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Effect of fatty acyl domain of phospholipids on the membrane-channel formation of *Staphylococcus aureus* alpha-toxin in liposome membrane

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By use of carboxyfluorescein-loaded multilamellar liposomes prepared from synthetic phosphatidylcholine (PC) or sphingomyelin and cholesterol in a molar ratio of 1:1, we studied whether or not fatty acyl domain of the phospholipids affects the membrane-damaging action (or channel formation) of *Staphylococcus aureus* alpha-toxin on the phospholipid-cholesterol membranes. Our data indicated: (1) that toxin-induced carboxyfluorescein-leakage from the liposomes composed of saturated fatty acyl residue-carrying PC and cholesterol was decreased with increasing chain length of the acyl residues between 12 and 18 carbon atoms, although toxin-binding to the liposomes was not significantly affected by the length of fatty acyl residue; (2) that unsaturated fatty acyl residue in PC or sphingomyelin molecule conferred higher sensitivity to alpha-toxin on the phospholipid-cholesterol liposomes, compared with saturated fatty acyl residues; and (3) that hexamerization of alpha-toxin, estimated by SDS-polyacrylamide gel electrophoresis, occurred more efficiently on the liposomes composed of PC with shorter fatty acyl chain or unsaturated fatty acyl chain. Thus, hydrophobic domain of the phospholipids influences membrane-channel formation of alpha-toxin in the phospholipid-cholesterol membrane, perhaps by modulating packing of phospholipid, cholesterol and the toxin in membrane.

Introduction

Staphylococcal alpha-toxin is a water-soluble 33 kDa polypeptide secreted by most pathogenic *Staphylococcus aureus*. It is hemolytic, dermonecrotic and lethal for laboratory animals. The toxin has also been shown to induce membrane damage on artificial membranes and a variety of mammalian cells including human platelets and peripheral monocytes [1–5]. It is now considered that the multiple biological actions of alpha-toxin are due to the transmembrane-channel activity of hexameric toxin assembled in the membrane of target cells [6–10].

By use of multilamellar liposome as a model system, we previously showed that alpha-toxin specifically bound to the choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin, and induced leakage of internal marker from liposomes [11]. Thus, polar head group of phospholipid plays an important role in the binding and/or incorporation of alpha-toxin into membrane. Our proposal of choline-containing phospholipids as the binder molecules for alpha-toxin is cosistent with the assymmetrical and preferential distribution of these lipids in the outer leaflet of the cytoplasmic membrane of erythrocytes and other types of cell.

In this paper, we studied involvement of hydrophobic domain of the choline-containing phospholipids in the membrane-channel formation of alpha-toxin. As model membranes having curvatures in the size comparable to that of erythrocytes and other types of cell, we

Abbreviations: PC, phosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DAPC, diarachidoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; OPPC, β-oleoyl-γ-palmitoylphosphatidylcholine; POPC, β-palmitoyl-γ-oleoylphosphatidylcholine; CF, carboxyfluorescein; T_m, the temperature of gel-liquid crystalline phase transition; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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employed multilamellar liposomes prepared from synthetic PC (or sphingomyelin), which carried saturated fatty acyl residues of different chain lengths or unsaturated fatty acyl residues, and cholesterol in a molar ratio of 1 : 1.

Materials and Methods

Chemicals

Dilauroyl-L-α-phosphatidylcholine (DLPC), dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), distearoyl-L-α-phosphatidylcholine (DSPC) and dioleoyl-L-α-phosphatidylcholine (DOPC) were kindly supplied from Nippon Oil and Fats Co. Ltd. (Tokyo). Diarachidoyl-L-α-phosphatidylcholine (DAPC), dilinoleoyl-L-α-phosphatidylcholine, β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine (OPPC), β-palmitoyl-γ-oleoyl-L-α-phosphatidylcholine (POPC) and cholesterol were purchased from Sigma Chemicals Co. Ltd. (St. Louis, MO). *N*-Palmitoyl-D-sphingomyelin and *N*-oleoyl-D-sphingomyelin were from Avanti Biochemicals (Birmingham, AL). Cholesterol was recrystallized twice from methanol before use.

Carboxyfluorescein (CF) was purchased from Eastman Kodak (Rochester, NY), and purified as described by Weinstein et al. [12].

Staphylococcal alpha-toxin

Alpha-toxin was purified from the culture supernatant of *Staphylococcus aureus* Wood 46 as described previously [11], and stored at −80°C. Before use, the toxin preparation was chromatographed on a column of Sephadex G-75 to eliminate the toxin hexamer spontaneously generated.

Liposomes

Multilamellar liposomes were prepared as described previously [11]. Mixture of PC (1 μmol) or sphingomyelin (1 μmol) and cholesterol (1 μmol) in chloroform solution was evaporated to dryness at 45–60°C under reduced pressure. The lipid film formed on the wall of a conical-bottomed flask was put in vacuum for 30–60 min, and dispersed in 0.1 ml of 0.1 M CF solution or 1 ml of 10 mM phosphate buffer (pH 7.2), supplemented with 0.85% NaCl by vortexing at 45–50°C or above the temperature of gel-liquid crystalline phase transition (T_m) of the PC. Multilamellar vesicles were collected by centrifugation of the lipid dispersion at 22000 × *g* for 15 min at 4°C. Mean diameters of the liposomes, determined by Microtrac SPA (Leeds and Northrup, St. Petersburg, FL), were in the range between 4.70 and 6.24 μm.

Assay of membrane damage by alpha-toxin as CF release from liposomes

In a 96-well U-bottomed microplate (Nunc Co. Ltd., Roskilde, Denmark), 25 μl of serial dilutions of alpha-toxin were mixed with 25 μl of CF-loaded liposome, and incubated at 25°C for 30 min. Fluorecence intensity was measured with a microplate fluorometer MTP-32 (Corona Electric Co., Katsuda, Japan) at the exitation wave length of 490 nm and the emmision wave length of 530 nm. 100% CF release was defined as the fluorescence intensity that was gained upon exposure of liposomes to 1% Triton X-100 at 25°C for 30 min. Concentrations of liposomes were adjusted on the basis of either phosphate amounts or the fluorescence intensity obtained upon 100% CF release.

Binding of alpha-toxin to liposomes

Mixtures of alpha-toxin (180–200 μg: 5.5–6.1 nmol) and liposome (1 μmol of phosphate) in 500 μl of 10 mM phosphate buffer (pH 7.2), supplemented with 0.85% NaCl (phosphate-buffered saline) were incubated at 25°C for 30 min. After the incubation, toxin-liposome complexes were collected by centrifugation at 23000 × *g* for 20 min, and then washed twice by centrifugation. The liposome-bound toxin was solubilized at 37°C for 1 h in the 10 mM phosphate buffer (pH 7.2), containing 1% Triton X-100. Aliquots of the solubilized toxin were subjected to protein determination, essentially according to Lowry's method using bovine serum albumin containing 1% Triton X-100 as a standard. White precipitate, if formed, was removed by the centrifugation at 23000 × *g* for 15 min.

Binding of the toxin to liposome was also estimated by use of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli's method [13]: Portions of the liposome-bound toxin obtained as described above were boiled for 5 min in the presence of 1% SDS and 2% 2-mercaptoethanol [13], and electrophoresed on 13% polyacrylamide slab gel. Under the conditions, all toxin molecules were mobilized as 33-kDa monomers, because toxin hexamers, if formed, were dissociated to monomers. The gel was stained with Coomassie brilliant blue R-250 as described by Fairbanks et al. [14], and subjected to densitometry. As a standard, certain amounts of the toxin (1–10 μg protein, determined by Lowry's method) were electrophoresed, stained and subjected to densitometry, as described above.

Hexamer formation of alpha-toxin in liposome membranes assessed on SDS-polyacrylamide gel electrophoresis

Alpha-toxin (200 μg: 6.1 nmol) and liposome (0.1 μmol phosphate) in 500 μl of phosphate-buffered saline were incubated for 1 h at 25°C. Toxin-liposome complexes formed were collected by centrifugation at

22000 × *g* for 15 min at 4°C, and washed by subsequent centrifugation to remove residual unbound toxin. Liposome-bound toxin was solubilized in 1% Triton X-100 for 1 h at 25°C, and thereafter subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [13] on 10% polyacrylamide without heating for 5 min at 100°C of the solubilized toxin in the presence of 1% SDS and 2% 2-mercaptoethanol.

The gel was stained as described above, and subjected to densitometry for determination of % hexamerization. % Hexamerization was defined as follows: (Area of hexamer/area of monomer and hexamer) × 100.

Micellaneous

Phosphorus was determined according to the method of Gerlach and Deuticke [15]. Cholesterol was determined with an assay kit from Nissui Pharmaceuticals Co. Ltd. (Tokyo).

Results

To elucidate whether or not the fatty acyl domain of phospholipids plays important role in the membrane-channel formation by alpha-toxin, we measured membrane-damaging action of alpha-toxin on various multilamellar liposomes composed each of PCs carrying saturated acyl residues of different chain lengths and cholesterol in a molar ratio of 1 : 1. Membrane damage by alpha-toxin was assessed as leakage of internal carboxyfluorescein (CF) from the liposomes upon exposure to various doses of the toxin at 25°C for 30 min. Binding of the toxin to liposome was estimated by either the modified Lowry's method or SDS-PAGE after solubilizing the liposome-bound toxin as described in Materials and Methods, and essentially similar results were obtained by the two methods.

As shown in Fig. 1A, alpha-toxin induced CF release from the liposomes composed of saturated fatty acyl residue-carrying PC and cholesterol in a dose-dependent manner. More importantly, the toxin-induced CF-release from the liposomes was decreased with increasing chain length of the fatty acyl residue between 12 and 18 carbon atoms (Fig. 1A), although binding of the toxin to liposome was not significantly affected by the length of fatty acyl residue (Table I). These results indicated that hydrophobic domain of PC was involved in the post-binding step(s) of membrane-channel formation by alpha-toxin, and that chain length of fatty acyl residue of PC profoundly affected the membrane-channel formation even when the membranes contained 50 mol% cholesterol. In the case of the liposome prepared from diarachidoylphosphatidylcholine (DAPC) and cholesterol, however, the sensitivity of the membrane to alpha-toxin was higher than that of the liposome composed of distearoylphosphatidylcholine (DSPC) or dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Fig. 1 and Table I).

To study the effect of unsaturated fatty acyl chain in PC molecules on the toxin-membrane interaction, we measured toxin-induced CF-release from the liposomes composed of unsaturated acyl residue-carrying PC and cholesterol in a molar ratio of 1 : 1. As shown in Fig. 1B and Table I, the liposomes composed of dioleoylphosphatidylcholine or dilinoleoylphosphatidylcholine and cholesterol released 50% of the internal CF upon exposure to alpha-toxin at the concentration of 3.3 or 3.5 μg/ml, respectively. Thus, the liposomes prepared from unsaturated fatty acyl residue-carrying PC and cholesterol were much more sensitive to alpha-toxin

TABLE I

Binding of alpha-toxin to various PC-cholesterol liposomes and concentration of the toxin required for inducing 50% CF-release from the liposomes

Liposome	Bound toxin [a] (%)	Concentration of toxin required [b] for inducing 50% CF release (μg/ml)	T_m of PC [c] (°C)
DLPC-Chol [d] MLV [e]	6.5	15	0
DMPC-Chol MLV	5.8	55	23
DPPC-Chol MLV	6.3	700	41
DSPC-Chol MLV	6.1	> 1000	55
DAPC-Chol MLV	5.3	160	75
DOPC-Chol MLV	9.8	3.3	−22
OPPC-Chol MLV	9.1	6.7	−7.9
POPC-Chol MLV	8.7	9.1	−0.8

[a] % Binding of alpha-toxin to liposomes was determined as described in Materials and Methods. Mean values obtained from three independent experiments are presented.

[b] Concentrations of the toxin required for inducing 50% CF-release were determined from the data of Fig. 1.

[c] T_m of the PC were from the references [24–26].

[d] Chol, cholesterol.

[e] MLV, multilamellar vesicle.

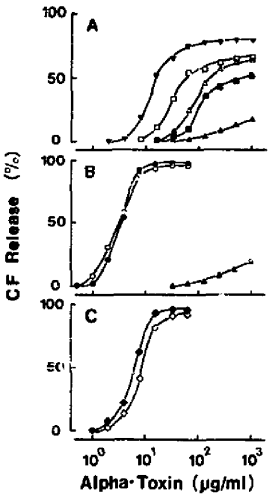

Fig. 1. Effect of chain length (A) and unsaturation (B and C) of fatty acyl residues of PC on the sensitivity of PC-cholesterol liposomes to alpha-toxin. (A) CF-loaded liposomes each composed of dilauroylphosphatidylcholine (DLPC; ▼), dimyristoylphosphatidylcholine (DMPC; □), dipalmitoylphosphatidylcholine (DPPC; ■), distearoylphosphatidylcholine (DSPC; ▲) or diarachidoylphosphatidylcholine (DAPC; △) and cholesterol in a molar ratio of 1:1 were exposed to various doses of alpha-toxin at 25°C for 30 min. Leakage of CF from the liposomes was measured as described in Materials and Methods. Assays were performed in duplicate for determination of the CF leakage. (B) CF release from the liposome composed of dioleoylphosphatidylcholine (DOPC; ○) or dilinoleoylphosphatidylcholine (●) and cholesterol was measured for various doses of the toxin. For comparison, toxin-induced CF-release from the DSPC-cholesterol liposome (▲) is also illustrated. (C) Liposomes composed of β-oleoyl-γ-palmitoylphosphatidylcholine (◆) or β-palmitoyl-γ-oleoylphosphatidylcholine (◇) and cholesterol were used as the target membranes. Mean values obtained from three independent experiments were plotted for all illustrations.

than the liposomes prepared from PC carrying saturated fatty acyl residues of the same chain length.

We further examined whether the position of unsaturated acyl chain in PC molecule affects the sensitivity of PC-cholesterol membranes to alpha-toxin. To answer this question, we used liposomes composed of β-oleoyl-γ-palmitoylphosphatidylcholine (OPPC) or β-palmitoyl-γ-oleoylphosphatidylcholine (POPC) and cholesterol in a molar ratio of 1:1. As shown in Fig. 1C and Table I, the OPPC-cholesterol liposome was slightly more sensitive to alpha-toxin than the POPC-cholesterol liposome. Further, the CF-release from both the liposomes occurred much more efficiently than that from DSPC-cholesterol liposome, but slightly less efficiently than that from the DOPC-cholesterol liposome. These results indicated that single unsaturated fatty acyl residue at any position was adequate to confer high sensitivity to alpha-toxin on the PC-cholesterol membrane.

Effect of *N*-acyl residue of sphingomyelin on the sensitivity of sphingomyelin-cholesterol membrane to alpha-toxin was studied by using liposomes prepared from *N*-palmitoylsphingomyelin or *N*-oleoylsphingomyelin and cholesterol in a molar ratio of 1:1. As shown in Fig. 2, alpha-toxin induced 50% CF release from the former and the latter liposome at the concentration of 30 and > 1000 μg/ml, respectively, i.e., the liposomes composed of unsaturated fatty acyl residue-carrying sphingomyelin and cholesterol was much more sensitive to alpha-toxin than that prepared from saturated acyl chain-carrying sphingomyelin and cholesterol.

We also studied the relationship between the toxin-induced CF-leakage from liposomes and hexamerization of the toxin in the liposome membranes. Alpha-toxin was incubated for 1 h at 25°C with each of the liposomes composed of DOPC, DLPC or DPPC and cholesterol in a molar ratio of 1:1. Liposome-bound toxin collected by centrifugation was solubilized in 1% Triton X-100 at 25°C, thereafter subjected to SDS-PAGE as described in Materials and Methods. Hexameric toxin was apparently resistant to the exposure to 1% Triton X-100 and 0.1% SDS during the solubilization and electrophoresis, respectively, as described by previous studies [6,16]. Boiling of the solubilized toxin (in the presence of 1% SDS and 2% 2-mercaptoethanol) prior to electrophoresis was omitted to avoid dissociation of toxin hexamer formed. As shown in Fig. 3, efficiency of hexamerization of the toxin in liposome membranes was in the order: DOPC > DLPC > DPPC. Average values of % hexamerization of the toxin in liposome membrane (obtained from three independent experiments) were 73, 34 and 12 for DOPC-, DLPC- and DPPC-cholesterol liposome, respectively. The results indicated a good correlation between hexamer

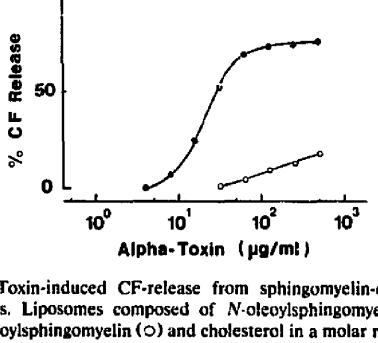

Fig. 2. Toxin-induced CF-release from sphingomyelin-cholesterol liposomes. Liposomes composed of *N*-oleoylsphingomyelin (●) or *N*-palmitoylsphingomyelin (○) and cholesterol in a molar ratio of 1:1 were incubated with various doses of alpha-toxin at 25°C for 30 min. After the incubation, CF release was measured as described in Materials and Methods. Representative data from three independent experiments were illustrated.

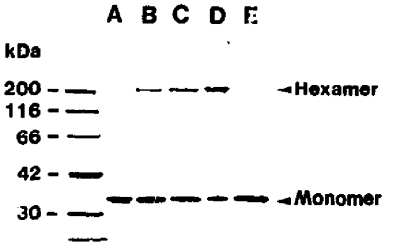

Fig. 3. Hexamerization of alpha-toxin in liposome membranes assessed by SDS-PAGE. Alpha-toxin was incubated for 1 h at 25°C with liposome composed of DPPC (B), DLPC (C) or DOPC (D) and cholesterol in a molar ratio of 1:1. Liposome-bound toxin was solubilized in 1% Triton X-100, and subjected to SDS-PAGE on 10% polyacylamide gel without heating for 5 min at 100°C in the presence of 1% SDS and 2% 2-mercaptoethanol, as described in Materials and Methods. Native toxin (A) or the toxin exposed to 1% Triton X-100 for 1 h at 25°C (E) were electrophoresed on the same gel.

formation of alpha-toxin and the toxin-induced membrane-damage.

By use of liposome and planar membrane, Menestrina et al. [17,18] reported that divalent cations such as Zn^{2+}, Ca^{2+} and Mg^{2+} inhibited the channel-forming activity of alpha-toxin, and that degree of the inhibitory effect of the divalent cations was in the order of Zn^{2+}, Ca^{2+} and Mg^{2+}. To make sure that the toxin-induced CF-release from liposomes reflects the channel-formation by alpha-toxin, we examined whether these divalent cations inhibited the toxin-induced CF-release in a similar fashion. As shown in the Fig. 4, Zn^{2+}, Ca^{2+} and Mg^{2+} inhibited the toxin-induced CF-release from DOPC-cholesterol liposome, and degree of the inhibitory effect was in the order: $Zn^{2+} > Ca^{2+} > Mg^{2+}$.

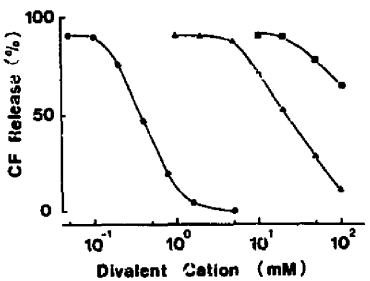

Fig. 4. Inhibitory effect of divalent cations on the toxin-induced CF-release. In the presence of Zn^{2+} (●), Ca^{2+} (▲) or Mg^{2+} (■), CF-loaded liposome composed of DOPC and cholesterol was exposed to alpha-toxin of a given concentration that induced 90% CF release in the absence of the divalent cations. CF release was measured as described in Materials and Methods, except for use of 10 mM Tris-HCl buffer (pH 7.2), supplemented with 0.85% NaCl instead of phosphate-buffered saline to avoid formation of precipitates from phosphate and divalent cation. Mean values from three independent experiments were plotted.

Similar results were obtained with the other liposomes used in this study (data not shown). These results suggested that the toxin-induced CF-leakage from liposomes described above occurred through the membrane-channel formed by alpha-toxin.

Discussion

The data described above indicated that chain length and unsaturation of the fatty acyl residues in PC and sphingomyelin affected the membrane-channel formation or hexamerization of staphylococcal alpha-toxin in the phospholipid-cholesterol membranes, even when the membranes contained cholesterol at 50 mol%. Since it is generally accepted that cholesterol fluidizes PC membrane at concentrations over 30 mol% [19], it is not likely that the hydrophobic domain of PC or sphingomyelin affected the membrane-channel formation (or hexamerization) of alpha-toxin by fluidizing overall the cholesterol-containing membrane. Therefore, we assume that the toxin interacted with the hydrophobic domain of the phospholipids in a rather direct but as yet unspecified manner. Alternatively, the saturated fatty acyl residues of shorter chain length or the unsaturated fatty acyl residues might confer on the cholesterol-containing membrane a more fluid microenvironment, which could not be well characterized by the so-far-developed methods such as differential thermal calorimetry. In this context, it should be noted that sensitivity of the cholesterol-containing membrane to the toxin was apparently in a good correlation with the phase transition temperature (T_m) of PC from gel to liquid crystalline state (Table I), except for the case of DAPC-cholesterol liposome. Although we are not able to account for the correlation at present, we presume that the toxin-bound PC-cholesterol membrane might exhibit moderate phase transition that can not be detected by differential thermal calorimetry and X-ray crystallography. Incidentally, previous studies by use of Raman spectroscopy [20], fluorescence photobleaching method [21] and viscosity measurement [22] detected moderate thermotrophic phase transition of erythrocyte membrane which contains cholesterol at arround 30 mol%.

The effect of the chain length of fatty acyl residues of PC might be explained as follows: PC, cholesterol and alpha-toxin are most tightly packed in the DSPC-cholesterol membrane. Thereby toxin molecules have least probability to associate each other, resulting in the lowest level of membrane-channel formation. With decreasing the length of fatty acyl residues of PC, however, packing of the three components is becoming looser. Under the less-packed conditions, lateral and/or rotational motion is conferred to toxin molecules, leading to the more efficient formation of the membrane-channel of alpha-toxin. The deviation of

DAPC-cholesterol liposome in the sensitivity to alpha-toxin is due to the packing problem at the center of the PC-cholesterol bilayers, because of the difference in the molecular length between arachidoyl chain and cholesterol. Similar explanation is applicable to the enhancing effect of unsaturated fatty acyl residues: Unsaturated fatty acyl residue in PC at any position or in sphingomyelin makes the phospholipid-cholesterol membrane less tightly packed, giving to the lipid bilayer certain microenvironment suitable for inducing the membrane-channel formation of alpha-toxin.

Hildebrand et al. recently proposed dual mechanism of alpha-toxin binding to target cells [23]. By use of radiolabelled, hemolytically-active toxin, they showed that there exist two distinct binding sites for alpha-toxin on erythrocytes, i.e. high-affinity binding sites on rabbit erythrocytes, to which the toxin binds in a fashion of the ligand–receptor interaction, and low-affinity binding sites on both rabbit and human erythrocytes, to which the toxin of high concentrations binds rather nonspecifically [23]. However, it is as yet unspecified what molecular species of erythrocyte membrane compose the two distinct binding sites. Choline-containing phospholipids are possible candidate molecules for a part of the high affinity binding sites and/or for the low affinity binding sites. Furthermore, it is feasible that the choline-containing phospholipids are involved in the binding and/or incorporation of alpha-toxin into lipid bilayer even when the toxin initially binds to certain specific receptor other than the phospholipids.

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