ and incubated for 5 min at 37 °C. 1 ml aliquots of the reaction mixture were added at fixed intervals to 5 ml of a chloroform:methanol:HCl mixture (66:33:1 vol. ratio). After phase separation, the aqueous or upper phase was removed and DOPG was added to the chlorophorm phase as a quantification standard. Then the solvents from the chloroform lower phase were evaporated thoroughly. The lipid film was resuspended in 750 µl of $CDCl_3$-d:methanol:200 mM EDTA-Cs (10:4:1 vol ratio) and then transferred to 5-mm NMR tubes. Data acquisition was performed in a Bruker AV500 spectrometer (Rheinstetten, Germany) operating at 202.4 MHz for P, with a 5-mm wide-band probe and a gradient in the Z-axis, at 25 °C. The experiments were performed with the *zgig* sequence (Bruker) and a delay time of 15 s between scans. Data were processed with a 1 Hz exponential factor [29,30].

3. Results

3.1. Computational studies

Residues E4, A3, Y56 and F66 were described to be important for phospholipid head group interaction with BcPLC (Fig. S2A) [15]. First a control docking experiment was run to determine the reliability of the program. We docked the non-hydrolysable ligand (3)-3,4-di-n-hexanoyloxybutyl-1-phosphocholine (DHPC) from PDB ID 1P6D into the active site of BcPLC and compared the result with the crystal structure. From the resulting docked model, the ligand has equivalent distances (see Table 1) and structure as compared to the template with the exception of the phosphate group (P-group) being buried 0.5 Å deeper into the active site (Fig. S2B). Attempts to keep water molecules in the active site that might be involved in the hydrolysis of the ester linkage led to unreasonable results. Moreover, the conformation of the docked ligand shows only slight differences in the acyl chains as compared to the template (Fig. S2B). In fact, when comparing the structures of the BcPLC protein crystallized with two different non-hydrolysable ligands (PDB ID 1P6D and 1P6E), the position of the phosphate group does not change even though the active site allows different conformations for the head group and acyl chains (Fig. S2C). We therefore required the docking solutions to reproduce the P-group/protein interaction, given the strong resemblance of the non-hydrolysable substrate docked structure in the BcPLC active site and the crystal structure from 1P6D.

Special attention was given to the position of the phosphate group and the distances between it and the CpPLC hydrolytic residues D56 and D130 (corresponding to D55 and D122 in BcPLC, respectively), the Zn ions and the residues that coordinate with the three Zn ions as they are essential for protein activity.

First we docked a D5PC molecule in the active site of CpPLC. Residues D2, G3 and Y57 interact with the lipid head group in agreement with our template (Fig. 2A). Asp and Tyr keep similar distances to the choline moiety of the phospholipid, 3.91 Å and 4.30 Å respectively (less than 0.5 Å difference from the template) (Table 1). A3 in the template is quite far from the headgroup (6.1 Å), but in our docking results (it corresponds to G3) it is situated about 2 Å closer and is participating in the phospholipid headgroup interaction. The distances between the aminoacids responsible for phosphodiester linkage cleavage (D55 and D122 in BcPLC vs. D56 and D130 in CpPLC) and the phosphate differ from the template by less than 0.5 Å. Moreover, the dihedral angles showed a maximum difference of 4° to the template. These minimal differences can be attributed to the absence of water in the protein active site.

When the ligand is 5SM, despite having the same headgroup as PC, this phospholipid has a ceramide backbone instead of diacylglycerol. The amide linkage from ceramide interacts with residue Y65 responsible of ester linkage interaction in glycerophospholipids. However, residue Y57 is not involved in the interaction with the choline moiety (Fig. 2B) which might cause a lower affinity of CpPLC for SM than for PC, in agreement with the experimental data from Urbina et al. [27,28]. [1]

[1] Note in order to avoid misunderstandings: –Y57 is a CpPLC residue that interacts with the phospholipid head group, it corresponds to Y56 in BcPLC. –Y65 is a CpPLC residue that interacts with one of the ester linkages of the phospholipids but with the amide linkage in SM, it corresponds to F66 in BcPLC.

Table 1
Docking results. List of distances in Å from docking results. Template distances in gray.

Distances (Å)			Distances (Å)					
Residues	Template (PcPLC-DHPC) (PDB ID 1P6D)	Control (PcPLC-DHPC)	Residues	CpPLC-D5PC	CpPLC-5SM	CpPLC-D5PE	CpPLC-D5PG	CpPLC-D5PI
A3 — choline	6.10	5.95	**G3 — choline**	4.10	4.75	4.7*	–	2.58#
E4 O1 — choline	3.99	3.84	**D2 O1 — choline**	3.91	3.62	2.7*	–	2.7#
E4 O2 — choline	4.83	4.74	**D2 O2 — choline**	5.28	5.07	4.1*	–	–
Y56 — choline	4.57	4.47	**Y57 — choline**	4.30	–	–	–	3.18#
N55 O1 — P-group	3.78	3.76	**D56 O1 — P-group**	4.07	3.92	4.10	3.90	4.0
N55 O2 — P-group	3.60	3.56	**D56 O2 — P-group**	3.77	3.56	3.70	3.54	3.69
D122 O1 — P-group	3.90	3.42	**D130 O1 — P-group**	4.07	3.57	3.70	3.84	3.92
D122 O2 — P-group	4.07	3.72	**D130 O2 — P-group**	3.70	3.79	3.40	4.00	4.05

–, No distances measured.
* Distances to ethanolamine headgroup.
\# Distances to closest hydroxyl groups from D5PG polar headgroup.

Since D5PE has a smaller headgroup than PC, it can settle deeper in the cavity. While D2 and G3 are still required to stabilize the headgroup, Y57 appears to have no role in the interaction with the ligand (Fig. 2C), in agreement with the mutagenesis results obtained with BcPLC where Y56 was not found to be necessary for interaction with PE [15,16]. Docking results from D5PE in CpPLC show shorter distances for the headgroup-interacting residues (up to 2 Å difference for D2) due to the unmethylated state of the PE amino group (Table 1). The cleavage site remains in the same position, due to the electronegativity of the phosphate group. The three zinc atoms attract the lipid phosphate group and stabilize it in order to keep the cleavage site accessible to the catalytic residues D56 and D130. The conformation of the phospholipid is almost the same as in the template (less than 0.5 Å r.m.s.), adding to the reliability of the results. In fact, dihedral angles between the D56 and D130 side chain oxygens and the phosphate group reveal no significant differences.

When phosphorylglycerol is the polar headgroup of the ligand, it is found to establish further hydrogen bridges with residue D56 of the macromolecule. G3 and Y57 are not required anymore to interact with the phospholipid. D2 is required for PG interaction according to our docking results (Fig. 2D). Besides this, D56 takes part in the headgroup interaction through a hydrogen bridge and no longer interacts with the phosphate group (Fig. 2D). The D5PG head and phosphate groups compete to interact with D56, which could explain the lack of enzymatic activity against these phospholipids [4,19]. Despite the phosphate group being found in the same position as in the template, the dihedral angles differ by up to 18°, which might also decrease the enzyme activity.

The inositol headgroup in the D5PI ligand can also establish additional hydrogen bridges with the interacting residues. To accommodate the inositol headgroup, D2, G3 and Y57 establish electrostatic interactions including hydrogen bridges with D2 and G3 (Fig. 2E). These interactions shorten the distances between these residues and the ligand as compared with the template (2.7 Å for D2, 2.58 Å for G3 and 3.18 Å for Y57), see Table 1. Ligand conformation is very similar to that of the template. All dihedral angles showed little difference with the template (<3°). As for the D5PE ligand, the D5PI docking results suggest that PI is a good CpPLC substrate candidate.

Although D5PS is known to be a substrate for BcPLC [15,16], our docking results showed a competition between the carboxyl and phosphate moieties of the D5PS head group, leading to inconclusive data on whether D5PS is a substrate candidate for CpPLC.

In summary, in all docking experiments (D5PS excepted) the same residues (D2, G3 and Y57) are involved in lipid headgroup interaction, and the dihedral angles of the catalytic residues with the phosphate group show in most cases little difference with the template. When the phospholipid has a large headgroup (PC, SM, PI), residues D2, G3 and Y57 accommodate the phospholipid headgroup, but when this headgroup is relatively small (PG, PE), Y57 does not seem to be necessary for the interaction. No major differences were detected for any of the phospholipids tested as compared to the template. Furthermore, the assigned correspondence between the aminoacid residues of CpPLC and the template was also confirmed by our docking results. The strong similarity between BcPLC and CpPLC suggests that the latter might not be as specific as it has been described to be in the literature [4,9,31].

3.2. Experimental studies

In order to verify the docking data, enzyme activities were assayed on model membranes (LUV) of different lipid compositions.

3.2.1. Phosphatidylcholine, sphingomyelin and phosphatidylethanolamin

Our previous data had shown that CpPLC is able to hydrolyse PC and SM in liposomes [27]. We tested the CpPLC activity on different lipid mixtures of PC (or SM) and PE that were previously shown to support a measurable activity [28], but we searched specifically for the hydrolysis of the individual phospholipids in the mixture. We assayed the α-toxin PLC activity on liposomes composed of DOPC:PE and DOPC:PE:Chol respectively at 2:1 and 2:1:1 M ratios in buffer A at pH 5, at 37 °C and 20 ng/ml toxin. pH 5 was used because previous measurements had shown that α-toxin PLC activity on PC was optimum at this value [27]. At different times, the reaction was stopped and an aliquot of the reaction mixture was lipid-extracted and loaded onto a silicagel plate and separated as indicated under Methods. After plate staining with sulphuric acid the spots were quantified (Fig. 3). For the phospholipid amounts in our assays (0 to 30 nmol), a linear relationship was found between lipid amount and intensity of the spot for every phospholipid quantified.

In the first stages of the assay (after 10 min) the enzyme hydrolyses more DOPC than PE but at later stages the trend is reversed both in the presence and absence of Chol. Comparing Fig. 3A and B shows that Chol activates α-toxin as observed previously [28].

In order to validate the densitometric measurements the experiment with DOPC:PE at 2:1 M ratio was repeated but phosphorus contents of TLC spots were quantified by a chemical (Fiske) method. Both the densitometry method used above and the TLC + phosphorus assay led to similar results after 60 min (Table S1). Thus under these conditions α-toxin can cleave at least one substrate other than PC or SM, namely PE, in contrast to current opinion. The observation that cleavage does occur even in the absence of cholesterol also contradicts previous conclusions made by other authors [32].

In the presence of SM, in LUVs composed of SM:PE:Chol at 2:1:1 M ratio, α-toxin was also able to hydrolyse PE (Fig. 4). Again at the late stages of hydrolysis, the proportion of cleaved PE was even higher than that of SM. Note that in this and other experiments carried out in the presence of SM, assays were performed at pH = 7, at which SMase activity of CpPLC is optimal [27].

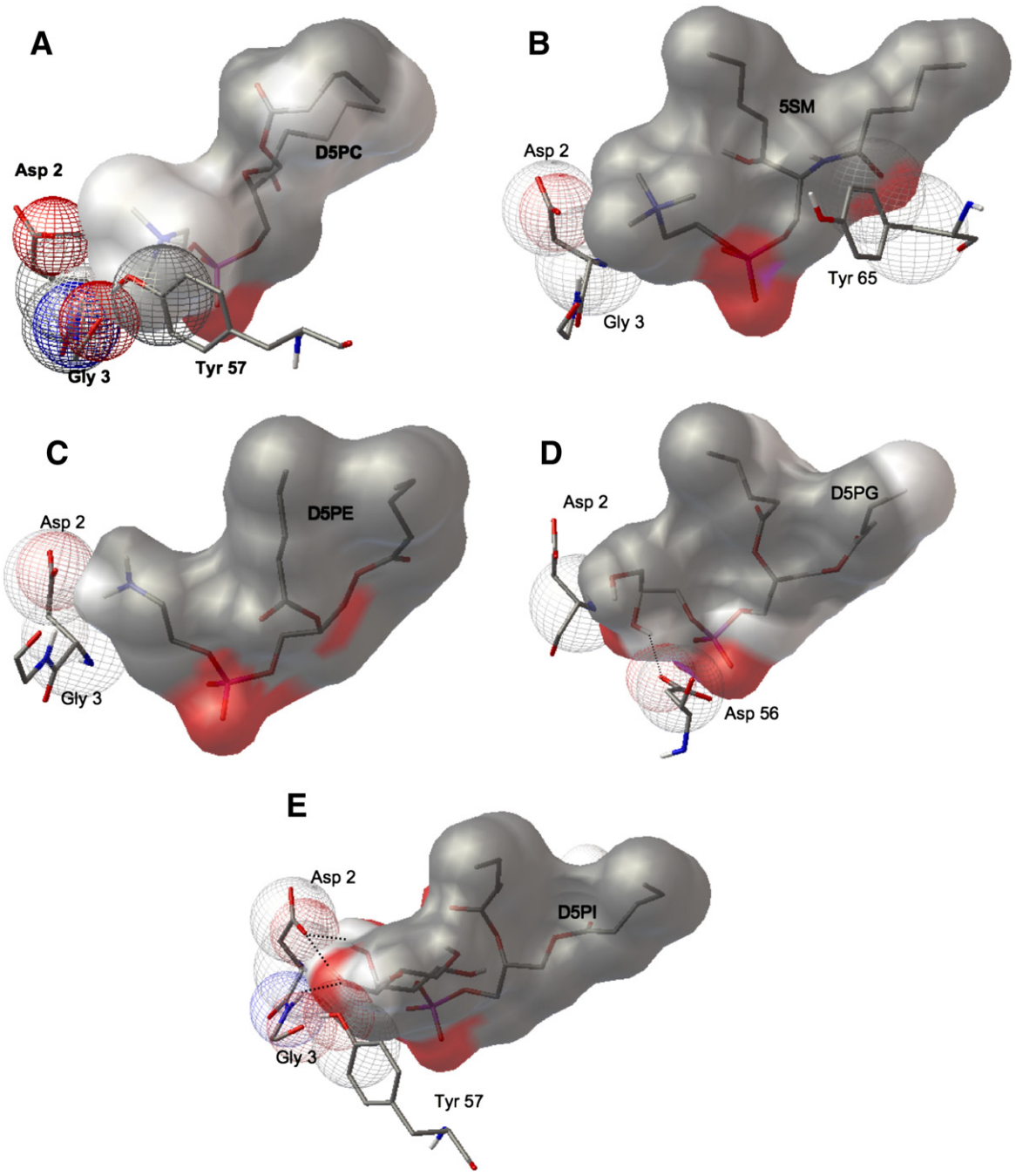

Fig. 2. Docking results. CpPLC residues involved in phospholipids polar head group interaction: D5PC (A), 5SM (B), D5PE (C), D5PG (D), D5PI (E). Hydrogen bonds are represented as dotted lines.

To test for the relative preference of PE, PC and SM as CpPLC substrates, an equimolar PE:PC:SM:Chol mixture was incubated with the enzyme and the mixture composition determined by quantitative ^{31}P-NMR. This technique has been used successfully in the quantitative analysis of phospholipid mixtures without chromatographic separation, since it is possible to obtain a separate signal for every phospholipid class [12,29,30]. The hydrolytic activity of the toxin was assayed on LUV. The reaction was stopped after 60 min and the reaction mixture was quantified (Fig. 5). DOPG was used as an external standard. The spectra revealed the hydrolytic activity of the toxin on the three substrates used in the following order of preference: DOPC≈PE>SM. Thus ^{31}P-NMR confirms that, at least in the late stages of hydrolysis, CpPLC cleaves PE in addition to PC and SM, as predicted by the docking studies.

3.2.2. Negatively charged phospholipid

Despite the fact that PG is not found in significant amounts in mammalian cell membranes, we checked the hydrolytic activity of the α-toxin on this phospholipid because of its net charge and the nature of the polar head group, which is structurally very different from the choline moiety and its different methylated forms. α-Toxin from *C. perfringens* was able to hydrolyse DOPG in the presence of DOPC in the mixtures: DOPC:DOPG (2:1) (Fig. 6A), DOPC:DOPG (1:1) (Fig. 6B) and DOPC:DOPG:Chol (2:1:1) (Fig. 6C). In these cases there was a clear preference for PC over PG, even after long incubation times, in agreement with the docking difficulties found in the *in silico* experiment. Chol improves PG hydrolysis in agreement with previous work from our group [28] where we demonstrated the activating effect of Chol on the

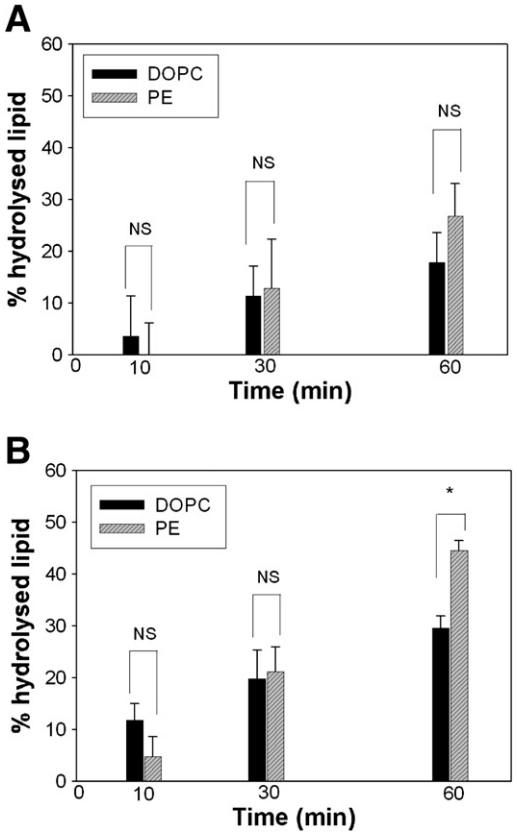

Fig. 3. Hydrolysis of egg phosphatidylethanolamine by α-toxin in the presence of DOPC. (A) LUVs are composed of DOPC:PE at 2:1 M ratio and (B) DOPC:PE:Chol at 2:1:1 M ratio. Assays are carried out at 0.3 mM liposome concentration, 20 ng/ml enzyme, in buffer A at pH 5 and 37 °C. Hydrolysed lipid is quantified by the TLC + densitometry method. Mean values ± S.D. of four independent experiments. Statistical significance (Student's *t*-test): *, $p<0.05$.

α-toxin hydrolytic activity in a dose-dependent manner. The assays carried out with LUVs composed of DOPC:DOPG (2:1) were repeated, and the amounts of the different phospholipids were quantified with the TLC + chemical phosphorus assay after 60 min with similar results (Table S2).

When activities were assayed with DOPG:Chol (2:1) LUV, detectable amounts of phospholipid were hydrolysed by the toxin (Fig. 7). Note that in all cases the percentage of cleaved PG is lower than those found for PC or PE or SM in comparable experiments (Fig. 3), again in agreement with the *in silico* observations.

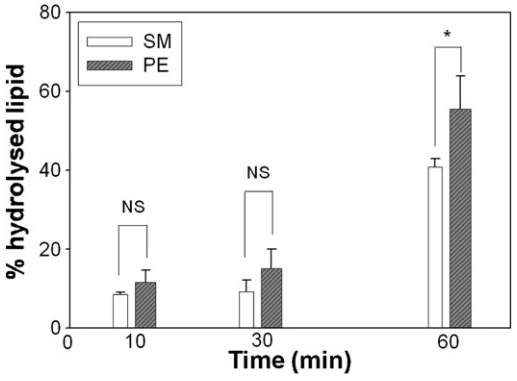

Fig. 4. Hydrolysis of egg phosphatidylethanolamine by α-toxin in the presence of SM. LUVs are composed of SM:PE:Chol at 2:1:1 M ratio. Assays are carried out at 0.3 mM liposome concentration, 80 ng/ml enzyme, in buffer A at pH 7 and 37 °C. Lipid hydrolysis is determined by the TLC + densitometry method. Mean values ± S.D. of three independent experiments. Statistical significance (Student's *t*-test): *, $p<0.05$.

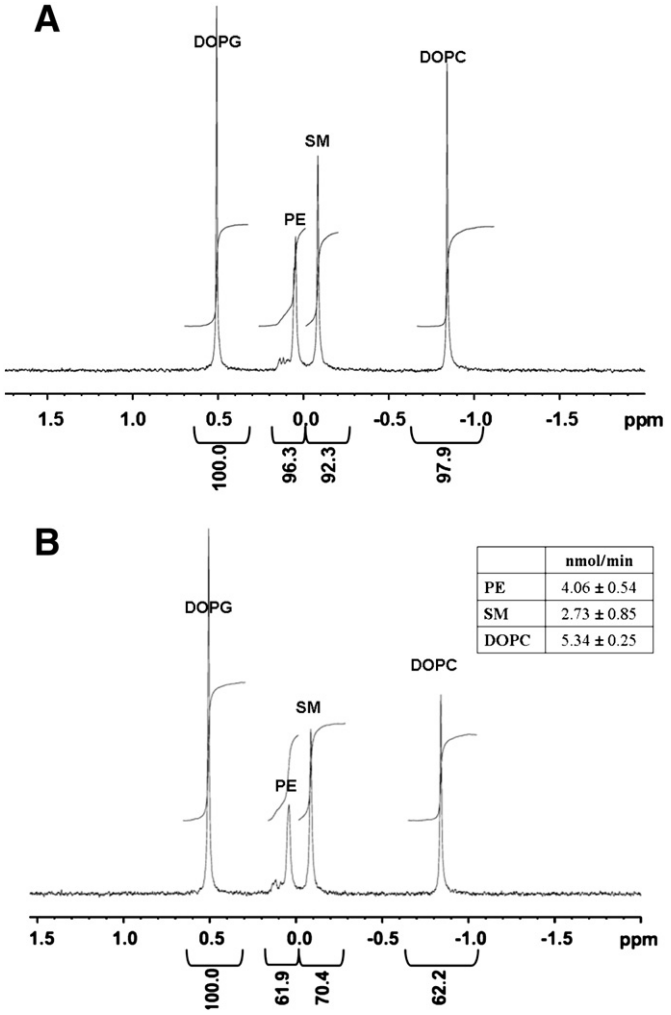

Fig. 5. Quantitative ^{31}P-NMR assay of α-toxin activity on LUVs. Representative NMR spectra showing the chemical shift of the different components of LUVs before (A) and after 60 min enzyme incubation (B). LUVs are composed of DOPC:SM:PE:Chol at 1:1:1:1 M ratio. DOPG is used as an external standard. Numbers under each lipid indicate their amount relative to DOPG in that particular assay. Assays are carried out at 5 mM liposome concentration, 334 ng/ml enzyme, in buffer A at pH 5 and 37 °C.

As far as we knew, PI was no substrate for CpPLC. Our previous work [28] showed that this lipid has no significant effect on the overall hydrolytic activity of α-toxin in model membranes. However, the toxin does hydrolyse liver PI in the presence of DOPC and SM (Table 2), to a similar extent as its more canonical substrates. PI docking had been observed to be also very similar to that of PC in the *in silico* studies.

According to the literature PS is a BcPLC substrate [15,16] and we were expecting the same for α-toxin. However, the initial docking results were inconclusive for this lipid. Nevertheless, when we checked the activity in the presence of DOPC (Fig. 8) or SM (Table 3), indeed a hydrolytic activity was detected. New docking attempts showed a competition between the carboxyl and phosphate moieties in the head group, preventing the docking of the phosphate in the active site. Although this may be an artifact of the docking parameters, we can therefore not relate the hydrolytic activity of the toxin on this ligand to specific interactions with the head group.

4. Discussion

The main conclusion in this paper is that the phospholipase of *C. perfringens* α-toxin, that was believed to cleave only PC and SM, degrades in fact a wide variety of phospholipids. A number of considerations are pertinent in the context of this observation.

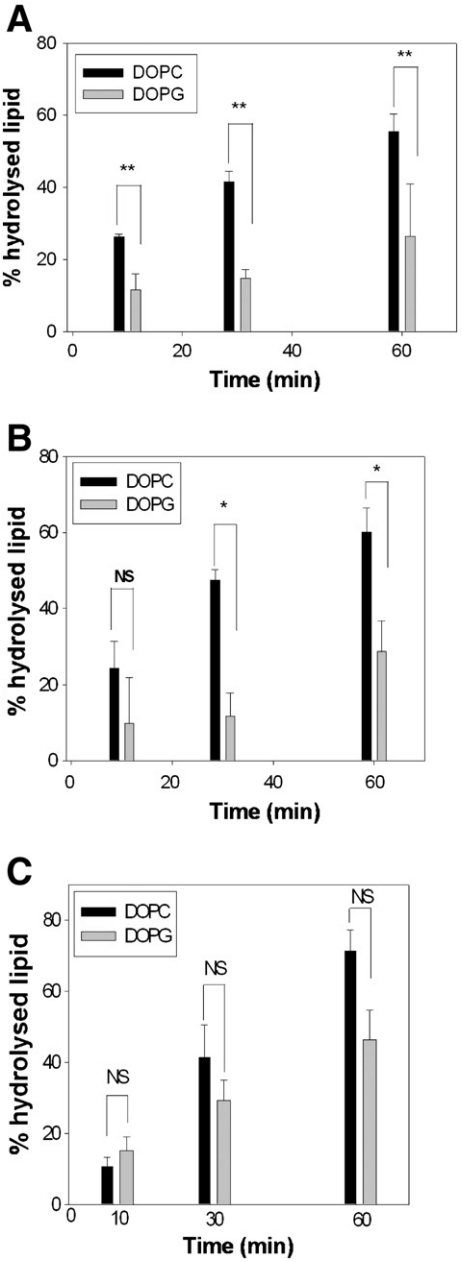

Fig. 6. Hydrolysis of dioleoylphosphatidylglycerol (DOPG) by α-toxin in the presence of DOPC. LUVs are composed of DOPC:DOPG at 2:1 M ratio (A), DOPC:DOPG at 1:1 M ratio (B) and DOPC:DOPG:Chol at 2:1:1 M ratio (C). Assays are carried out at 0.3 mM liposome concentration, 20 ng/ml enzyme, in buffer A at pH 5 and at 37 °C. The hydrolysed lipid is quantified by the TLC + densitometry method. Mean values ± S.D. of three independent experiments. Statistical significance (Student's *t*-test): *, $p<0.05$; **, $p<0.01$.

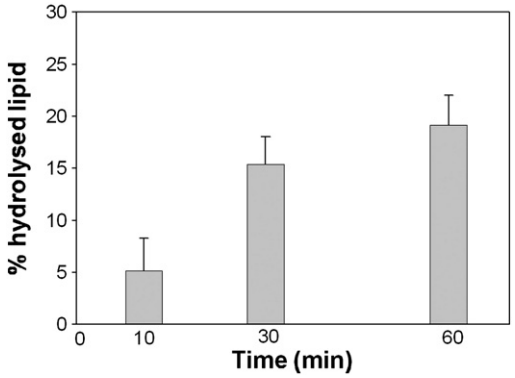

Fig. 7. Hydrolysis of dioleoylphosphatidylglycerol (DOPG) by α-toxin. LUV composition is DOPG:Chol at 2:1 M ratio. Assays are carried out at 0.3 mM liposome concentration, 80 ng/ml of enzyme, in buffer A at pH 5 and 37 °C. The lipid hydrolysed is quantified by the TLC + densitometry method. Mean values ± S.D. of three independent experiments.

4.1. The combination of in silico and in vitro studies

CpPLC is an amphitropic protein, it can exist freely in solution but binds the membrane in order to exert its biological function [33]. The hydrolytic phosphodiesterase reaction requires that the phospholipid headgroup is somehow extracted or isolated from other equivalent molecules in the bilayer, and accommodated within the active site of the enzyme. The degree of affinity of the phospholipid headgroup for the active site will largely control the catalytic efficiency. In our study, a simple bioinformatic tool such as molecular docking has provided results of high heuristic value, with virtually all the experimental observations (Figs. 5–9, Table 1) having been anticipated by the calculations. This in turn provides a firm basis for further predictions concerning CpPLC and related phospholipases, and furthermore, for the development of new specific enzyme inhibitors and the design of new drugs. Docking programs find their most important applications in virtual database screening approaches in which hundreds of thousands of molecules are docked into the binding pocket to identify plausible binders [34]. The CpPLC has historically been associated with the ability of the bacterium to cause gangrene and gastrointestinal disease (e.g. food poisoning and necrotic enteritis) in humans, whereas in other animals gastrointestinal and enterotoxemic diseases occur more frequently [35,36]. Various workers have investigated the efficiency of a toxoid vaccine in protection against necrotic enteritis in poultry [37,38]. These results indicated that vaccination with a *C. perfringens* type A α-toxoid can help to control losses related to this disease, but this vaccine it is not a 100% protection against the bacteria. Therefore, structure-based drug design methods could help us to design new effective inhibitors for these pathogenic bacteria, which are becoming of significant economic importance worldwide due to the pathologies it causes in animals.

4.2. Our results in light of previous studies

There is a significant, if not extensive, literature concerning CpPLC, and the number of publications dealing with structurally related enzymes, e.g. the *B. cereus* phospholipases C, is already sizeable. Previous authors have considered that α-toxins, including CpPLC, were devoid of activity on phospholipids other than PC or SM. Evidently the latter view must be corrected in the light of the above results.

Not only studies from other laboratories, but also our own require some reinterpretation. In particular in our recent paper CpPLC activity was measured on mixtures of PC or SM with e.g. PE, or PI, and the results were interpreted as if phospholipids other than PC or SM would not act as substrates [28]. However, in light of the results presented here it appears that, even if the absolute values of enzyme activities may have

Table 2
Hydrolysis of liver phosphatidylinositol (PI) by α-toxin in the presence of DOPC and SM. Assays are carried out at 1 mM liposome concentration, 66 ng/ml enzyme and pH 5 for PLC activity and 266.6 ng/ml and pH 7 for SMase activity, in buffer A for 30 min at 37 °C. Lipid hydrolysis is determined by the TLC + densitometry method. Mean values ± S.D. of three independent experiments.

LUV composition	Lipid	Hydrolysed lipid (mol%)
DOPC:PI:Chol 2:1:1	DOPC	56.1 ± 2.05
	Liver PI	46.52 ± 6.1
SM:PI:Chol 2:1:1	SM	24 ± 7
	Liver PI	22.2 ± 3.7

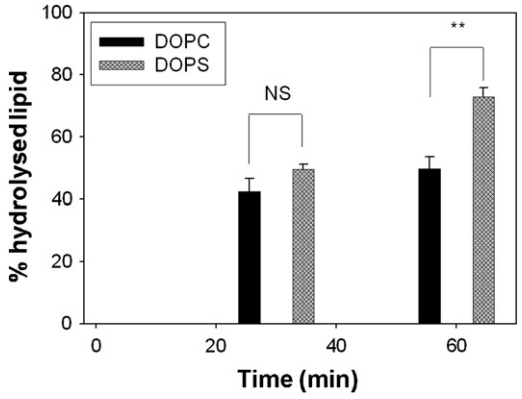

Fig. 8. Hydrolysis of dioleoylphosphatidylserine (DOPS) by α-toxin in the presence of DOPC. LUVs are composed of DOPC:DOPS:Chol at 2:1:1 M ratio. After 1 min incubation, Ca^{2+} is added (3 mM final concentration). Assays are carried out at 1 mM liposome concentration, 66 ng/ml enzyme and pH 5, in buffer A for 30 min at 37 °C. Lipid hydrolysis is determined by the TLC + densitometry method. Mean values ± S.D. of three independent experiments. Statistical significance (Student's *t*-test): **, $p<0.01$.

to be computed again, the main conclusions about certain lipids activating the lipase activity while others being inhibitory, or just neutral, remain fully valid. In addition, the present results confirm and clarify some observations and suggestions made in our previous studies. In particular, we had found that the PLC and SMase activities of α-toxin, while being obviously located in the same protein, were different enough (maximum rates, optimum pH, effects of inhibitors/activators) to suggest that α-toxin would contain two active centers, one for PLC and one for SMase, partially overlapping in the same region of the protein [28]. Our molecular docking experiments predict a different pattern of docking for ceramide-containing molecules than for glycerolphosphatides, entailing in the former case absence of participation of residue Y57 for headgroup interaction. This could provide a simple, testable explanation for the observed results, namely only a single active site to which sphingomyelin and phosphatidylcholine bind in a slightly different way, phosphatidylcholine binding more efficiently, thus being hydrolysed at higher rates. Another possible explanation for a clear preference for phosphatidylcholine could be found at the membrane level. Even though both SM and PC have the same polar head group function, the hydrophobic part of the molecules differ greatly. The interfacial regions of SM and PC differ significantly from each other, SM has the amide group, a free hydroxyl on C3 and the trans-double bond between C4 and C5 [39]. The amide and hydroxyl groups can act as hydrogen bond donors and acceptors, whereas the amide carbonyl in SM only can act as hydrogen acceptor [40]. PC with two ester carbonyls has only hydrogen bond accepting features. These interfacial differences give SMs the unique ability to form both intra- and intermolecular hydrogen bonding [41] and therefore more glued to the bilayer. In a three component bilayer membrane (PC/SM/Chol) in a NMR study, it was observed that cholesterol interacted more strongly with SM as compared to PC [42,43]. Such SM propensity to establish hydrogen bonds with other membrane components may prevent easy substrate extraction from the membrane for CpPLC or other protein. However, CpPLC has an extra domain at the C-terminal compared to BcPLC. CpPLC C-domain is an eight-stranded, antiparallel, β-sandwich domain, which is an analog of the eukaryotic C2 domains [44] and removal of this domain reduces, but does not abolish, sphingomyelinase activity [45,46]. This extra domain might allow the protein to remain in the membrane for longer periods favoring the SM extraction from the bilayer. This is a domain absent in BcPLC, as is the SMase activity.

Table 3
Hydrolysis of dioleoylphosphatidylserine (DOPS) by α-toxin in the presence of SM. After 1 min incubation, Ca^{2+} is added (3 mM final concentration). Assays are carried out at 1 mM liposome concentration, 266.6 ng/ml enzyme and pH 7, in buffer A for 30 min at 37 °C. Lipid hydrolysis is determined by the TLC + densitometry method. Mean values ± S.D. of three independent experiments.

LUV composition	Lipid	Hydrolysed lipid (mol%)
SM:DOPS:Chol 2:1:1	SM	17.8 ± 0.8
	DOPS	27.7 ± 6.1

Chol is an important membrane component for the activity of α-toxin. Nagahama et al. (1996) concluded that the presence of Chol was essential for α-toxin-vesicle interaction, and consequently for α-toxin activity. In pure egg PC monolayers, *C. perfringens* α-toxin showed a negligible activity at lateral pressures lower than 30 mN/m compared to the activity developed by α-toxin in the presence of Chol [31]. However, under our experimental conditions α-toxin was able to hydrolyse different phospholipids with no Chol in the bilayer. This is in agreement with our own previous observations [27] in which we demonstrate the activating effect of cholesterol due to its intrinsic negative curvature.

In these experiments we have used phospholipids with different acyl chain lengths (see Material and methods), in agreement with previous experiments [28], and in order to obtain a good and measurable activity. The same principle was applied for the pH and protein concentration [27,28]. These factors might affect the fluidity and packing of the bilayer and by consequence the toxin activity, which might explain some differences with previous publications. However, these differences cannot account for the demonstrated low specificity of the α-toxin.

4.3. Comparative and adaptive aspects

Numerous bacteria release extracellular toxins with phospholipase and sphingomyelinase activities. For example, *B. cereus* produces at least two phospholipases C and one sphingomyelinase [13–15], *Pseudomonas aeruginosa* produces a multifunctional toxin (PlcH) with phospholipase C, sphingomyelinase and sphingomyelin synthase activity [12,32,47], and a *L. monocytogenes* toxin also exhibits phospholipase and sphingomyelinase activities [2,10]. These toxins have often pathogenic relevance. Indeed, *C. perfringens* α-toxin is the main virulence factor for gas gangrene in humans, and *L. monocytogenes* PLC toxin is involved in the intercellular transfection of the bacteria.

While there is no significant sequence homology to known eukaryotic gene data bases, antibodies raised towards BcPLC cross-react with proteins in mammalian cells that hydrolyse PC at considerable rates [48]. This information along with the fact that these proteins are not essential for bacterial replication, has led to the suggestion that these genes come from horizontal transfer from eukaryotic hosts [49]. In fact, many of these toxins are structurally homologous to mammalian phospholipases. Their role is basically defense and eukaryotic membrane disruption, in order to obtain nutrients or to scape the phagosome or endosome. This could be the case for *C. perfringens, P. aeruginosa* and *L. monocytogenes*, to which specific phospholipase genes might have been transferred, and have since lost specificity. In contrast to previously published data, α-toxin is able to hydrolyse other phospholipids than SM and PC in model membranes. This wider substrate specificity might allow *C. perfringens* to infect a greater variety of host tissues of which membrane composition can differ from one cell type to another. Moreover, this low specificity may help to disrupt intracellular membrane types that vary in composition and help the bacterium to escape the early phagosome and from the host cell cytoplasm, thus increasing α-toxin tissue damaging ability.

C. perfringens α-toxin at high concentrations induces massive degradation of PC and SM in membranes, followed by membrane disruption. However, small amounts of toxin cause limited hydrolysis of PC and SM, generating diacylglycerol and ceramide, respectively. These events activate various signal transduction pathways, which lead to the uncontrolled production of several intercellular mediators as follows. The toxin causes contraction of isolated rat ileum and aorta tissue through the activation of phospholipid metabolism in membranes [50,51]. Notably,

the toxin activates the arachidonic acid cascade in isolated rat aorta [52]. It was found that the toxin-induced contraction is related to the production of thromboxane A2 from arachidonic acid. Later, similar results were reported for PLC produced by other microorganisms [53]. It is therefore likely that bacterial PLC mimics the actions of endogenous PLC in eukaryotic cell membranes, demonstrating its relevance as a tool for the study of eukaryotic signaling pathways.

4.4. Initial enzyme rates and substrate specificity

The reason why PE, PI, PG, and perhaps other phospholipids have never been suggested as CpPLC substrates in the past is not obvious and CpPCL is not the only example. One of our laboratories has recently reported that PlcHR$_2$, a phospholipase C from *P. aeruginosa* and totally unrelated to CpPLC, is not specific for PC and SM as it had been described, but can also hydrolyse PE, PG or CL [12]. Among the reasons for this oblivion we can venture at least two. One is the fact that these enzymes are often tested with soluble, artificial substrates, or with natural lipids in mixed micelles with surfactants, and these conditions are very different from the physiological situation of an enzyme binding a lipid bilayer. Alternatively, or perhaps additionally, only the initial stages of enzyme activity are considered worthy of attention. This is strictly true for kinetic studies when measurement of initial rates is required, but not necessarily for studying other aspects of enzyme behavior. In our case there are examples, e.g. Fig. 3A, in which the presumed non-substrate PE is only hydrolysed after 10 min, so that its cleavage would be missed in initial rate measurements. The same happened with *P. aeruginosa* PlcHR$_2$, acting on PC/PE mixtures [12]. We conclude that the full exploration of a given enzyme activity goes well beyond the initial stages of hydrolysis. It is likely that for the early stages of α-toxin activity, in which the main effects occur through the generation of lipid signal diacylglycerol and ceramide, PC and SM are the main substrates. However, at later stages of α-toxin attack, virtually all membrane phospholipids can be degraded.

From the structure of the docked complex and by site-directed mutagenesis of the residues suggested to play roles in the binding to the substrate head group (D2, G3 and Y57), we could study the modulation of the selectivity of CpPLC as was successfully done for BcPLC [47]. Moreover, the homology of CpPLC with BcPLC and with the other members of the bacterial phospholipase C family, gave us the opportunity to set a paragon for the specificity of other phospholipases that have the same motif in their sequence.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2011.06.008.

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