Mechanism of Membrane Damage by *Clostridium perfringens* Alpha-Toxin

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Received March 23, 1998. Accepted May 11, 1998

Abstract: The effect of *Clostridium perfringens* alpha-toxin on liposomes prepared from phosphatidylcholine (PC) containing the fatty acyl residues of 18 carbon atoms was investigated. The toxin-induced carboxyfluorescein (CF) leakage and phosphorylcholine release from multilamellar liposomes increased as the phase transition temperature of the phosphatidylcholines containing unsaturated fatty acyl residues decreased. However, there was no difference between the sensitivity of the different phosphatidylcholines solubilized by deoxycholate to the phospholipase C (PLC) activity of the toxin. However, the toxin did not hydrolyze solubilized distearoyl-\(L\)-\(\alpha\)-phosphatidylcholine (DSPC) or phosphatidylcholine containing saturated fatty acyl residue, and caused no effect on liposomes composed of DSPC. These results suggest that the activity of the toxin is closely related to the membrane fluidity and double bond in PC. The N-terminal domain of alpha-toxin (AT\(_{1:26}\)) and variant H148G did not induce CF leakage from liposomes composed of dioleoyl-\(L\)-\(\alpha\)-phosphatidylcholine (DOPC). H148G bound to the liposomes, but AT\(_{1:26}\) did not. However, the C-terminal domain (AT\(_{21:33}\)) conferred binding to liposomes and the membrane-damaging activity on AT\(_{1:26}\). These observations suggest that the membrane-damaging action of alpha-toxin is due to the binding of the C-terminal domain of the toxin to the double bond in the PC in the bilayer and hydrolysis of the PC by the N-terminal domain.

Key words: *Clostridium perfringens*, Alpha-toxin, Liposome, Phospholipase C, Phosphatidylcholine, Fatty acyl residue

*Clostridium perfringens* alpha-toxin is thought to be an important factor in gas gangrene induced by organisms (8, 16). This toxin, which has PLC and sphingomyelinase activities, causes membrane damage to a variety of erythrocytes and mammalian cells (16, 20). We have reported that toxin-induced hemolysis is due to the activation of phospholipid metabolism systems through GTP-binding proteins and that the initial step of hemolysis is the hydrolysis of phospholipids by the enzymatic activities of alpha-toxin in the membrane (13–15). Furthermore, the hemolytic activity of alpha-toxin could not be separated from the enzymatic activities of the toxin (9). We investigated the mechanism of toxin-induced CF leakage from liposomes composed of cholesterol and PC containing between 12 and 20 saturated or unsaturated fatty acyl residues (10). This result suggested that membrane fluidity of PC liposomes, which is affected by the saturation of PC and length of the fatty acyl residues, is closely related to the membrane-damaging activity of alpha-toxin (10). Therefore, to clarify the relationship between the membrane-damaging activity of alpha-toxin and unsaturated fatty acyl residue of PC, first, we investigated the effect of the double bond in PCs containing fatty acyl residues of 18 carbon atoms on the activity of the toxin.

The N-terminal two-thirds of alpha-toxin shows sequence homology with the entire *B. cereus* PLC (20). The *B. cereus* PLC is known to hydrolyze PC, but to be nontoxic for mice. Titball et al (18, 19) reported that the N-terminal domain (AT\(_{1:26}\)) hydrolyzes PC and sphingomyelin, but is no longer hemolytic and lethal. We have reported that one zinc ion, which is linked to histidine-148 and glutamic acid-152 in alpha-toxin, is essential for the catalytic site of PLC (9, 12), and that H148G, which is not hemolytic and lethal, binds to sheep erythrocytes (9). Later, our finding was supported by the results obtained by Kameyama et al (5) and Guillouard et al (3).

Abbreviations: AT\(_{1:26}\), N-terminal domain of alpha-toxin; AT\(_{21:33}\), C-terminal domain of alpha-toxin; CF, carboxyfluorescein; DC, sodium deoxycholate; DEPC, dielaidoyl-\(L\)-\(\alpha\)-phosphatidylcholine; DSPC, distearoyl-\(L\)-\(\alpha\)-phosphatidylcholine; DOPC, dioleoyl-\(L\)-\(\alpha\)-phosphatidylcholine; OSPC, \(\beta\)-oleoyl-\(\gamma\)-stearoyl-\(L\)-\(\alpha\)-phosphatidylcholine; PC, phosphatidylcholine; PLC, phospholipase C; SOPC, \(\beta\)-stearoyl-\(\gamma\)-oleoyl-\(L\)-\(\alpha\)-phosphatidylcholine; Tm, phase transition temperature.

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These observations indicate that the N-terminal domain of the toxin is essential for its enzymatic activity.

Second, to investigate the role of the N- and the C-terminal domains of this toxin on its action, we analyzed the membrane-damaging action and binding to liposomes of the N-domain and/or C-domain of alpha-toxin.

**Materials and Methods**

**Materials.** Phosphatidylcholines, DOPC, dielaidoyl-

L-α-phosphatidylcholine (DEPC), β-oleoyl-γ-stearoyl-L-

α-phosphatidylcholine (OSPC), β-stearoyl-γ-oleoyl-L-

α-phosphatidylcholine (SOPC) and DSPC were purchased from Sigma Chemicals Co., Ltd. (St. Louis, Mo., U.S.A.). Cholesterol and sodium deoxycholate (DC) were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Plasmids and purification of wild-type alpha-toxin and variant H148G toxin. The 1.3 kb sequence containing wild-type alpha-toxin or variant H148G gene was subcloned from pT7Blue into the pHY300PLK (E. coli-B. subtilis shuttle vector) Smal site and transformed into B. subtilis strain ISW1214 to purify the proteins. The expression and purification of wild-type toxin and variant H148G were performed as described previously (2, 9, 11).

Expression and purification of AT1376, AT1246 and AT251370. Oligonucleotides (26 mers) for use in the PCR were designed from the nucleotide sequence of the alpha-toxin gene. They were synthesized with 6 additional nucleotides at the 5’ ends, which contained restriction endonuclease sites. The 1.3 kb DNA fragment containing alpha-toxin gene was used as the template DNA. A DNA fragment, which would encode the AT1376, AT1246 or AT251370, was generated after 20 amplification cycles. PCR products were purified using a Geneclean kit (Bio 101 Inc., Calif., U.S.A.), and were subjected to restriction digestion using BglII and SalI. The DNA fragments were ligated with BamHI and SalI-digested pGEX-5X-1 (Pharmacia-LKB Biotechnology). Transformants were screened, and a colony which contained recombinant plasmids (pGEXAT1376, pGEXAT1246, or pGEXAT251370) was isolated. The determination of nucleotide sequences was performed using a dye deoxy termination kit (Applied Biosystems), as described previously (9). When GST-fusion proteins were proteolytically cleaved with Factor Xa, two amino acids (Gly-Ile) were introduced at the N-terminal ends of AT1376, AT1246 and AT251370, encoded by vector.

One milliliter of overnight culture of E. coli JM109 containing the plasmid (pGEXAT1376, pGEXAT1246 or pGEXAT251370) was inoculated into 100 ml of L-broth containing ampicillin 100 µg/ml and incubated at 37 C with shaking. After 3 hr, IPTG was added to a final concentration of 0.6 mM, and the cultures were incubated for an additional 4 hr at 30 C with shaking. After centrifugation (6,000×g, 20 min), the cell pellet was suspended in buffer A (20 mM Tris-HCl, 0.9% NaCl, pH 7.5) and sonicated for 5 min. After another centrifugation (10,000×g, 30 min, 4 C), the supernatant was mixed with 2 ml of glutathione-Sepharose 4B (Pharmacia) previously washed three times with buffer A. The mixture was stirred for 2 hr at 4 C, packed into a chromatography column, and the column washed with 20 ml of buffer A followed by 10 ml of buffer A containing 5 mM reduced glutathione. To generate AT1376, AT1246 and AT251370 fusion, the protein was cleaved for 12 hr at room temperature with Factor Xa according to the manufacturer’s data sheet. The mixture was applied to a 1 ml mini-column of glutathione-Sepharose and the column eluted with 3 ml of buffer A. Each fraction was analyzed by SDS-PAGE.

**Other procedures.** Liposomes containing CF were prepared as described previously (10). CF release was monitored by a procedure described previously (10). The binding of alpha-toxin to liposomes and release of phosphorylcholine from PC by the toxin were performed as described previously (10). Protein determination was carried out according to the method of Lowry et al (6).

**Results**

**Effect of the Double Bond in the Fatty Acyl Residue of PC on the Sensitivity of Liposomes to Alpha-Toxin**

To investigate the effect of the double bond in the fatty acyl residues in positions 1 or 2 of PC on the sensitivity of PC liposomes to the membrane-damaging action of alpha-toxin, liposomes composed of PC containing fatty acyl residues of 18 carbon atoms (DOPC, DEPC, SOPC or DSPC) were incubated with various concentrations of alpha-toxin at 37 C for 30 min. Figure 1 shows that a toxin concentration in the range of 0.1 to 1.0 ng/ml induced CF leakage from liposomes composed of DOPC, and in the range of 10 to 100 ng/ml from liposomes composed of DSPC or SOPC. The sensitivity of liposomes containing DSPC to the toxin was not different from that of liposomes composed of SOPC. CF leakage from liposomes composed of DEPC was induced by the toxin in doses over 50 ng/ml. However, alpha-toxin at the concentration of 2 µg/ml caused no CF leakage from liposomes composed of DSPC.

**Effect of the Double Bond in Fatty Acyl Residue of PC on Hydrolysis of PC by Alpha-Toxin**

To determine whether the toxin hydrolyzes PC containing stearoyl, oleoyl or elaidoyl residue in the liposomes, we measured the phosphorylcholine released by
treating the liposomes composed of PC containing these fatty acyl residues with the toxin (Fig. 2). Toxin at concentrations of 10 to 100 ng/ml dose-dependently induced phosphorylcholine release from liposomes composed of DOPC, concentrations of 50 to 500 ng/ml induced release from liposomes composed of OSPC or SOPC, and concentrations over 200 ng/ml induced release from liposomes composed of DEPC. However, toxin at the concentration of 2 µg/ml caused no release of phosphorylcholine from liposomes composed of DSPC. These results were in agreement with those of the toxin-induced CF leakage from liposomes composed of these PCs. On the other hand, as shown in Fig. 3, a toxin concentration over 20 ng/ml hydrolyzed DC-solubilized PCs containing unsaturated fatty acyl residues in a dose-dependent manner. There were no significant differences in the sensitivity of DOPC, OSPC or DSPC to alpha-toxin. However, the toxin did not hydrolyze DC-solubilized DSPC under the conditions described.

**Effect of the Double Bond in Fatty Acyl Residue of PC on the Binding of Alpha-Toxin to Liposomes**

H148G at the concentration of 100 µg/ml caused no CF release from liposomes composed of DOPC (data not shown). The variant toxin was incubated with liposomes composed of PC containing fatty acyl residues (DOPC, DEPC, OSPC, SOPC or DSPC) at 37 C for 30 min. The liposomes were washed by centrifugation (22,000 × g, 20 min) and assayed on SDS-PAGE after solubilizing in 1% Triton X-100. As shown in Fig. 4, the band of 43 kDa was observed from liposomes composed of DOPC treated with the variant toxin at doses
over 0.5 µg/ml, and increased with increasing doses of H148G (0.25 to 2.0 µg/ml). The band from liposomes composed of SOPC or OSPC was detected at doses of the variant toxin over 1 µg/ml. In addition, the band from liposomes composed of DEPC was detected at 2.0 µg/ml of H148G (Fig. 4). The observation shows that binding of the toxin to liposomes composed of these PCs was in the following order: DOPC > SOPC = OSPC > DEPC. On the other hand, no band was detected for DSPC liposomes, even when incubated with 10 µg/ml of the variant toxin (data not shown). The incubation of liposomes with alpha-toxin at 37°C changed from liposome suspension to oil layer on the surface of the reaction solution, indicating that the toxin hydrolyzes PC in the liposomes and disrupts them. Therefore, the binding of toxin to the liposomes was not detected under these conditions (10). The toxin was incubated with liposomes composed of these PCs at 4°C for 30 min, and the binding of the toxin to the liposomes was investigated. The results were the same as those obtained with the variant toxin. Furthermore, H148G inhibited the binding of toxin to the liposomes under these conditions (data not shown).

**Effect of AT1,246 on Liposomes Composed of DOPC**

To investigate the membrane-damaging activity of AT1,270 and AT1,246, these proteins were incubated with CF-loaded liposomes composed of DOPC in the presence of 2 mM Co²⁺ and 1 mM Ca²⁺ at 37°C for 30 min. AT1,270 in concentrations over 100 ng/ml induced CF release from liposomes, indicating that the addition of Gly-Ile at the N-terminal end of alpha-toxin causes a significant reduction in membrane-damaging activity as compared with that of the wild-type toxin (10). AT1,246 hydrolyzed PC, about 30-fold less than the phospholipase C activity of the wild-type form (data not shown). However, AT1,246 at the concentration of 100 µg/ml induced no CF leakage from the liposomes. Titball et al (18, 19) reported that the N-terminal domain (AT1,246) alone showed no hemolytic activity. Therefore, our result is in agreement with the result of the mentioned authors. However, when CF-loaded liposomes were incubated with various concentrations of AT1,246 in the presence of AT251-370 (10 µg/ml) at 37°C, CF leakage from the liposomes increased with increasing doses of AT1,246 (Fig. 5). AT251-370 (10 µg/ml) bound to liposomes, but alone caused no release of CF from the liposomes (data not shown). In addition, Fig. 5 shows that the membrane-damaging activity of AT1,246 in the presence of AT251-370 was similar to that of AT1,270, indicating that the C-terminal domain is essential for the action of AT1,246 on liposome membranes. The
binding of wild-type alpha-toxin, \( \text{AT}_{120}, \text{AT}_{1246}, \text{AT}_{251-370} \) or \( \text{AT}_{1246} \) in the presence of \( \text{AT}_{251-370} \). On liposomes composed of DOPC was tested. Liposomes were incubated with these proteins at 4°C for 30 min. After centrifugation, liposomes were assayed on SDS-PAGE after solubilizing in 1% Triton X-100. The bands of the wild-type form (about 43 kDa), \( \text{AT}_{1270} \) (about 43 kDa), \( \text{AT}_{251-370} \) (about 12 kDa) and \( \text{AT}_{1246} \) (about 25 kDa) in the presence of \( \text{AT}_{251-370} \) were observed on SDS-PAGE, but there was no band for \( \text{AT}_{1246} \) in the absence of \( \text{AT}_{251-370} \) (data not shown). In addition, the binding of the wild-type toxin was indistinguishable from that of \( \text{AT}_{1270} \) (data not shown).

**Discussion**

The data presented here indicate a dependence of the membrane-damaging action of alpha-toxin on the phase transition temperature (Tm) of phosphatidylcholines containing the fatty acyl residues of 18 carbon atoms. The toxin induced the leakage of internal CF from liposomes composed of DOPC (Tm = −22 C) (21), DEPC (Tm = 10 C) (1), OSPC and SOPC (Tm = 6 C) (1) in the following order: DOPC > OSPC = SOPC > DEPC. It therefore appears that the specificity of liposomes composed of PC to the toxin decreases as the Tm of the PC used increases. Furthermore, both the toxin binding to liposomes and hydrolysis of PC in liposomes by the toxin were closely related to the Tm of the PC in the liposomes. The result shows that the membrane-damaging activity (the binding and action) of the toxin is completely dependent on the membrane fluidity of the liposomes.

DC-solubilized PCs containing unsaturated fatty acyl residue (DOPC, OSPC, SOPC and DEPC) showed the same sensitivity to the enzymatic activity of alpha-toxin. On the other hand, the toxin was unable to hydrolyze DC-solubilized PC containing saturated fatty acyl residue, DSPC. These observations show that the toxin is able to attack equally PCs containing unsaturated fatty acyl residue in positions 1 or 2, but is unable to hydrolyze PC containing saturated residues in positions 1 and 2, showing that the double bond in the fatty acyl residue of PC is essential to the PLC activity of the toxin. It is interesting that the hydrolysis of PC by the toxin is not dependent on positions 1 and 2, and the configuration of the double bond (cis and trans). We therefore assume that the toxin specifically recognizes the double bond in the fatty acyl residue of PC, and perhaps binds to the residue. We can’t explain the difference between the sensitivity of liposomes and DC-solubilized PC to the toxin. However, it is possible that the hydrolysis of PC in the liposomes by the toxin under the detection limit causes marked CF release from the liposomes. Accordingly, CF release from liposomes composed of DOPC may be more sensitive to the toxin as judged by comparing the Tms of the PCs.

The N-terminal domain (\( \text{AT}_{1246} \)) hydrolyzed DOPC, but was unable to bind to liposomes composed of DOPC and disrupt liposomes, indicating that the disappearance of the activity of \( \text{AT}_{1246} \) is due to the absence of protein binding to the liposomes. The crystallography of \( \text{B. cereus} \) PLC (4, 17) and site-directed mutagenesis of \( \text{B. cereus} \) PLC (7) and alpha-toxin (9, 12), suggest that the catalytic site and the binding site for substrates such as PC are in the N-terminal domain. However, the N-terminal domain was unable to bind to the PC in liposomes, suggesting that the binding site of the N-terminal domain with the erythrocyte membrane. Our results showed that the C-terminal domain (\( \text{AT}_{251-370} \)), which alone bound to liposomes, conferred binding to liposomes and membrane-damaging activity on \( \text{AT}_{1246} \). It is likely that the interaction of the C-terminal domain with the PC in the bilayer induces specific binding of the N-terminal domain to PC. H148G that is unable to hydrolyze PC could bond with liposomes composed of DOPC, but was unable to destroy liposomes, indicating that the enzymatic activity of the toxin is essential for membrane-damaging activity. These observations indicate that a combination of the binding to the liposomes, caused by the C-terminal domain of alpha-toxin, and PC hydrolysis, caused by the N-terminal domain of the toxin, is required for membrane-damaging activity. Alpha-toxin seems to have two hydrophobic regions in the C-terminal domain (251-370 amino acid residues) as judged by the hydropathy of the toxin. Furthermore, binding of the toxin to liposomes was dependent on membrane fluidity. Accordingly, the C-terminal domain appears to interact with the hydrophobic region, the fatty acyl residue of the PC buried in the bilayer.

It is therefore concluded that the membrane-damaging activity of the toxin is due to the special accessibility of the C-terminal domain of alpha-toxin to the unsaturated fatty acyl residue of the PC in the bilayer, specific binding of the N-terminal domain to PC and then cleavage of the phosphodiester bond in position 3 of PC by the N-terminal domain of the toxin.

We are grateful to Tatsuji Yasuda for continuous interest and encouragement. We thank Keiko Kobayashi and Tomoko Taniguchi for competent technical assistance. This research was...
supported in part by a grant from the Ministry of Education, Science, Sports and Culture, Japan.

References


