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Effect of unsaturated bonds in the *sn*-2 acyl chain of phosphatidylcholine on the membrane-damaging action of *Clostridium perfringens* alpha-toxin toward liposomes

Masahiro Nagahama [a], Akiko Otsuka [a], Masataka Oda [a], Rajesh K. Singh [b], Zyta M. Ziora [b], Hiroshi Imagawa [b], Mugio Nishizawa [b], Jun Sakurai [a,*]

[a] *Department of Microbiology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan*

[b] *Department of Chemistry of Functional Molecule, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan*

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Abstract

Clostridium perfringens alpha-toxin degrades phosphatidylcholine (PC) in the bilayer of liposomes and destroys the membrane. The effect of the type and position of unsaturation in the fatty acyl chain of PC (18:0/18:1 PC) synthesized on the toxin-induced leakage of carboxyfluorescein (CF) from PC liposomes was examined. Differential scanning calorimetry showed that the phase transition temperature (T_m) was minimal when the triple bond was positioned at C (9) in the *sn*-2 acyl chain. The toxin-induced CF leakage decreased with the migration of the bond from C (9) to either end of the acyl chain in PC. The PC containing the *cis*-double bond had a similar T_m to that with the triple bond, but a lower value than the PC containing the *trans*-double bond. Furthermore, the toxin-induced leakage from liposomes composed of PC containing the *cis*-double bond resembled that with PC having the triple bond and was greater than that from liposomes with PC having the *trans*-double bond. The binding of a H148G mutant to PC liposomes showed a reciprocal relationship in terms of the T_m value of PC containing the triple bond. These results indicate that the toxin-induced membrane damage is closely related to membrane fluidity in liposomes.

Keywords: *Clostridium perfringens*; Alpha-toxin; Membrane fluidity; Phase transition; Temperature; Liposome

1. Introduction

Clostridium perfringens produces alpha-toxin, which is an important virulence factor in gas gangrene [1–3]. Alpha-toxin is hemolytic, dermonecrotic, and lethal. Furthermore, it has phospholipase C (PLC) and sphingomyelinase (SMase) activities [1–3]. The toxin has been shown to damage the membranes of various mammalian cells [4–6] as well as artificial membranes [7]. We have reported that toxin-induced hemolysis of rabbit erythrocytes is due to the activation of systems for the metabolism of glycerophospholipid through GTP-binding proteins, and that the toxin activated endogenous SMase via a GTP-binding protein in sheep erythrocytes [4–6]. Furthermore, we reported that the toxin independently induced both production of diacylglycerol through activation of endogenous PLC and phosphorylation of PDK1 via the TrkA receptor signaling pathway in rabbit neutrophils [8]. The initial step in the toxin-induced hemolysis is the hydrolysis of phosphatidylcholine by the enzymatic activities of alpha-toxin in the membrane [4–6].

We reported that alpha-toxin induced leakage of CF from liposomes composed of cholesterol and PC containing unsaturated fatty acyl residues or shorter chains of saturated fatty acyl residues (12 and 14 carbon atoms), and that the toxin-induced release of CF decreased as the chain of the acyl residues of PC increased in length [7]. Therefore, it is possible that the membrane-damaging effect of alpha-toxin on liposomes is related to the T_m of PC in PC-cholesterol liposomes. On the

Abbreviations: DSC, differential scanning calorimetry; T_m, phase transition temperature; CF, carboxyfluorescein; PC, phosphatidylcholine; PCs, phosphatidylcholines; c, double bond of the *cis* type; t, double bond of the *trans* type; y, triple bond; PLC, phospholipase C; SDC, sodium deoxycholate

* Corresponding author. Tel.: +81 88 602 8483; fax: +81 88 655 3051.
E-mail address: sakurai@ph.bunri-u.ac.jp (J. Sakurai).

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other hand, it is known that cholesterol increases the fluidity of the bilayer at temperatures below T_m and decreases the fluidity at temperatures above T_m [9]. Therefore, an accurate analysis has not been made of the relationship between the toxin-induced release of CF and membrane fluidity of liposomes.

The effect on liposomes of an unsaturated bond in the *sn*-2 acyl chain of PC has not yet been fully investigated. Notably, very little is known about the liposomes composed of PC containing a triple bond in fatty acid residues. We analyzed the T_m values of PC analogues containing a triple bond (y), a *cis*-double bond (c), and a *trans*-double bond (t), and compared the binding of the toxin to and the action of the toxin toward liposomes composed of PC containing a triple bond without cholesterol and liposomes composed of PC containing a *cis*-double bond or *trans*-double bond. Here we clarify the relationship between the activity of alpha-toxin and PC analogues in liposomes.

2. Materials and methods

2.1. Materials

Dimyristoyl-L-α-phosphatidylcholine (C14:0/C14:0 PC), dipalmitoyl-L-α-phosphatidylcholine (C16:0/C16:0 PC), distearoyl-L-α-phosphatidylcholine (C18:0/C18:0 PC), 5(6)-carboxyfluorescein diacetate (CF) and sodium deoxycholate (SDC) were purchased from Sigma (St. Louis, MO).

2.2. Expression and purification of alpha-toxin

A 1.3-kb fragment of the wild-type alpha-toxin or *H148G* gene was subcloned from pUC19 into the pHY300PLK (*Escherichia coli–Bacillus subtilis* shuttle vector) *Sma*I site and transformed into *B. subtilis* ISW1214 to purify these proteins [10,11]. Expression and purification of the wild-type alpha-toxin and H148G were performed as described previously [10,11].

2.3. Synthesis of monounsaturated phosphatidylcholines

A series of phosphatidylcholine analogues with *sn*-2 octadecynoyl residues containing triple bond (18:0/18:1y PCs), *cis*-octadecenoyl residues (18:0/18:1c PCs), and *trans*-octadecenoyl residues (18:0/18:1t PCs) were prepared by the condensation of *sn*-2 stearoyl L-α-lysophosphatidylcholine (supplied from Sigma) with corresponding carboxylic acids by using 1,3-dicyclohexylcarbodiimide (1.5 equiv.) in the presence of 4-dimethylaminopyridine hydrochloride (1.3 equiv.) [12]. Preparation of octadecynoic acids was achieved by acetylide coupling reaction to give tetrahydropyranyl ether-protected octadecynols and following direct Jones oxidation. Alkynoic acids were reduced to the corresponding *cis*-octadecenoic acids by the catalytic hydrogenation in the presence of Lindlar catalyst. On the other hand, Birch reduction (lithium in liquid ammonia) provided the corresponding *trans*-octadecenoic acids. All reactions were carried out under an Ar atmosphere to avoid lipid oxidation. The detail of synthesis is shown in the Supplementary file.

2.4. DSC measurements

DSC measurements were performed with a Mettler-Toledo DSC 822e differential scanning calorimeter (Switzerland) using empty hermetically sealed aluminum pans as a reference. The liposomal suspension was carefully placed (the lipid content; 1.5 mg) and sealed in the aluminum hermetic pans. The scan rate was set at 10 °C/min. Thermodynamic data were analyzed with Mettler-Toledo STARe Software 6.20 to determine the peak temperature (T_m). The analysis was repeated four times. The thermograms of DSC and detailed thermodynamic parameters of PC were described in supplementary data (Supplementary Fig. 2 and Supplementary Table 2).

2.5. Other procedures

Liposomes containing CF were prepared as described previously [7]. CF's release was monitored by a procedure described previously [7]. The binding of H148G to liposomes was performed as reported previously [7]. Hydrolysis of sodium deoxycholate (SDC)-solubilized PC by the toxin was also performed as described previously [13].

3. Results

3.1. Effect of the location of the unsaturated bond on the phase transition behavior of sn-1 saturated/sn-2 monounsaturated phosphatidylcholines

The T_m of the gel-to-liquid crystalline phase transitions for PC was measured by differential scanning calorimetry using an aqueous dispersion of synthesized PC (14 molecular species of *sn*-1 saturated/*sn*-2 triple-monounsaturated phosphatidylcholine (C18:0/C18:1y PCs), 9 molecular species of *sn*-1 saturated/*sn*-2 *cis*-monounsaturated phosphatidylcholine (C18:0/C18:1c PCs) and 9 molecular species of *sn*-1 saturated/*sn*-2 *trans*-monounsaturated phosphatidylcholine (C18:0/C18:1t PCs)). Thermograms of DSC and detailed thermodynamic parameters of PC were described in Supplementary Fig. 2 and Supplementary Table 2. Table 1 lists T_m values of C18:0/18:1y PCs. The value was minimal when the triple bond was positioned at C (9), which is the middle of the *sn*-2 acyl chain. The value increased steadily with the migration of the triple bond from the middle to either end (C (4) or C (17)) of the acyl chain, resulting in an asymmetrical, inverted bell-shaped profile. Interestingly, the increase in T_m was more pronounced toward the carboxyl end. Next, the effect of the positions of the *cis*- or *trans*-monodouble bond of PC (C18:0/18:1c PCs or C18:0/C18:1t PC) on T_m was analyzed. DSC shows that the T_m value was minimal when the *cis*- or *trans*-double bond was located at the middle of the *sn*-2 acyl chain, and progressively increased with the migration of the *cis*- or *trans*-double bond toward either end of the chain.

Table 1
Phase transition temperature of *sn*-1 saturated/*sn*-2 monosaturated phosphatidylcholine

Location of unsaturated bond	T_m (°C)		
	Triple bond (C18:0/C18:1y)	*cis*-Double bond (C18:0/C18:1c)	*trans*-Double bond (C18:0/C18:1t)
4	35.44	31.96	44.66
5	30.84	26.03	38.11
6	22.79	21.94	34.67
7	16.83	17.48	31.48
8	12.55	–	–
9	6.88	7.55	27.19
10	8.73	–	–
11	9.13	10.12	29.55
12	15.48	–	–
13	17.29	17.59	37.93
14	26.42	–	–
15	32.96	36.20	44.44
16	45.50	42.92	49.12
17	54.78	–	–

The T_m value of PC species composed of the *cis*-double bond resembled that of PC species composed of the triple bond. On the other hand, the PC species composed of the *trans*-double bond had a higher T_m than that composed of the triple bond or the *cis*-double bond. Koynova and Caffrey [14] reported inverted bell-shaped profiles in the plot of T_m versus the position of the *cis*-double bond in the *sn*-2 acyl chain of *sn*-1 saturated/*sn*-2 monounsaturated PCs. Our result was well consistent with their report.

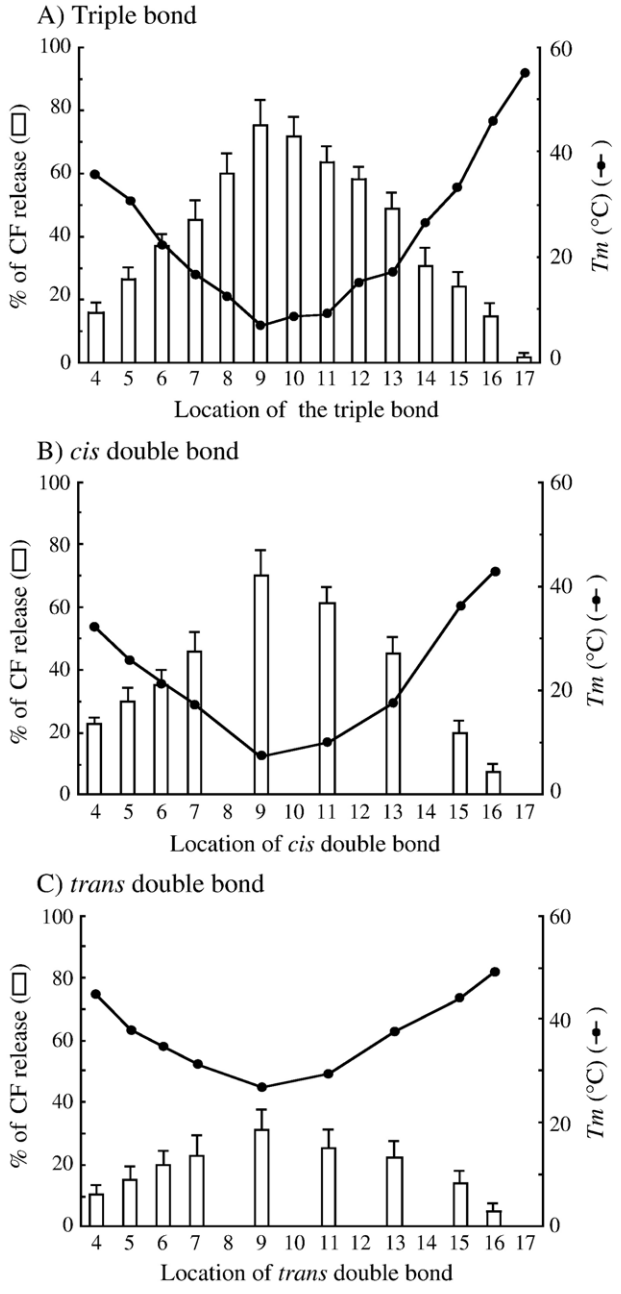

Fig. 1. Relationship between the T_m of PC and the membrane-damaging activity of alpha-toxin. CF-loaded liposomes composed of various PCs were exposed to alpha-toxin (50 ng/ml) in the presence of 1 mM $CaCl_2$ at 37 °C for 30 min. Leakage of CF from the liposomes was measured as described previously [7]. Mean values obtained from three independent experiments were plotted for all illustrations.

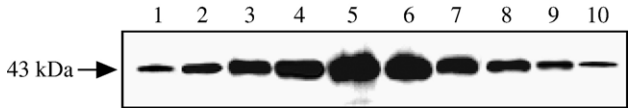

Fig. 2. Binding of H148G to liposomes composed of various PCs. The liposomes composed of various PCs were incubated with H148G (1 μg/ml) in the presence of 1 mM $CaCl_2$ at 37 C for 30 min. After washing, the liposomes were electrophoresed and subsequently electroblotted to PVDF membranes. The immunoblot analysis was performed by using anti-alpha-toxin antiserum. Lanes: 1, 18:0/18:1y5 PC; 2, 18:0/18:1y6 PC; 3, 18:0/18:1y7 PC; 4, 18:0/18:1y8 PC; 5, 18:0/18:1y9 PC; 6, 18:0/18:1 y11 PC; 7, 18:0/18:1y12 PC; 8, 18:0/18:1y13 PC; 9, 18:0/18:1y14 PC; 10, 18:0/18:1y15 PC.

3.2. Effect of the location of the unsaturated bond in acyl chain of PCs on the sensitivity of liposomes composed of synthetic PCs to alpha-toxin

To investigate the sensitivity to alpha-toxin of liposomes composed of synthetic PCs, CF-loaded liposomes were incubated with the toxin in the presence of 1 mM $CaCl_2$ at 37 °C for 30 min. As shown in Fig. 1A, the toxin-induced release of CF from liposomes was greatest when the triple bond was located in the middle of the *sn*-2 acyl chain. The toxin-induced release decreased with the migration of the triple bond from the middle to either end of the chain, resulting in an asymmetrical, bell-shaped profile. As shown in Fig. 1B and C, the membrane-damaging activity of alpha-toxin toward liposomes composed of PC containing the triple bond was similar to that toward PC containing the *cis*-double bond in the *sn*-2 acyl chain. However, less CF was released from the liposomes composed of PC containing the *trans*-double bond than those composed of PC containing the triple or the *cis*-double bond. These results support our report [7] in that the membrane-damaging activity of alpha-toxin depended on the T_m of PC in liposomes.

3.3. Effect of the location of unsaturated bond in the acyl chain of PCs on the binding of alpha-toxin to liposomes

We previously reported that H148G caused no release of CF from liposomes, but possessed the ability to bind liposomes [10]. The variant toxin was incubated with liposomes composed of PC containing the triple bond in the presence of 1 mM $CaCl_2$ at 37 °C for 30 min. The liposomes were washed by centrifugation (22,000×*g*, 20 min), electrophoresed, and subsequently electroblotted to PVDF membranes. The immunoblot analysis using anti-alpha-toxin antibody showed that the band (about 43 kDa) was most intense when the variant toxin was incubated with liposomes composed of PC in which the triple bond is positioned at C (9) (Fig. 2). The intensity of the band gradually decreased as the triple bond migrated to either end of the *sn*-2 acyl chain. When the variant toxin was incubated with liposomes composed of PC containing the *cis*-double bond, the intensity of the band resembled that for the triple bond (data not shown). However, the band was less intense when the liposomes were composed of PC containing the *trans*-double bond rather than the triple bond or *cis*-double bond (data not shown). The result indicates that the binding of the toxin to

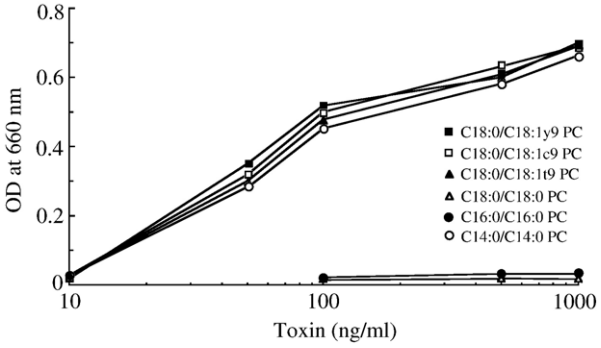

Fig. 3. Hydrolysis of sodium deoxycholate-solubilized PCs with the *sn*-1 saturated chain/*sn*-2 unsaturated bond chain by alpha-toxin. Sodium deoxycholate-solubilized phosphatidylcholine was incubated with various concentrations of alpha-toxin in the presence of 1 mM $CaCl_2$ at 37 °C for 30 min. Phosphorylcholine's release was assayed as described previously [7]. Mean values obtained from four independent experiments were plotted for all illustrations. Symbols: C18:0/C18:1y9 PC (■), C18:0/C18:1c9 PC (□), C18:0/C18:1t9 PC (▲), C18:0/C18:0 PC (△), C16:0/C16:0 PC (●), C14:0/C14:0 PC (○).

liposomes composed of PC is dependent on the T_m of PC and the activity of the toxin.

3.4. Effect of the location of unsaturated bonds in sn-1 saturated/sn-2 monounsaturated PCs on the hydrolysis of PC by alpha-toxin

To determine whether the PLC activity of the toxin is affected by the location of unsaturated bonds in the *sn*-2, the toxin was incubated with SDC-solubilized PC at 37 °C. As shown in Fig. 3, at concentrations over 10 ng/ml, the toxin hydrolyzed SDC-solubilized PC in a dose-dependent manner. There were no significant differences in the sensitivity of PC containing the triple bond and the *cis*-double bond, and the *trans*-double bond in the *sn*-2 acyl chain to alpha-toxin. On the other hand, SDC-solubilized C14:0/C14:0 PC (T_m=23 °C) was also hydrolyzed by the toxin, but the toxin did not hydrolyze SDC-solubilized C18:0/18:0 PC (T_m=55 °C) and C16:0/C16:0 PC (T_m=41 °C) under the conditions described. Therefore, it appears that an unsaturated bond in the *sn*-2 chain of PC is not essential to the activity of the toxin and the activity of the toxin was dependent upon T_m value of PC under the experimental condition.

4. Discussion

The present study demonstrates that the alpha-toxin-induced damage of PC liposomes depends on membrane fluidity. The gel-to-liquid crystalline phase transition behavior exhibited by PC is modulated by many internal factors, most notably the variation in the length and chemical structure (unsaturated bonds) of the hydrocarbon chain of PC. In addition, Koynova and Caffrey [14] reported that the T_m value of the main phase transition for aqueous dispersions of monomeric PC is critically dependent on the location of the carbon–carbon double bond in acyl chains of PC. It therefore appears that the position of the unsaturated bond in acyl chains of PC has a profound effect on the gel-to-liquid-crystalline phase transition behavior. We prepared various PCs (C18:0/C18:1) with a unsaturated bond in the *sn*-2 acyl chain. DSC showed that the T_m value was minimal when a triple bond, *cis*-double bond, or *trans*-double bond was located near the geometric center of the acyl chain (18 carbon atoms), and progressively increased as the bond migrated toward either end of the chain, indicating that the T_m of PC is closely dependent on the location of the unsaturated bond in the *sn*-2 acyl chain.

The T_m value of PC containing an unsaturated bond in the *sn*-2 acyl chain ranked in the following order: triple bond=*cis*-double bond>*trans*-double bond. The triple, and the *cis*- and *trans*-double bonds are known to be structurally different. The *cis*-double bond produces a bend in the acyl chain in which has been thought to interfere with the highly ordered packing of acyl chains in the bilayer membrane of PC liposomes, the interference lowering the T_m value. Koynova and Caffrey [14] reported that the position of the double bond along the fatty acyl chain influenced the T_m, suggesting that a bend interferes with the interaction among fatty acyl chains in PC. Moreover, experimental studies show that in the membrane, a double bond of the *cis* conformation interferes with hydrocarbon chain packing and disrupts the interaction among chains in the bilayer. This substantially lowers T_m values of chains with the *cis* bond located near their center. Several reports [15–17] showed that the structural and dynamic properties of PC with the *trans*-double bond are similar to those of saturated PC, whereas PC with a *cis*-double bond behaves differently. However, T_m value was minimal when *trans*-double bond was located near the center of the acyl chain, indicating that the position of the *trans*-double bond along the fatty acyl chain influences the T_m. Therefore, it is likely that the properties of PC with the *trans*-double bond are different from those of saturated PC. The *trans*-double bond is thermodynamically more stable than the *cis*-double bond because of its zigzag conformation, showing that the effect is greater for lipid bilayers composed of PC containing *cis*-double bond than *trans*-double bond.

We first elucidated that the effect of the triple bond in the *sn*-2 acyl chain on the interaction between molecules of PC is fundamentally the same as that of the *cis*-double bond, and significantly different from that of the *trans*-double bond. It is reasonable that the *trans*-double bond behaves more like the saturated acyl chain as it has an analogous zigzag conformation, while the *cis*-double bond disturbs the packing due to its bend. Since the triple bond contains two more π-electrons than the double bond, some electronic repulsion is expected in that more interference with packing is induced. However, the triple bond behaved similarly to the *cis*-double bond, suggesting that the electronic repulsive effect is not as large as expected. Thus, it appears that the linear conformation of the triple bond is the factor interfering with the packing, similar to the effect of the bend caused by the *cis*-double bond. Accordingly, we concluded that the effect of the triple bond is similar to that of the *cis*-double bond based on the linear conformation that interferes with the packing as with the effect of the *cis*-double bond. The expected electronic repulsive interaction is not the primary factor

disturbing the packing of the acyl chain. It is likely that the effect of the location of the unsaturated bond along the acyl chain in PC on the stability of membranes is associated with a maximum local perturbation of the van der Waals force between chains. When the unsaturated bond occupied the middle of the acyl chain, the T_m value was lowest. It is speculated that the longest effective length for the interaction of acyl chain-acyl chains in PC is minimized. As the unsaturated bond moves toward the carboxy or methyl terminus of the chain, there is a longer region that is not interrupted by the unsaturated bond and interacts with the chain, leading to a higher transition temperature. It is concluded that the PC structural elements that contribute to bulk fluidity and phase behavior are likely to be the van der Waals force between acyl chains.

We have reported that acrylodan-labeled C-domain variants (S263C and S365C) bind to liposomes and exhibit a marked blue shift, suggesting internalization of the C-domain of the toxin into the hydrophobic environment in liposomes [13]. Several papers have reported that the C-domain plays an important role in binding to membranes [18–20]. We reported that Tyr-57 and -65 in the N-domain of the toxin are inserted into the membrane, and that the N-domain specifically binds to membranes and hydrolyzes phospholipids [21]. The data presented here indicated that the sensitivity of liposomes to the toxin correlates well with the T_m value of the PC. It has been reported that membranes of liposomes composed of PC are fluidized over or at the T_m value of PC in liposomes and that an increase in membrane fluidity correlates well with a decrease in the T_m of PC used in liposomes [22,23]. Our result shows that the binding of the toxin to liposomes increased with a decrease in the T_m of the PC in liposomes, suggesting that an increase in membrane fluidity promotes the binding of the toxin to liposomes. It appears that an increase in membrane fluidity results in the toxin being inserted into the bilayer of the membrane. Accordingly, it is concluded that the membrane-damaging action of alpha-toxin is closely related to the membrane fluidity of liposomes.

SDC-solubilized PCs containing an unsaturated bond in the acyl chain (C18:0/C18:1y9, C18:0/C18:1c9 or C18:0/C18:1t9) showed the same sensitivity to PLC activity of alpha-toxin as that containing a saturated bond in the acyl chain (C14:0/C14:0 PC). The toxin was unable to hydrolyze SDC-solubilized C18:0/C18:0 PC and C16:0/C16:0 PC. These observations show that the toxin attacks equally PCs possessing lower T_m than the incubation temperature (37 °C). Therefore, the hydrolysis of PC in the membrane by the toxin seems to depend on the access of the toxin to PC in the bilayer, supporting that membrane fluidity has an important role in the activity of the toxin.

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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.2007.08.016.

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