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Effects of bilayer composition and physical properties on the phospholipase C and sphingomyelinase activities of *Clostridium perfringens* α-toxin

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ABSTRACT

α-Toxin, a major determinant of *Clostridium perfringens* toxicity, exhibits both phospholipase C and sphingomyelinase activities. Our studies with large unilamellar vesicles containing a variety of lipid mixtures reveal that both lipase activities are enhanced by cholesterol and by lipids with an intrinsic negative curvature, e.g. phosphatidylethanolamine. Conversely lysophospholipids, that possess a positive intrinsic curvature, inhibit the α-toxin lipase activities. Phospholipids with a net negative charge do not exert any major effect on the lipase activities, and the same lack of effect is seen with the lysosomal lipid bis (monoacylglycero) phosphate. Ganglioside GT1b has a clear inhibitory effect, while the monosialic ganglioside GM3 is virtually ineffectual even when incorporated at 6 mol % in the vesicles. The length of the lag periods appears to be inversely related to the maximum (post-lag) enzyme activities. Moreover, and particularly in the presence of cholesterol, lag times increase with pH. Both lipase activities are sensitive to vesicle size, but in opposite ways: while phospholipase C is higher with larger vesicles, sphingomyelinase activity is lower. The combination of our results with previous structural studies suggests that α-toxin lipase activities have distinct, but partially overlapping and interacting active sites.

1. Introduction

α-Toxin, a major pathogenic determinant of *Clostridium perfringens*, is a zinc metallophospholipase [1]. The structure of the toxin (370 aa residues) includes a catalytic domain at the amino-terminal part of the protein sequence (N-domain) and a bilayer-binding domain at the carboxy-terminal part (C-domain). The C-domain of α-toxin resembles the structures of eukaryotic phospholipid-binding C2 domains [2]. The toxin has a phosphatidylcholine phospholipase C (PLC) and a sphingomyelinase (SMase) activity [3]. Both hydrolytic activities have been separately characterized on phospholipids in the form of large unilamellar vesicles (LUV), in the absence of detergents, in a previous report from our laboratories [4]. Oda et al. [5] have identified the α-toxin SMase activity as an early step in the toxin-induced haemolysis of sheep erythrocytes.

An interesting problem that has not received sufficient attention in the past is how the presence of lipids other than phosphatidylcholine (PC) or sphingomyelin (SM), i.e. non-substrate lipids would influence the α-toxin activities, and whether or not both hydrolytic activities would be modified in parallel. In the past, most experiments with varying lipid compositions [6,7] had addressed the question of the toxin specificity for substrates, and concluded that only PC and SM were hydrolyzed. Nevertheless Nagahama et al. [7] observed that cholesterol increased α-toxin binding to liposomes composed of PC. Nagahama et al. [8] further described the requirement of unsaturated fatty acyl chains for the C-domain to become inserted in the bilayer. One of our laboratories [9] has shown that gangliosides, particularly those rich in sialic acid, exert a protective effect on cultured cells against α-toxin attack, in agreement with previous studies on lipid monolayers by Bianco et al. [10]. In comparison with the scarcity of studies on the lipidic environment effects on α-toxin PLC and SMase activities, the corresponding investigations on other bacterial lipases are relatively abundant [11–16]. In general lipases appear to be influenced by lipids other than their substrates or end-products, perhaps through changes in the bilayer physical properties.

In the present contribution we have studied the effects of a variety of lipids on the PLC and/or SMase activities of α-toxin. Lipid intrinsic curvature, net charge and intracellular location have been taken into account. Moreover, the role of bilayer curvature has been explored.

Abbreviations: BMP, bis (monoacylglycero)phosphate; BSA, bovine serum albumin; Chol, cholesterol; DOPA, dioleoyl phosphatidic acid; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DOPG, dioleoyl phosphatidylglycerol; DOPS, dioleoyl phosphatidylserine; LBPA, lysobisphosphatidic acid; PI, phosphatidylinositol; PLC, phospholipase C; SMase, sphingomyelinase

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The ensemble of the results shows that α-toxin PLC and SMase activities are individually and delicately modulated by the lipid and bilayer environment.

2. Materials and methods

2.1. Materials

Wild type recombinant *C. perfringens* α-toxin from the strain 8-6 expressed in *Escherichia coli* was purified as described [17]. Fatty acid-free bovine serum albumin (BSA), monosialoganglioside GM3 and trisialoganglioside GT1b were purchased from Sigma. Egg SM, cholesterol (Chol), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylserine (DOPS), dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidic acid (DOPA), dioleoylphosphatidylethanolamine (DOPE), phosphatidylinositol from bovine liver (PI), lysobisphosphatidic acid (LBPA or Oleoyl BMP) and bis(monomyristoylglycero) phosphate (Myristoyl BMP) were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2. Methods

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 solution was frozen in liquid nitrogen and thawed at 37 °C 10 times. Symmetric LUVs were prepared by the extrusion method as described by Nieva et al. [18]. Liposomes to be used in PLC and SMase activity assays were routinely prepared in buffer A (10 mM Tris–HCl, 0.9% w/v NaCl, 3 mM $CaCl_2$, 0.005 mM $ZnSO_4$). For enzyme dilution buffer A was used (10 mM Tris–HCl, 0.9% w/v NaCl, 3 mM $CaCl_2$, 0.005 mM $ZnSO_4$, 0.1 % w/v BSA, pH 7.5). For the preparation of liposomes with DOPS or DOPA in their composition buffer B was used (10 mM Tris–HCl, 0.9% w/v NaCl, 0.005 mM $ZnSO_4$). Before adding protein to these liposome samples, calcium must be present in the aqueous medium. To reach the 3 mM final concentration, buffer B (10 mM Tris–HCl, 0.9% w/v NaCl, 750 mM $CaCl_2$, 0.005 mM $ZnSO_4$) was prepared.

SMase and PLC activities of α-toxin on LUVs have been shown to induce extensive vesicle aggregation [4]. A good correlation was found in the previous study between enzyme activity measurements performed with a chemical method, i.e. water-soluble phosphorous assays, and those carried out following changes in light scattering. Only the latter method was used in the present study, for reasons of convenience, except for the studies as a function of vesicle size, in which formation of water-soluble phosphate was assayed as a function of time, as indicated previously [4]. Changes in light scattering of LUV suspensions caused by the toxin were measured using a SLM Aminco Bowman Series 2 (SLM-Aminco, Spectronic Instruments) spectrofluorimeter, setting both excitation and emission wavelengths at 500 nm [19].

The total volume of each LUV sample was 1000 μl, and substrate concentration (either PC, or SM, or PC + SM) was 300 μM. α-toxin concentration was 20 ng/ml for PLC assays, 80 ng/ml for SMase assays, and 250 ng/ml for assays of the combined PLC and SMase activities. Enzyme concentrations 20 and 80 ng/ml were used to obtain data comparable to those of our previous publication [4]. These are very low α-toxin concentrations, selected to measure initial enzyme rates. This gave rise to very lengthy latency periods and reaction times. The combined PLC and SMase activities were studied with 250 ng/ml α-toxin in order to obtain convenient times of reaction. Consequently, enzyme rates can only be compared within each group of measurements, e.g. measurements of PLC activity can be compared among them, but not with data of PLC + SMase activities. The reactions occurred at 37 °C with continuous stirring for approximately 600–1800 s. Maximum slopes were measured on the resulting "light scattering versus time" plots.

3. Results

3.1. Cholesterol effects

The studies by Nagahama et al. [7] and our own preliminary observations [4] had suggested that cholesterol enhanced the hydrolytic activity of α-toxin. This was tested systematically under conditions when the PLC and SMase activities of α-toxin could be assayed either independently or together. PLC activity can be assayed independently from SMase on bilayers in which PC is the only substrate lipid. Assays are performed at the optimum pH 5.0 [4]. Relevant results are summarized in Table 1. For pure DOPC bilayers, or for mixtures with PE or PG, addition of cholesterol causes a marked increase in PLC activity and a decrease in the length of the latency period, or lag time. Both cholesterol effects are dose-dependent, at least up to 33 mol% cholesterol, as shown in Fig. 1 for the lipid composition DOPC:DOPG:cholesterol.

The SMase activity of α-toxin was assayed at its optimum pH 7.0 on LUV containing SM but no PC. The results are summarized in Table 2. Vesicles consisting of pure SM were not hydrolyzed by α-toxin at 37 °C to any measurable extent after 1 h, thus the effect of cholesterol can only be observed on SM:DOPE or SM:DOPG mixtures. With SM:DOPE SMase behaves in the same way as PLC, cholesterol increasing enzyme rate and decreasing lag time. SM:DOPG however is interesting because in this case cholesterol decreases by ≈60% the enzyme rate, as well as the lag time. Note that in DOPC:DOPG mixtures cholesterol had a clear activating effect (Fig. 1). The difference may be related to different charged amino acids being involved in PC and SM hydrolysis.

SM, in mixtures with cholesterol and other lipids, is known to give rise to lateral phase separation of liquid-ordered, liquid-disordered, and gel phases under certain circumstances [20,21]. In our case, giant unilamellar vesicles were prepared with either SM:DOPC:Cholesterol (1:1:1 mol ratio), SM:DOPC:Cholesterol:PI (23.5:23.5:33:20) or SM:DOPC:Cholesterol:DOPS (23.5:23.5:33:20), and examined by confocal microscopy after DiI staining [20]. Only the ternary mixture exhibited lateral phase separation at room temperature, the remaining two been homogeneous in appearance. At 37 °C, the assay temperature for all the measurements in this paper, all vesicles were homogeneous (J. Sot unpublished data). Thus lateral phase separation has not been considered in the analysis of our results, although strictly speaking the confocal microscopy observations do not exclude the formation of nanometric domains.

The effect of cholesterol was also tested on bilayers containing both SM and PC, so that both PLC and SMase activities could be simultaneously measured (Table 3). Again cholesterol increased enzyme activity and decreased lag times. The same activatory effects were seen on more complex mixtures SM:DOPC:PI (45:45:10) and (40:40:20) with and without cholesterol (Table 3), but in the

Table 1
Phospholipase C activity of *C. perfringens* α-toxin acting on bilayers of different compositions. Enzyme activity was assayed as an increase in light scattering of LUV suspensions at pH 5.

LUV composition (mol ratio)	Maximum slope (s^{-1})·10^{-4}	Lag phase (min)
pure DOPC	0.09 ± 3.8e-3	10 ± 0.63
DOPC:Chol (2:1)	2.17 ± 0.23	3.1 ± 0.4
DOPC:DOPE (2:1)	8.56 ± 1.36	1.6 ± 0.87
DOPC:DOPE:Chol (2:1:1)	16.89 ± 1.42	0.84 ± 0.26
DOPC:DOPE:lysoPC (2:1:1)	0.11 ± 0.04	14.5 ± 1.8
DOPC:DOPG (2:1)	2.33 ± 0.33	3.76 ± 0.29
DOPC:DOPG:Chol (2:1:1)	13.98 ± 2.06	1.46 ± 0.21

Average values of 3 independent measurements ± S.E.M.

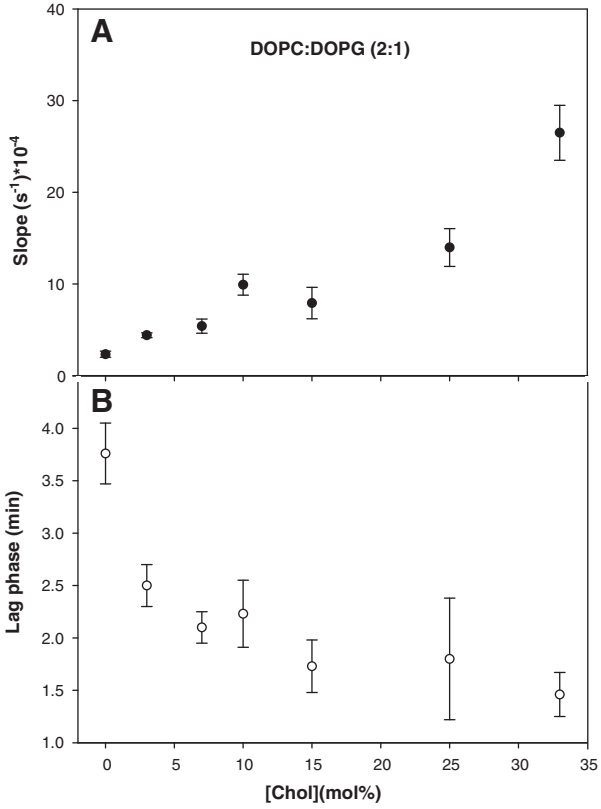

Fig. 1. Effect of cholesterol. Phospholipase C activities of *C. perfringens* α-toxin acting on bilayers composed of DOPC:DOPG (2:1) and different amounts of cholesterol. (A) Maximum slopes, measured after the lag period, of the "light-scattering vs. time" plots. (B) Lag times. Assay conditions as stated under Methods. α-toxin concentration was 20 ng/ml. Average values ± S.D. (n = 3).

presence of PI the enhancement of enzyme rate was not matched by a decrease in lag time. Again this anomaly may reflect some particular effect of negatively charged bilayers on α-toxin SMase activity (further experiments with charged lipids below).

The origin of latency periods in lipases was studied in detail by Basañez et al. [22] for the case of *Bacillus cereus* phospholipase C. These authors found that, during the lag period, the enzyme was working slowly, until a critical amount of the end-product diacylglycerol was formed, about 10 mol%. Then a kinetic switch operated, and the enzyme activity started at full speed. The same phenomenon has been observed for $PlcHR_2$, a PLC/SMase from *Pseudomonas aeruginosa* [16] and, in our previous paper [4], for α-toxin. The observation that cholesterol increases enzyme rates and lowers lag times suggests that cholesterol exerts its activating effect even during the latency period, making the enzyme work faster so that the critical amounts of diacylglycerol and/or ceramide are produced in a shorter time.

The question on how does cholesterol increase α-toxin hydrolytic activities is a complex one, considering the manifold effects of cholesterol on the physico-chemical properties of lipid bilayers.

Table 2
Sphingomyelinase activity of *C. perfringens* α-toxin acting on bilayers of different compositions. Enzyme activity was assayed as an increase in light scattering of LUV suspensions at pH 7.

LUV composition	Slope (s^{-1})·10^{-4}	Lag phase (min)
SM:DOPE (2:1)	0.27 ± 0.06	8.79 ± 1.05
SM:DOPE:Chol (2:1:1)	4.94 ± 0.49	3.66 ± 0.24
SM:DOPE:lysoPC (2:1:1)	0.02 ± 0.002	27.88 ± 3.13
SM:DOPG (2:1)	0.82 ± 0.07	15.53 ± 3.2
SM:DOPG:Chol (2:1:1)	0.33 ± 0.03	2.15 ± 0.35

Average values of 3 independent measurements ± S.E.M.

Table 3
Combined phospholipase C + sphingomyelinase activities of *C. perfringens* α-toxin acting on bilayers of different compositions. Effect of cholesterol. The combined enzyme activities were assayed as an increase in light scattering of LUV suspensions at pH 7.

LUV composition	Slope (s^{-1})·10^{-6}	Lag phase (min)
SM:DOPC (1:1)	1.77 ± 0.06	1.38 ± 0.06
SM:DOPC:Chol (1:1:1)	6.02 ± 0.68	0.27 ± 0.08
SM:DOPC:PI(45:45:10)	3.32 ± 0.21	0.6 ± 0.12
SM:DOPC:PI:Chol (28.5:28.5:10:33)	6.18 ± 0.5	0.73 ± 0.105
SM:DOPC:PI (40:40:20)	3.7 ± 0.26	0.66 ± 0.019
SM:DOPC:PI:Chol (23.5:23.5:20:33)	5.7 ± 0.15	1.8 ± 0.03

Average values of 3 independent measurements ± S.E.M.

Nagahama et al. [7] observed that cholesterol enhances α-toxin binding to liposomes. Cholesterol as well as other "negative-curvature lipids" have been reported to facilitate membrane protein insertion [23,24]. In order to ascertain whether or not the negative intrinsic curvature of cholesterol could be a factor explaining its activating effect on α-toxin hydrolase a number of assays were performed in the presence of lipids with a positive or negative intrinsic curvature.

3.2. Lipid intrinsic curvature

The intrinsic curvature of a lipid monolayer can be defined as the spontaneous curl that the monolayer would adopt in the absence of external perturbations. A positive curvature is defined as that in which the polar head groups of the lipid molecules are on the convex side of the monolayer, conversely when the monolayer curvature is negative the polar head groups are on the concave side. The intrinsic curvature of a monolayer is given by the geometry of the lipids involved i.e. by the ratio of the cross-area of the head group to that of the hydrophobic moiety. For PC or SM this ratio is about 1, and the spontaneous curvature is ≈0. PE or cholesterol have a smaller polar group, the ratio is <1, and the spontaneous curvature is negative. The opposite is true of lysoPC, with a ratio >1, and a positive spontaneous curvature [25,26].

As shown in the previous section cholesterol, a lipid with a negative intrinsic curvature, has in general an activating effect on both PLC and SMase activities of α-toxin. DOPE is a phospholipid with a characteristic negative intrinsic curvature [27]. Its enhancing effect on PLC activity is clearly seen in Table 1, DOPC:DOPE mixtures support a higher activity than pure DOPC, and enzyme activity on DOPC:DOPE:Cholesterol is higher than on DOPC:Cholesterol. The corresponding lag times are reduced. A similar situation is observed with the α-toxin SMase activity (Table 2). SM alone did not allow any measurable activity [4], while a clear activity was reproducibly observed on SM:DOPE (2:1) bilayers (Table 2). Note however that in this case pure SM would be largely in the gel phase at the experimental temperature, thus PE would have here the dual effect of introducing negative curvature and fluidizing SM.

If the enhancing effects of PE and cholesterol were due, at least in part, to their intrinsic negative curvature, the enzyme activities should be inhibited, and the latency periods lengthened, in the presence of lipids with an intrinsic positive curvature in the bilayer. This is what was observed when lysoPC, a typical positive-curvature lipid, was present in the bilayers. For both enzyme activities lysoPC reverted the effects of cholesterol, respectively on DOPC:DOPE and SM:DOPE bilayers (Tables 1 and 2). The reason by which negative-curvature lipids enhance the α-toxin lipase activities is unclear at present. In the absence of direct experimental evidence we can speculate that the relatively small areas of the polar head groups in lipids with a spontaneous negative curvature may help the α-toxin to become partially inserted into the lipid bilayer prior to exerting its hydrolytic activities. There are several examples of membrane protein insertion modulated by the intrinsic curvature of the host lipids [15,23–25,28]. Most lipases appear to operate on the two-stage principle of (i) insertion, (ii) hydrolysis [29,30]. It is reasonable to assume that

factors, e.g. negative-curvature lipids, that appear to facilitate insertion will equally favour an enhanced lipase activity.

3.3. Lipid negative charge

Several common phospholipids exhibit a net negative charge under physiological conditions, e.g. PG, PI, PS. The effects of these phospholipids were tested on the lipase activities of α-toxin. Both PC and SM were present in the bilayers used in these experiments, so that the observed lipase activity corresponds to the sum of PLC and SMase activities. Measurements were carried out at various pH in the 5–8 range, in order to explore as well the pH effects.

The effect of DOPG, a negatively charged phospholipid with ≈0 intrinsic curvature, is shown in Fig. 2. In the absence of DOPG (Fig. 2A, left) the combined PLC + SMase activities appear to increase with pH in the 5–7 range. This is not surprising because PLC activity is about 5-fold higher than SM-ase activity and PLC activity is optimum at pH ≈ 7 [4]. DOPG reverts this trend, and at 20 mol % DOPG in the bilayers (Fig. 2A, right) the activity decreases with increasing pH. At 10 mol % DOPG the activity appears to be independent from pH (Fig. 2A, centre). The effect of 20 mol % DOPG on the pH behaviour of the toxin was studied in more detail as shown in Fig. 2B. DOPG has the effect of clearly shifting the optimum pH range of the enzyme towards lower pH values, i.e. when the lipid net charge is smaller. At pH<6, when enzyme activity increases, the lag times reach a zero value (Fig. 2C). From the data in Fig. 2 it can be concluded that, at pH 7, DOPG does not appear to induce significant changes on the combined lipase activities of α-toxin except for a slight decrease at 20 mol% DOPG.

PI is another negatively-charged phospholipid with ≈0 intrinsic curvature. Its effect on SM:DOPC:Chol bilayers appears to be small, as in the case of DOPG. There are no significant effects of PI on the enzyme activity in the 5–7 pH range (Fig. 3A), although lag times increase with pH. In the absence of cholesterol however, when the activity is lower than in its presence under all conditions, PI increases slightly the α-toxin lipase activity at the two pH values tested, 6 and 7 (Fig. 3B), while lag times decrease correspondingly.

PS is interesting because, in addition to its net negative charge at neutral pH, it has a small positive intrinsic curvature [31]. Detection of lipase activity through changes in light scattering is complicated in this case by the fact that PS-containing vesicles were aggregated in the presence of the Ca^{2+} in the assay buffer [32]. Thus for these vesicles the assay buffer was Ca^{2+}-free, and the cation was only added to the diluted vesicle suspension in the assay cuvette. Ca^{2+} addition caused an instantaneous vesicle aggregation, i.e. increase in light scattering (Fig. 4A). After equilibrium was reached, α-toxin was added and the lipase activity was measured as the additional, slow increase in light scattering in the presence of the enzyme. The separate addition of vesicles, Ca^{2+}, and enzyme had been previously used by one of our laboratories [32]. The effects of PS are summarized in Fig. 4B. A clear inhibitory effect is observed, almost no activity being detected at 30 mol% PS. This is in agreement with the above observations that negative-charge phospholipids do not have a major effect on the lipase activity, and that positive-curvature lipids exert a marked inhibitory effect. Note however that Ca^{2+}-binding might also have an effect on the PS intrinsic curvature.

PA represents the situation of a lipid with both negative intrinsic curvature [33] and negative electric charge. The phosphomonoester group of PA has a pK_{a2} around 8 [33,34]. The data in Fig. 5A show a dual effect of PA on the α-toxin combined PLC + SMase activities. At pH 8, when about 50 % of PA molecules are doubly ionized (—PO_4^{2-}) this lipid has a clear activating effect, while at pH 5–6, when the vast majority of the molecules bear a single charge [-$(PO_4H)^-$] PA inhibits the α-toxin lipase activity. The doubly charged form of the molecule follows the pattern observed with other lipids: intrinsic curvature (in this case negative) is more important than charge for modifying α-lipase activity. It is unusual however that the singly charged form displays such a clear

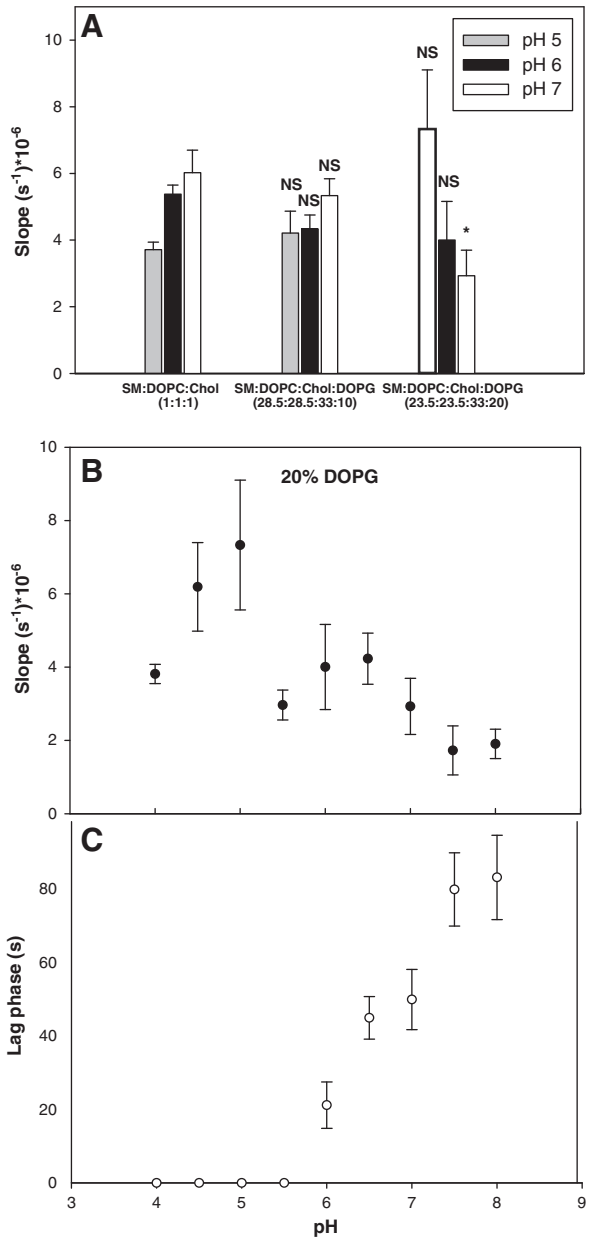

Fig. 2. Effect of PG. Combined phospholipase C and sphingomyelinase activities of *C. perfringens* α-toxin acting on bilayers composed of SM:DOPC:cholesterol (1:1:1) and different amounts of DOPG. (A) Maximum slopes, measured after the lag period, of the "light-scattering vs. time" plots. pH effect on the combined lipase activities for bilayers containing 20 ml % DOPC: maximum slopes (B), and lag times (C). Assay conditions as stated under Methods. α-toxin concentration: 250 ng/ml. Average values ± S.D. ($n = 3$). Significance of differences with respect to the control (no DOPG) experiment (Student's *t*-test): $^*p<0.05$; NS, non-significant.

inhibiting effect. The fact that the phosphomonoester (single charged) group of PA exhibits a tendency to form intra- or intermolecular hydrogen bonds [33] speaks in favour of a putative intermolecular H-bond network that would stabilize PA-rich membrane regions decreasing bilayer accessibility to the enzyme. While the molecular basis for the observed dual PA behaviour remains uncertain, the fact is that the presence of 10 mol% DOPA shifts the optimum pH of α-toxin lipase activity from ca. 7.0 to 8.0, as shown in Fig. 5.

3.4. Late endosomal lipids

C. perfringens is believed to enter the infected cell via endocytosis [35]. The bacterium then releases α-toxin and the lipase activity of the

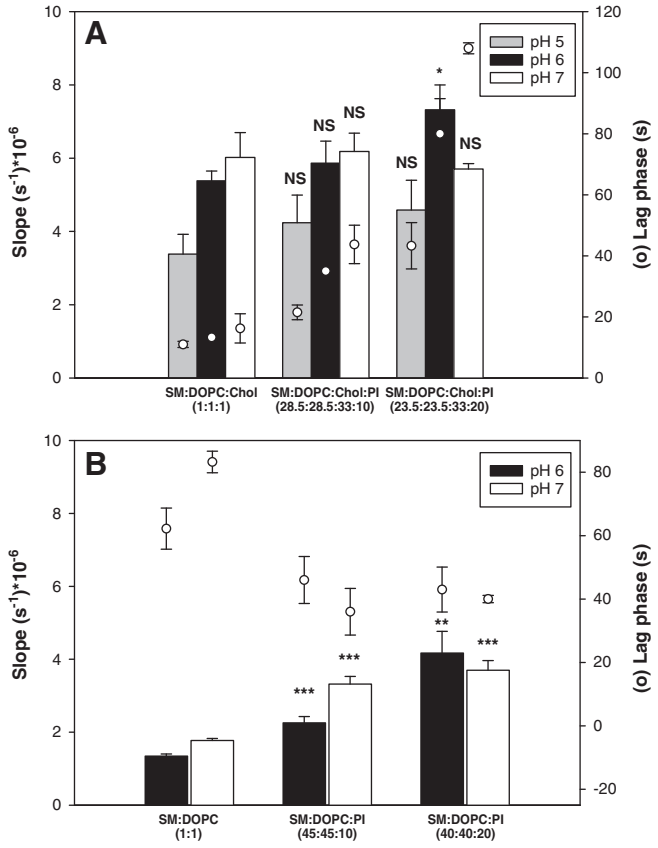

Fig. 3. Effect of PI. (A) Combined phospholipase C and sphingomyelinase activities of *C. perfringens* α-toxin acting on bilayers composed of SM:DOPC:cholesterol (1:1:1) and different amounts of PI. (B) As in A, but in the absence of cholesterol. Assay conditions as stated under Methods. α-toxin concentration: 250 ng/ml. Average values ± S.D. (n = 3). Significance of differences with respect to the control (no PI) experiment, (Student's *t*-test): $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; NS, non-significant.

latter helps the bacterium to escape from the endosomal compartment. Thus we considered of interest to explore the effect of lysobisphosphatidic acid, in which the late endosomal membrane is enriched [36] on the lipase activities of α-toxin. Two such lipids were used, namely dioleoyl and dimyristoyl bis (monoacylglycero)phosphate, respectively DOBMP and DMBMP. The physical properties of DOBMP have been described only recently [37]. At neutral pH pure DOBMP forms highly structured, clustered dispersions, while at acidic pH spherical vesicles are formed. Lipase assays have been performed at pH 5–7 in the presence and absence of cholesterol (cholesterol concentration in endosomal membranes is low [38]). In both cases DOBMP as well as DMBMP exert essentially similar effects, causing some activation at pH 6 and 7, and little or no effect, at pH 5 (Fig. 6). Note that in the presence of cholesterol, but not so much in its absence, lag times increase with pH in vesicles containing BMP. The pH of late endosomes appears to be close to that of the acidic lysosomes, i.e. ≈5. The role of BMP appears to be to facilitate production of budded vesicles rich in BMP, but this lipid does not seem to modify the lipase activities of α-toxin under conditions akin to those found in the endosome/lysosome.

3.5. Gangliosides

Observations from one of our laboratories [9] had indicated that gangliosides in the cell surface, and particularly those rich in sialic acids, protected the cell from α-toxin attack. We tried to reproduce those studies in model bilayers, and obtained essentially similar results. Fig. 7A shows the effect of GM3, a monosialoganglioside, on the combined PLC + SMase activities of α-toxin. No effect is seen up to GM3 concentrations of 6 mol%, when an increase in activity is recorded. Conversely, the trisialoganglioside GT1b at 3 mol% causes a drastic decrease in lipase activity (Fig. 7B). Long lag times are observed in the presence of 1 or 3 mol% GM3, although the origin of this observation is not understood at present. These results are in good agreement with the data by Flores-Díaz et al. [9] in which α-toxin activity was measured through the enzyme-dependent leakage of a fluorescent dye. An inhibitory effect of gangliosides on several phospholipases C has been observed by various authors [10,39–41].

3.6. Effect of vesicle radius

Vesicle radius, a parameter unrelated to the lipids spontaneous curvature, is an additional potentially important factor on the lipase activities. Ahyayauch et al. [15] showed that PI-PLC activity of *B. cereus* increased with decreasing vesicle radii. In our case, PLC and SMase activities were assayed separately, respectively on bilayers composed of DOPC:PE:cholesterol (2:1:1, mol ratio) and SM:PE:cholesterol (2:1:1, mol ratio), forming vesicles with average diameters (estimated by quasielastic light scattering) between ≈60 and 500 nm. For vesicles composed of DOPC:PE:cholesterol 200 nm was the largest size attainable by the extrusion technique. In these experiments enzyme activities were assayed directly as formation of water-soluble phosphorous [4] instead of using the indirect assay through changes in light scattering, because the latter could be unduly influenced by vesicle size. Interestingly, both activities showed opposite behaviours

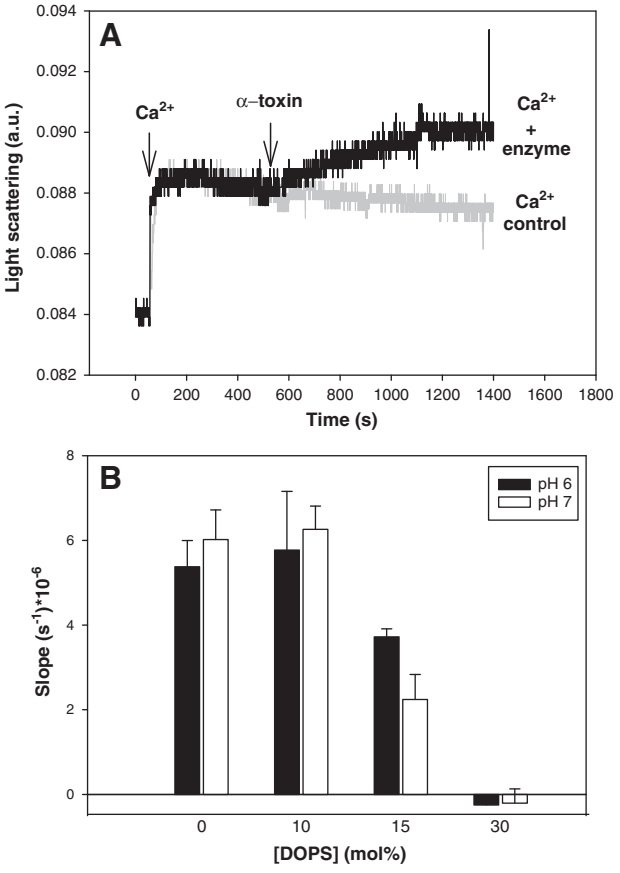

Fig. 4. Effect of PS. (A) Time course of the light scattering of a LUV suspension (SM:DOPC:cholesterol:PS, 28.5:28.5:33:10 mol ratio). After 1 min incubation, Ca^{2+} is added (3 mM final concentration) and when required, α-toxin is added after 500 s. Ca^{2+}-induced increase in light-scattering is subtracted from the total effect caused by Ca^{2+}-enzyme. (B) Combined phospholipase C and sphingomyelinase activities of *C. perfringens* α-toxin acting on bilayers composed of SM:DOPC:cholesterol (1:1:1) and different amounts of DOPS. Maximum slopes of the "light-scattering vs. time" plots as shown in (A). α-toxin concentration: 250 ng/ml. Average values ± S.D. (*N* = 3).

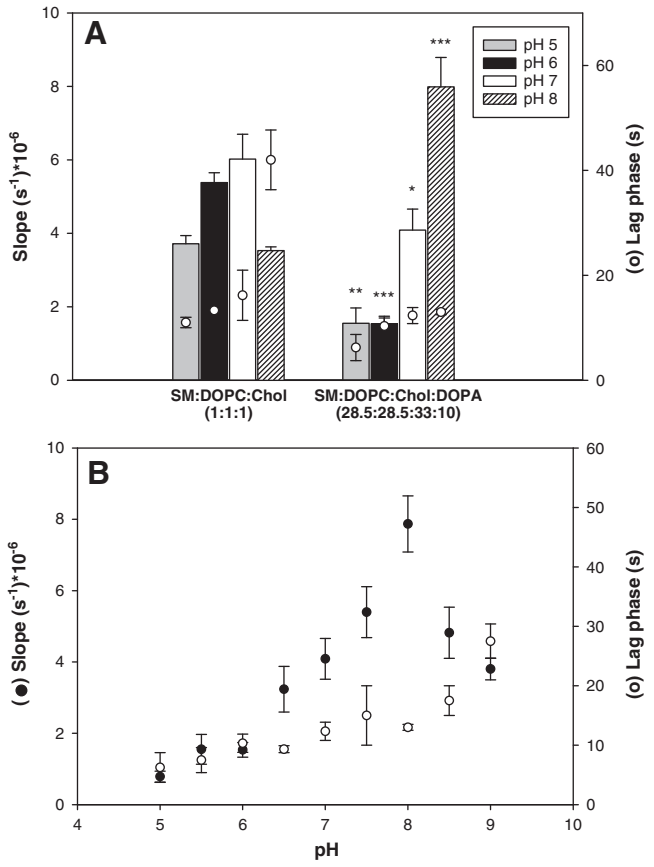

Fig. 5. Effect of PA. Combined phospholipase C and sphingomyelinase activities of *C. perfringens* α-toxin acting on bilayers composed of SM:DOPC:cholesterol (1:1:1) with or without 10 mol % DOPA. (A) Maximum slopes, measured after the lag period, of the "light-scattering vs. time" plots, and the corresponding lag times, at selected pH. (B) A scanning of enzyme activities (maximum slopes) and lag times over the 5–9 pH range. Assay conditions as stated under Methods. α-toxin concentration: 250 ng/ml. Average values ± S.D. ($n = 3$). Significance of differences with respect to the control (no PA) experiment, (Student's *t*-test): $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; NS, non-significant.

(Fig. 8). SMase decreased with increasing vesicle size up to 150 nm, then remained stable, while PLC increased with increasing vesicle size, reaching a plateau at 120 nm. In the absence of more extensive studies on the effect of vesicle radius on lipase activities, it is worth noting that in α-toxin the two lipase activities dependent, according to structural studies, on the same active centre [2], differ in behaviour with respect to vesicle radius.

4. Discussion

The above experiments describe many instances in which the PLC and or SMase activities of *C. perfringens* α-toxin are regulated by composition (cholesterol, phospholipids, sphingolipids) and physical properties (liquid intrinsic curvature, vesicle radius, bilayer electric charge). Two methodological aspects should be discussed first, namely the use of changes in light scattering to assay the enzyme activities, and the problems related to protein binding. PLC and SMase can be assayed following changes in liposome suspension turbidity because diacylglycerol and ceramide, respectively, end-products of PLC and SMase, form "patches" or domains on the vesicle surface that are relatively dehydrated, thus leading to vesicle aggregation when two such patches become in contact [11,12]. Extensive use has been made of this method to assay lipase activities [e.g. 4,11,13,15,22] in one of our laboratories. The procedure has the advantage of convenience, and of allowing a continuous monitoring of the activity. However it cannot be used to compare data from vesicles of different sizes (e.g. Fig. 8) because scattering is directly related to particle size. Another difficulty, that has been ignored in this paper for reasons of simplicity, is that different lipids may modify the physical properties of the bilayer [48], making it more or less amenable to intermembrane contacts irrespective of enzyme activity. For the basic compositions in this paper PC:Chol and SM:Chol, a direct correlation between enzyme hydrolytic activity and increased light scattering was demonstrated [4].

An additional aspect that has not been considered in the present study is that changes in lipid composition may alter enzyme binding to the membrane, independently from modifying enzyme activity. In this work, that was aimed at examining the effects of a large variety of lipids, activity and binding have not been experimentally dissected, i.e. the activities compared here are strictly speaking the combined effects of enzyme binding and lipid hydrolysis [15].

With these caveats in mind it can be stated that while previous studies had pointed at effects of individual lipids [6–8,10] our more systematic approach allows us to conclude that: (i) cholesterol enhances both the PLC and SMase activities, either individually assayed or when they act simultaneously (Tables 1–3, Fig. 1), (ii) lipids with intrinsic negative curvatures, e.g. DOPE (Tables 1 and 2), or PA at pH 8 (Fig. 5) increase PLC and SMase activities, while lipids with positive intrinsic curvatures, e.g. lyso PC (Tables 1 and 2), or PS (Fig. 4) exert inhibitory effects on the same lipase activities, (iii) the stimulatory effects of cholesterol can be largely explained in terms of its negative intrinsic curvature, (iv) the presence of phospholipids with a net negative charge in the headgroup does not exert any marked effect on

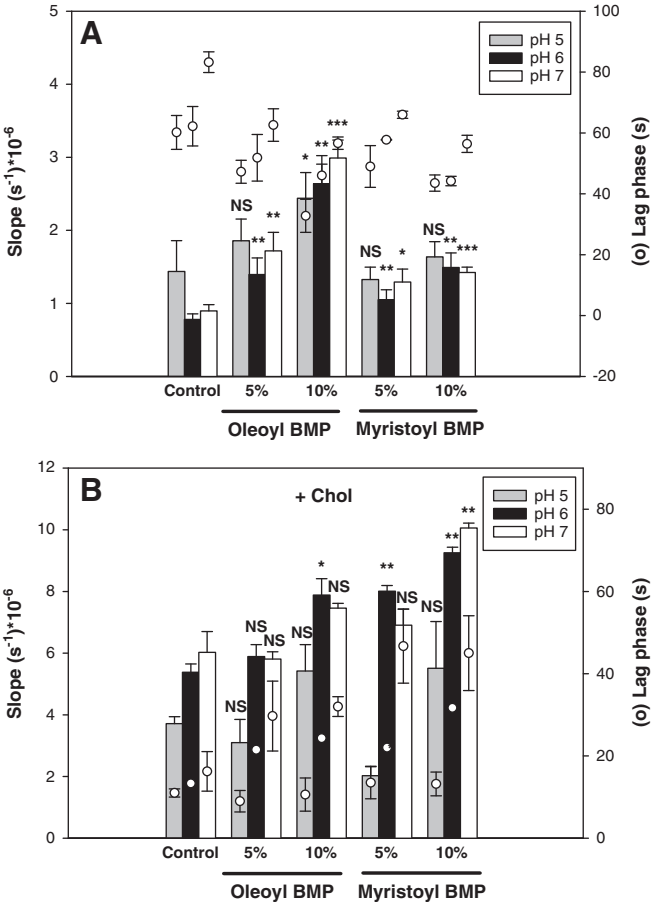

Fig. 6. Effect of BMP. Combined phospholipase C and sphingomyelinase activities of *C. perfringens* α-toxin acting on bilayers containing different amounts of oleoyl BMP or myristol BMP. (A) In the absence of cholesterol, control LUV composed of SM:DOPC (1:1). (B) In the presence of cholesterol, control LUV composed of SM:DOPC:cholesterol (1:1:1). Assay conditions as stated under Methods. α-toxin concentration: 250 ng/ml. Average values ± S.D. ($n = 3$). Significance of differences with respect to the control (no BMP) experiment, (Student's *t*-test): $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; NS, non-significant.

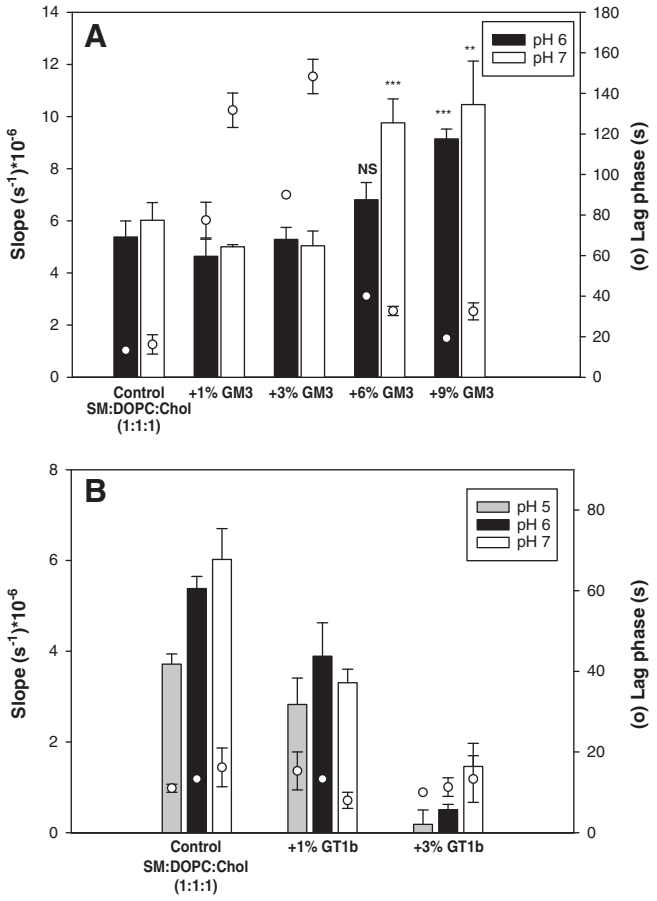

Fig. 7. Effect of gangliosides. Combined phospholipase C and sphingomyelinase activities of *C. perfringens* α-toxin acting on bilayers composed of SM:DOPC:cholesterol (1:1:1) and different amounts of (A) GM3, and (B) GT1b gangliosides. Assay conditions as stated under Methods. α-toxin concentration: 250 ng/ml. Average values ± S.D. ($n = 3$). Significance of differences with respect to the control (no ganglioside) experiment, (Student's *t*-test): $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; NS, non-significant.

the α-toxin lipase activities. The explanation of these effects at the level of bilayer and substrate interaction with individual amino acid residues in the protein remains an open area of study.

Other results in our contribution deserve some comment, namely the observed changes in lag times, and the dual enzyme activities of α-toxin. With respect to lag times, two generalizations can be made. One is that, with very few exceptions, lag times decrease when enzyme activities increase (see, e.g. Figs. 1 and 2). In previous studies [16,22] we have shown that during the lag phase the enzyme works at a low rate, until a critical amount of the end-product (diacylglycerol and/or ceramide) is formed at which point enzyme rates increase dramatically. The results in this paper generalise these observations to a large number of bilayer compositions, and indicate that the various compositions exert a qualitatively similar effect on the enzyme rates during the lag phase and at the optimum activity phase; i.e. those lipids that increase the maximum enzyme rates act similarly during the latency period, decreasing the lag times.

An additional property of lag times is more puzzling, consisting of an increased lag period at higher pH, unrelated to concomitant changes in enzyme activity (see e.g. Figs. 2, 3A and 6B). The phenomenon is more marked in the presence of cholesterol (compare e.g. Fig. 3A and B). At present we cannot offer any hypothesis to explain these observations. The fact that the pH dependence of lag times is less marked, or even nonexistent, in the presence of PA (Fig. 5) or gangliosides (Fig. 7) may be significant, since both PA and gangliosides are know to form extensive intra- and intermolecular H-bond networks. This might be consistent with enzyme activities during the lag phase being hindered by the deprotonation of a key residue, while such protonation/deprotonation events would be buffered or hampered (thus enzyme activities not inhibited) by the H-bond network at the substrate level. Note that lag times remain particularly short and invariant with PA at low pH (Fig. 5B) and with GT1b ganglioside (Fig. 7) both conditions that favour extensive H-bonding [34].

Conversely the presence of cholesterol would tend to weaken the H-bonding networks, thus facilitating the protonation/ deprotonation equilibria. A variety of protonable residues have been related to α-toxin membrane binding, e.g. Tyr 57, Tyr 65 [42], Tyr 307 [43], Lys 330 [2] or Tyr 331 [44].

A further aspect deserving separate discussion is the fact that two activities, PLC and SMase, appear to reside in the same active site of the toxin [2,43,45]: from the chemical point of view, both activities are very similar, being both lipid phosphodiesterases. α-toxin is not the only protein to possess both PLC and SMase activities, other examples being PlcHR from *P. aeruginosa* [46] or the PLC/SMase from *Listeria monocytogenes* [47].

However, at variance with those other enzymes, the PLC and SMase activities of α-toxin can be biochemically dissected, i.e. they can be shown to have similar but not identical properties. In our previous study we found different optimum pH for each of them, more acidic for PLC [4]. In the same study, PLC was shown to be more active than SMase under most conditions. In the present communication, we make the interesting observation that both activities react differently when vesicle radius is increased, SMase becoming less active while PLC activity is enhanced. These combined observations point towards a situation in which two active centres, one for PLC and one for SMase (partially) overlap in the same region of the α-toxin protein. Further experimentation will be required to discern the individual amino acid residues that are responsible for the observed peculiarities.

A comment concerning α-toxin in the framework of *Clostridium* pathology is pertinent. Like so many other similar lipase toxins, α-toxin appears to be directed mainly at degrading animal cell membranes, not protected by a cell wall, in order to obtain carbon and energy sources for the bacterium. From this point of view it makes sense that the enzyme natural substrates are PC and SM, and that it is activated by cholesterol, PC, SM and Chol being the major three lipids in the plasma membrane outer monolayer. But *C. perfringens* is also known to invade the cells in a non-lytic manner, via endo/lysosomes, from which it makes its way to the cytosol. While the lipase activities will undoubtedly help the bacterium in its escape route, it is surprising that the typical endosomal lipid BMP does not appear to modify the α-toxin activities. Finally, it should be taken into account that the lipid end-products of PLC and SMase are respectively

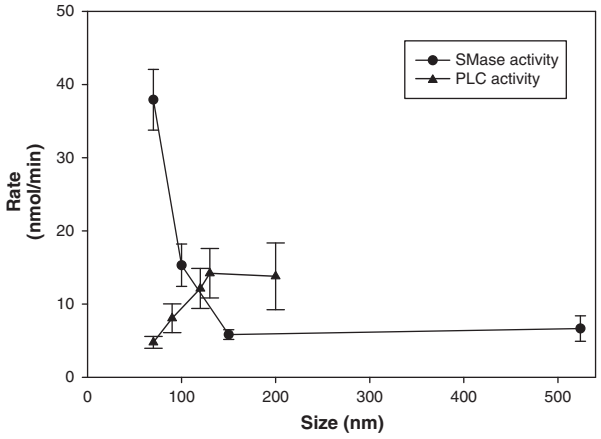

Fig. 8. Effect of vesicle size. The PLC (▲) and SMase (●) rates, measured, respectively, on vesicles of varying diameters composed of DOPC:PE:cholesterol (2:1:1 mol ratio) and SM:PE:cholesterol (2:1:1 mol ratio). α-toxin concentration was 20 ng/ml for PLC and 80 ng/ml for SMase activities. Average values ± S.D. ($n = 3$).

diacylglycerol and ceramide, two potent metabolic signals. Thus α-toxin may be unleashing complex and powerful metabolic responses in the host cell, of which little is known at present.

Acknowledgments

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