Oligomerization of *Clostridium perfringens* ϵ-Toxin Is Dependent upon Membrane Fluidity in Liposomes†

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ABSTRACT: *Clostridium perfringens* ϵ-toxin binds to receptors on MDCK cells and forms a heptamer in membranes. The mechanism behind the oligomerization of ϵ-toxin was studied using carboxyfluorescein (CF)-loaded liposomes composed of various phosphatidylcholines (PCs). The toxin caused CF to leak from liposomes in a dose-dependent manner. The toxin-induced leakage of CF, binding of the toxin to liposomes, and formation of a functional oligomer increased as the phase-transition temperature (T_m) of the PC used in the liposomes decreased. Surface plasmon resonance analysis using an HPA sensorchip (BIAcore) also revealed that the binding of the toxin to liposomes increased with a decrease in the T_m of the PC used in liposomes. The oligomer that was formed in 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine ([^{125}I]TID)-treated liposomes was labeled, indicating that it inserts into a hydrophobic region. Furthermore, the rate of ϵ-toxin-induced CF leakage was enhanced by treatment with phosphatidylethanolamine or diacylglycerol, which is known to favor a lamellar-to-inverted hexagonal (L−H) phase transition. We show that membrane fluidity in the liposome plays an important role in the binding of the toxin to liposomes, insertion into the hydrophobic region in the bilayer of liposomes, and the assembly process in the bilayer.

ϵ-Toxin is produced by *Clostridium perfringens* types B and D and is responsible for a rapidly fatal enterotoxemia in sheep and other animals, which causes heavy economic losses (*1*, *2*). The toxin is produced as an inactive prototoxin, which is converted to a highly active mature protein by proteolytic removal of N- and C-terminal peptides (*3*).

ϵ-Toxin is lethal and dermonecrotic. Growth of *C. perfringens* type D in host animals is accompanied by the release of large amounts of ϵ-toxin, which is absorbed through the intestine and diffuses to the whole body (*1*, *2*). It has been reported that ϵ-toxin increases vascular permeability and causes edema and congestion in various organs including lungs and kidneys (*2*). We reported that intravenous administration of ϵ-toxin simultaneously caused a rise in blood pressure and a fall in blood flow without any change of the heart rate and electrocardiogram reading (*4*). In addition, we have reported that the toxin caused contraction of rat-isolated ileum and aorta and that the toxin-induced contraction was due to an action mediated through the nervous system (*5*, *6*). Finne et al. (*7*) have demonstrated that ϵ-toxin is able to cross the blood-brain barrier and is accumulated in the brain. Furthermore, we have reported that the toxin exhibited high affinity for brain and synaptosomal membranes, showing that the binding to the brain is important to the lethal activity of the toxin (*8*, *9*). It was found that the toxin stimulated the release of dopamine in rat brain, suggesting that it activates the dopamine nerve system in the brain (*10*). Miyamoto et al. (*11*) reported that ϵ-toxin caused an excessive release of glutamate from hippocampal neurons. It therefore appears that these events induced by the toxin are related to the disruption of brain tissues and perivascular edema, which is probably responsible for neuronal damage.

A comparative analysis of the deduced amino acid sequences of ϵ-toxin (*12*) and other protein toxins, e.g., *Aeromonas hydrophila* aerolysin, *C. perfringens* perfringolysin O, and *Pseudomonas aeruginosa* cytotoxin, indicated that ϵ-toxin belongs to a family of bacterial pore-forming toxins (*13*). Cole et al. (*14*) reported that the crystal structure of ϵ-toxin was similar to that of aerolysin. Nagahama et al. (*15*) and Petit et al. (*16*) reported that ϵ-toxin formed a heptamer in Madin−Darby canine kidney (MDCK) cells, which are sensitive to ϵ-toxin, and synaptic membranes of rats and that the cytotoxic activity correlated with the formation of the oligomer and an efflux of K^+ (*15*). Furthermore, Miyata et al. (*17*, *18*) reported that cleavage of a C-terminal peptide was required for oligomerization of ϵ-toxin in the synaptosomal membrane and that the activated toxin formed an oligomer within the detergent-insoluble microdomains of MDCK cells. Petit et al. (*19*) reported that the diameter of the membrane pore formed by ϵ-toxin in the monolayer was about 2 nm in experiments involving the use of osmotic protectants with different hydrodynamic diameters, indicating that the toxin binds to artificial membranes and forms a functional oligomer. However, little is known

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about the factors important for the oligomerization of the toxin.

To investigate the initial interaction between ϵ-toxin and biological membranes, we employed liposomes as a model and examined the relationship between PC in the bilayer of the liposomes and the binding of the toxin to liposomes or formation of a functional oligomer in liposomes. We first demonstrate that the sensitivity of liposomes to the toxin is dependent upon the fluidity of the liposomes.

MATERIALS AND METHODS

Materials. 1,2-Dioleoyl-*sn*-glycerol (DAG)[1] was obtained from Avanti Polar Lipids (Alabaster, AL). Dilauroyl-L-α-phosphatidylcholine (DLPC), dimyristoyl-L-α-phosphatidylcholine (DMPC), dioleoyl-L-α-phosphatidylcholine (DOPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), dipalmitoleoyl-L-α-phosphatidylcholine (DPOPC), distearoyl-L-α-phosphatidylcholine (DSPC), dioleoyl-L-α-phosphatidylethanolamine (DOPE), oleoyl-L-α-lysophophatidylcholine (lyso-PC), and 5(6)-carboxyfluorescein diacetate (CF) were purchased from Sigma (St. Louis, MO).

Purification of ϵ Prototoxin and Activation of Prototoxin. Procedures for the culture of *C. perfringens* type D (NCTC 8346) and purification of ϵ ptototoxin were carried out as described previously (*4*). The purified prototoxin was activated by incubation at 37 °C for 30 min with 0.1% trypsin (Sigma) in 0.02 M phosphate buffer (pH 8.0).

Preparation of Liposomes. Multilamellar liposomes composed of PC were prepared according to previously described methods (*20*). Organic solvents were evaporated from PC (0.5 μmol) under nitrogen. The resulting films were kept under vacuum for at least 2 h to remove traces of the solvent. The lipids were then hydrated by vortexing at 40−50 °C in 100 mM CF in 20 mM Tris-HCl buffer (pH 7.5) containing 0.9% NaCl (TBS). The liposome suspensions were centrifuged at 22000*g* for 15 min at 4 °C to remove the unincorporated CF and washed 3 times by centrifugation. The resulting liposomes were suspended in 200 μL of TBS.

Assay of Marker Release from Liposomes. In a 96-well microplate, ϵ-toxin and 5 μL of CF−liposomes were mixed in TBS to give a total volume of 200 μL. After the mixture had been incubated at 37 or 4 °C for 1 h, the fluorescence intensity was measured with a MTP 32 microplate fluorometer (Corona Electric Co., Katsuda, Japan) at an excitation wavelength of 490 nm and an emission wavelength of 530 nm (*20*). The toxin-induced CF release was maximal after 60 min of incubation. A CF release of 100% was defined as the fluorescence intensity that was gained upon exposure of liposomes to 1% Triton X-100 at 37 °C for 30 min.

Measurement of ϵ-Toxin Binding to Liposomes. Liposomes (0.02 μmol of lipid) were incubated with ϵ-toxin (10 μg/mL) at 4 or 37 °C for 60 min. The liposomes were then sedimented at 22000*g* for 20 min at 4 °C, and the pellet was washed 3 times by centrifugation. The washed liposomes were dissolved in 20 μL of SDS−sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% 2-mercaptoethanol, 1% SDS, and 0.01% bromphenol blue) and boiled for 3 min prior loading on the gel. SDS−polyacrylamide gel electrophoresis (PAGE) was performed on 4−20% gradient gels at 30 mA for 1 h at 25 °C, and the protein bands were then electrophoretically transferred from the gel to a poly(vinylidene fluoride) membrane (Immobilon P; Millipore) in a buffer containing 20 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3) at 2 mA/cm^2 for 4 h at 25 °C. Residual binding sites on the membrane were blocked by 5% skim milk in 20 mM Tris-HCl buffer (pH 7.6) containing 0.9% NaCl and 0.1% Tween 20 for 2 h at 25 °C. To detect the band of ϵ-toxin by Western blotting, the membrane was incubated first with polyclonal anti-ϵ-toxin (rabbit) antibody and then with anti-rabbit immunoglobulin G-peroxidase conjugate (Amersham Biosciences, Picaway, NJ). The toxin on the membrane was visualized with the enhanced chemiluminescence Western blotting (immunoblotting) analysis system (Amersham Biosciences).

Surface Plasmon Resonance (SPR) Analysis. The PCs in the organic solvent were dried in glass vials under N_2 gas. The resulting films were kept under vacuum for at least 2 h to remove traces of the solvent. The lipids were then hydrated by vortexing at 40−50 °C in TBS and placed in a sonicating bath for 20 s at 25 °C. Suspensions containing the liposomes were extruded (20 passes) through 50 nm polycarbonate membranes in a Liposofast apparatus (Avestin, Inc., Ottawa, Ontario, Canada) at 25 °C. Liposomes were separated from unincorporated material by passage through a 1 mL Sepharose CL-4B column at 25 °C. SPR was measured with a BIAcore 3000 system using a HPA sensor chip (BIAcore, Uppsala, Sweden). The surface of the sensor chip is composed of long-chain alkanethiol molecules that form a flat, quasicrystalline hydrophobic layer. This sensor chip can immobilize lipids via surface lipophilic interaction. To assess liposome binding by the BIAcore, the sensor chip was cleaned with 25 μL of 20 mM CHAPS at a flow rate of 5 μL/min and then with 30% ethanol. Liposomes were injected at a flow rate of 1 μL/min for 400 min at 25 °C. After the injection of the liposomes, 10 mM NaOH was injected for 1 min at a flow rate of 5 μL/min to remove the multiple layers of lipids. After immobilization of the lipids, 20 μL of bovine serum albumin (1 mg/mL) was injected at a flow rate of 1 μL/min to coat the nonspecific binding sites. All analyses of interactions between PC and ϵ-toxin were performed in TBS at 37 °C and at a flow rate of 20 μL/min. The toxin was injected at various concentrations (10, 25, and 50 μg/mL) to determine the rate constants. The surface of the sensor chip was regenerated to remove bound toxin by injection of 10 mM Tris-HCl buffer (pH 9.0) for 1.5 min. This regeneration procedure did not result in a loss of binding capacity. The association and dissociation rate constants k_{on} and k_{off} were determined from sensorgram data by using the BiaEvaluation 3.0 software package. The equilibrium constant, K_D, was calculated from these kinetic constants.

Liposome−TID Photolabeling. Under photographic safety lighting, 6.5 μCi of [^{125}I]TID (5 μCi/μL solution in 3:1 ethanol/water; Amersham Biosciences) was added to 100 μL

[1] Abbreviations: CF, 5(6)-carboxyfluorescein diacetate; DAG, 1,2-dioleoyl-*sn*-glycerol; DLPC, dilauroyl-L-α-phosphatidylcholine; DMPC, dimyristoyl-L-α-phosphatidylcholine; DOPC, dioleoyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-L-α-phosphatidylcholine; DPOPC, dipalmitoleoyl-L-α-phosphatidylcholine; DSPC, distearoyl-L-α-phosphatidylcholine; DOPE, dioleoyl-L-α-phosphatidylethanolamine; lyso-PC, oleoyl-L-α-lysophophatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPR, surface plasmon resonance; TBS, 20 mM Tris-HCl buffer (pH 7.5) containing 0.9% NaCl; T_m, phase transition temperature; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; L−H, lamellar-to-inverted hexagonal.

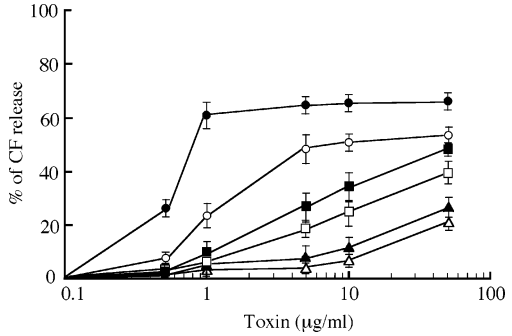

FIGURE 1: ϵ-Toxin-induced release of CF from liposomes. CF-loaded liposomes composed of DPOPC (●), DOPC (○), DLPC (■), DMPC (□), DPPC (▲), or DSPC (△) were exposed to various doses of ϵ-toxin at 37 °C for 60 min. The leakage of CF from liposomes was measured as described in the Materials and Methods. The values are the mean ± SEM ($n = 5$).

of liposome suspension in TBS and incubated at 37 °C for 10 min (*21*). This represented an excess of liposomes to ensure the complete incorporation of [^{125}I]TID into the hydrophobic phase of the lipids. The liposome suspensions were centrifuged at 22000*g* for 15 min at 4 °C to remove the unincorporated [^{125}I]TID and washed 3 times by centrifugation in TBS. ϵ-Toxin (10 μg/mL) was incubated with the [^{125}I]TID−liposome and then incubated for 30 min at 37 °C. Samples were illuminated with a long-wave UV fluorescent strip, precipitated by trichloroacetic acid, washed with ethanol and acetone, then separated by SDS−PAGE, and autoradiographed using a Fuji BAS 2000 system (Fuji Photo Film Co. Ltd., Tokyo, Japan).

RESULTS

ϵ-Toxin-Induced CF Leakage from Liposomes Composed of Various PCs. We reported that ϵ-toxin formed an oligomer on the MDCK cell membrane and that the oligomer caused a rapid release of K^+ from the cells and a subsequent loss of cell viability (*15*). To define the formation of the oligomer on artificial membranes, we examined the effect of ϵ-toxin on CF-loaded liposomes prepared from various PCs: DPOPC ($T_m = -36$ °C), DOPC ($T_m = -22$ °C), DLPC ($T_m = 0$ °C), DMPC ($T_m = 23$ °C), DPPC ($T_m = 42$ °C), and DSPC ($T_m = 55$ °C). As shown in Figure 1, these liposomes released the internal marker, CF, in a dose-dependent manner, when they were exposed to 0.5−10 μg/mL ϵ-toxin at 37 °C for 60 min, in the following order: DPOPC > DOPC > DLPC > DMPC > DPPC > DSPC. The results indicated that the toxin-induced CF release from the liposomes is dependent upon the T_m of the PCs used, although the sensitivity of DPOPC−liposomes to the toxin was about 100-fold lower than that of MDCK cells (*15*). On the other hand, the toxin had no effect upon the release of CF when these liposomes were exposed at 4 °C for 60 min (data not shown).

Binding of ϵ-Toxin to Liposomes. To clarify the relationship between the action of the toxin on liposomes and the T_m of PCs in liposomes, we studied the binding to liposomes and oligomerization of the toxin. After incubation of the toxin with liposomes at 4 or 37 °C for 60 min, the liposomes were washed by centrifugation (20000*g* for 20 min) and subjected to SDS−PAGE analysis after solubilizing in SDS−sample buffer and Western blotting with antiserum prepared against ϵ-toxin. When incubated at 4 °C, the toxin was observed as

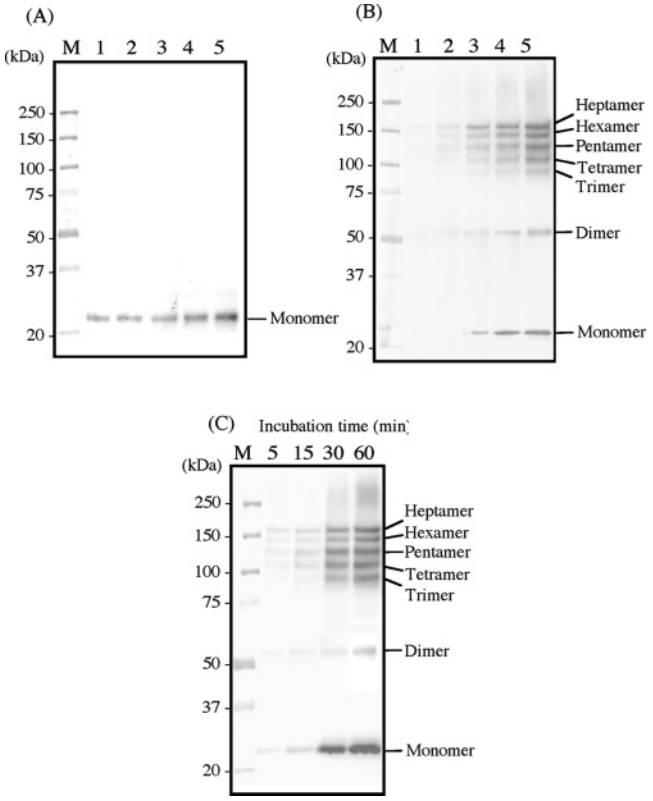

FIGURE 2: Binding of ϵ-toxin to liposomes. ϵ-Toxin (10 μg/mL) was incubated with liposomes composed of DSPC (lane 1), DMPC (lane 2), DLPC (lane 3), DOPC (lane 4), or DPOPC (lane 5) at 4 °C (A) or 37 °C (B) for 60 min. ϵ-Toxin (10 μg/mL) was incubated with liposomes prepared from DPOPC at 37 °C for various periods (C). The liposomes were washed and subjected to 4−20% gradient SDS−PAGE and Western blotting using anti-ϵ-toxin antibody as described in the Materials and Methods. M = prestained molecular-weight markers.

a monomer of about 30 kDa (Figure 2A), suggesting that no oligomerization occurs under the condition. As shown in Figure 2B, upon incubation at 37 °C, the order in terms of efficiency of oligomerization of the toxin in liposomes was as follows: DPOPC > DOPC > DMPC > DLPC > DSPC, indicating that ϵ-toxin forms an oligomer in liposomes in a T_m-dependent manner. These results show a good correlation between oligomerization of ϵ-toxin and the toxin-induced release of CF. The monomer and oligomer bands of ϵ-toxin were not detected when normal serum of rabbit was used (data not shown). When the toxin was incubated with liposomes composed of DPOPC (DPOPC−liposomes) at 37 °C, a dimer, trimer, tetramer, pentamer, and hexamer of the toxin were observed, although the binding of the toxin to the liposomes was significantly weaker than that to MDCK cells in which the heptamer only was observed (*15*). Miyata et al. (*17*) also found that ϵ-toxin formed a dimeric−heptameric toxin complex in synaptosomal membranes using ϵ-toxin derivatives. The binding of ϵ-toxin to DPOPC−liposomes was measured after incubation at 37 °C for various periods. As shown in Figure 2C, the formation of these oligomers increased in a time-dependent manner.

SPR Analysis of the Binding of ϵ-Toxin to Lipid Monolayers. To monitor the binding of ϵ-toxin to liposomes, liposomes composed of various PCs were immobilized on the surface of a HPA sensor chip (typically ∼5000 RU) and the toxin solution (TBS) was injected. As shown in Figure

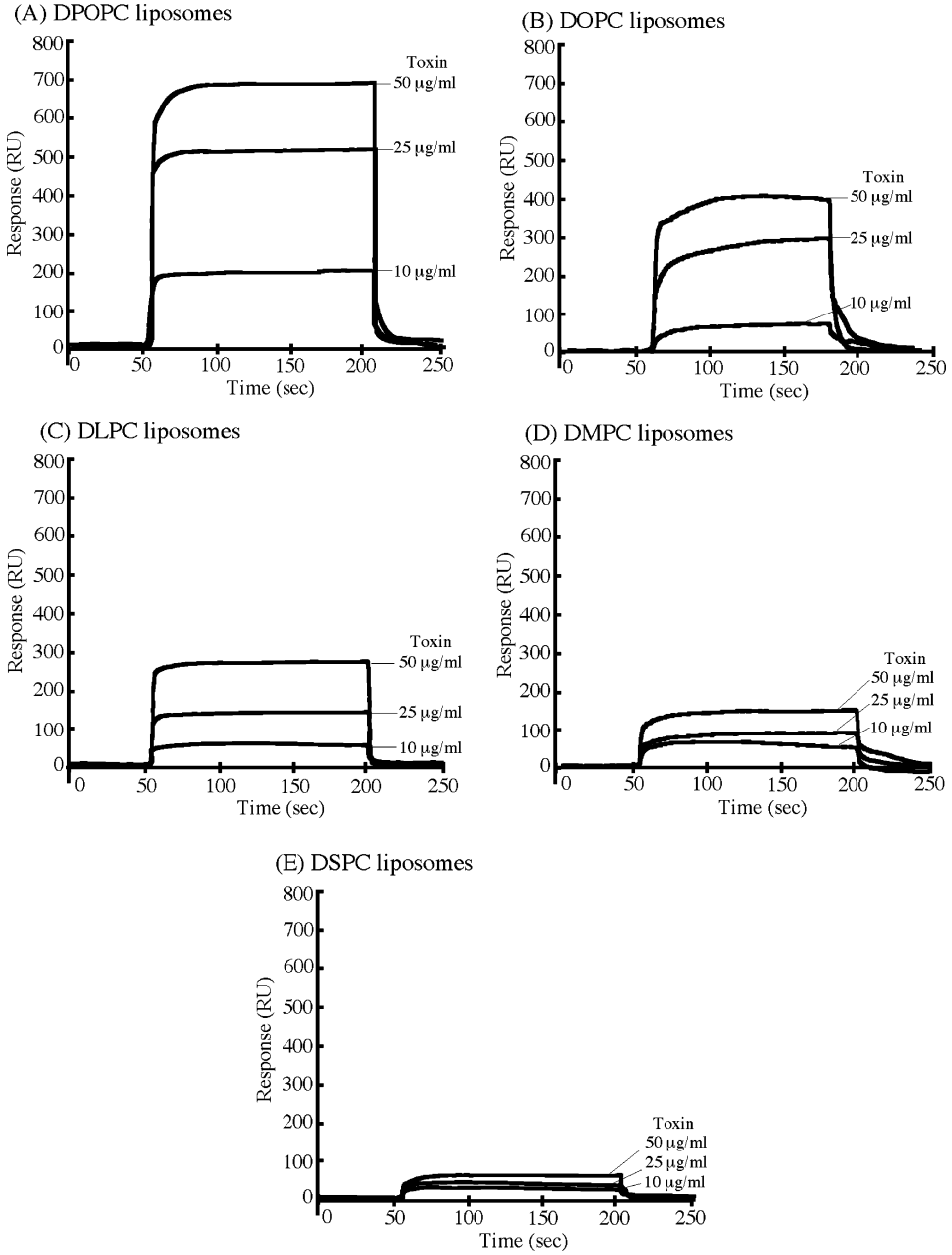

FIGURE 3: Kinetic analysis of the binding between ϵ-toxin and liposomes. Liposomes composed of DPOPC (A), DOPC (B), DLPC (C), DMPC (D), or DSPC (E) were bound to a HPA sensor chip. Injections of ϵ-toxin (10, 25, and 50 ng/mL) in TBS were made onto the surface of the liposome. The binding of the toxin was recorded in real time using a BIAcore 3000 system. The experiments were repeated 3 times, and the results for a representative experiment are shown.

3, the sensorgrams showed that binding of the toxin to liposomes decreased with an increase in T_m of the PC used. Furthermore, the K_D values derived from the Scatchard plots were calculated to be 4.23 μM for DSPC, 1.15 μM for DMPC, 0.81 μM for DLPC, 0.37 μM for DOPC, and 0.08 μM for DPOPC. The result shows that the binding of ϵ-toxin to liposomes is dependent upon the T_m of the PC used.

Insertion of the ϵ-Toxin Oligomer into Liposome Membranes. The insertion of ϵ-toxin into the bilayer of liposomes was studied by insertion-dependent labeling using the hydrophobic photoactivatable radiolabeled probe [^{125}I]TID. Hyland et al. (*21*) reported that the photoactive probe was spontaneously incorporated into the membrane bilayer at 37 °C and that it photolabeled only the transmembrane segments of inserted proteins, when used with the membrane system. [^{125}I]TID was incubated with a suspension of liposomes composed of DPOPC or DSPC at 37 °C for 10 min and washed, to allow incorporation into the hydrophobic phase. The [^{125}I]TID-treated liposomes were mixed with ϵ-toxin and incubated at 4 or 37 °C for 60 min. After SDS−PAGE of the samples, labeling of the toxin was analyzed by autoradiography (Figure 4). When the toxin was incubated with [^{125}I]TID-treated DPOPC or DSPC−liposomes at 4 °C, no labeled band was detected. On the other hand, some oligomers of the toxin were radiolabeled when the toxin was incubated with [^{125}I]TID-treated DPOPC−liposomes at 37 °C, whereas a barely detectable radioactive oligomer of ϵ-toxin was found when the toxin was incubated with [^{125}I]TID-treated DSPC−liposomes. The result suggests that oligomers of ϵ-toxin were inserted into the bilayer of liposomes at 37 °C. It therefore appears that the toxin insertion profile depends upon the T_m of the PC used.

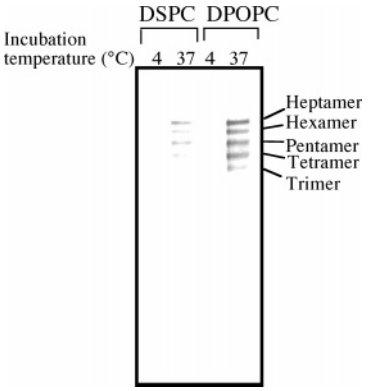

FIGURE 4: Insertion of the ϵ-toxin oligomer into the lipid bilayer of liposomes. ϵ-Toxin (10 μg/mL) was incubated with liposomes composed of DSPC or DPOPC, in which [^{125}I]TID was incorporated, at 4 or 37 °C for 60 min as described in the Materials and Methods. The membrane-bound toxin was solubilized and subjected to 4−20% gradient SDS−PAGE, followed by autoradiography.

Effect of Lipids That Favor the Lamellar-to-Inverted Hexagonal (L−H) Transition on ϵ-Toxin-Induced CF Leakage. Alonso et al. (*22*) reported that the inclusion of lipids that facilitate the L−H phase transition enhanced the formation of channels by aerolysin in lipid bilayers and aerolysin-induced leakage of an internal marker. Diacylglycerol (DAG), phosphatidylethanolamine (PE), and cholesterol are known to facilitate a transition from the L−H phase (*22*−*24*). To investigate the involvement of the inverted hexagonal phase in ϵ-toxin-induced CF leakage from liposomes, liposomes were prepared from binary mixtures of DPOPC and DAG, dioleoyl-PE (DOPE), or cholesterol. Figure 5A shows that addition of DAG, DOPE, and cholesterol enhanced the ϵ-toxin-induced leakage rate in a dose-dependent fashion. As shown in Figure 5B, DAG also increased the oligomerization of ϵ-toxin in a dose-dependent manner. DOPE and cholesterol also stimulated the formation of oligomers similar to DAG (data not shown).

Some lipids are known to hinder the formation of the inverted hexagonal or cubic phase (*22*−*24*). This is the case for the cone-shaped lipid lyso-PC, which favors micellar structures and forms very stable lamellar phases. We tested the effect of lyso-PC on the oligomerization of ϵ-toxin. For this purpose, the effect of lyso-PC on the toxin-induced CF release was explored using DPOPC−liposomes. As shown in Figure 6A, lyso-PC inhibited ϵ-toxin-induced CF leakage from liposomes in a dose-dependent manner. In addition, the oligomerization of ϵ-toxin decreased with an increase in lyso-PC in liposomes (Figure 6B).

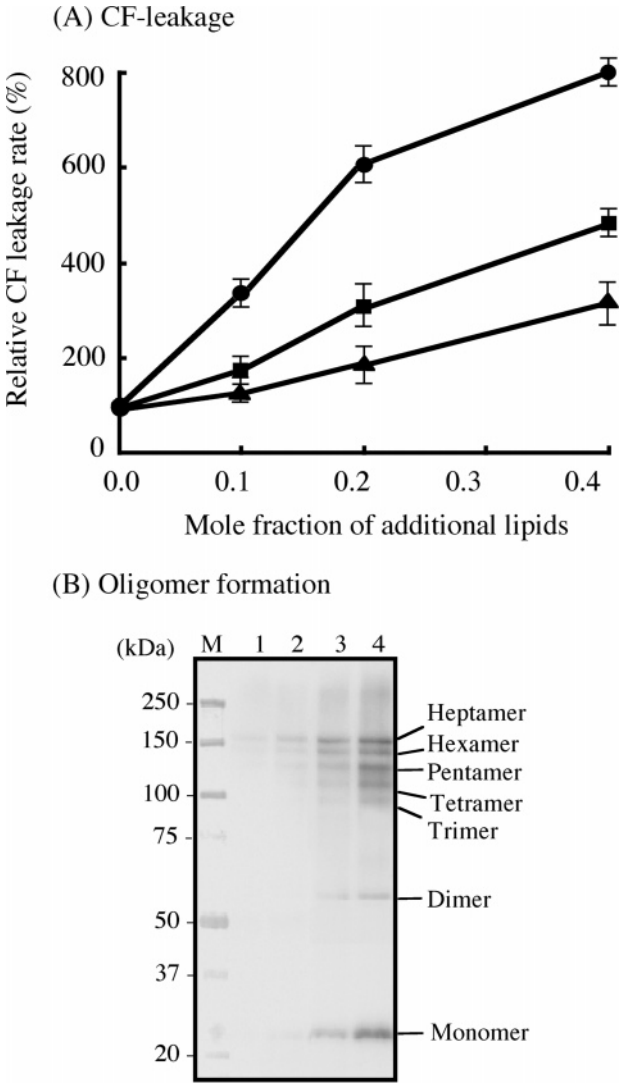

FIGURE 5: Effect of lipids that favor the L−H phase transition on the activity of ϵ-toxin. The CF-loaded liposomes were composed of DPOPC and DAG (●), PE (■), or cholesterol (▲) (A). The liposomes were incubated with ϵ-toxin (0.5 μg/mL) at 37 °C for 60 min. The values are the mean ± SEM (n = 5). ϵ-Toxin (0.5 μg/mL) was incubated with liposomes composed of DPOPC containing various proportions of diacylglycerol at 37 °C for 60 min (B). The liposomes were washed and subjected to 4−20% gradient SDS−PAGE and Western blotting using anti-ϵ-toxin antibody. The molar ratio of DAG/DPOPC was 0 (lane 1), 0.1 (lane 2), 0.2 (lane 3), or 0.4 (lane 4). M = prestained molecular-weight markers.

DISCUSSION

The present study provided evidence that ϵ-toxin induced the leakage of internal CF from liposomes composed of various PCs at 37 °C in the following order: DPOPC > DOPC > DLPC > DMPC > DPPC > DSPC, showing that the toxin-induced leakage increases with a decrease in the T_m of the PCs used in the liposomes. Furthermore, SDS−PAGE analysis and SPR analysis indicated that the binding of the toxin to liposomes and oligomerization of the toxin in liposomes were closely dependent upon the T_m of the PCs used as well as the release of CF from liposomes induced by the toxin. It has been reported that membranes of liposomes composed of PC are fluidized over or at the T_m of the PC used and that an increase in membrane fluidity correlates well with a decrease in the T_m of the PC used in liposomes (*25*). Furthermore, oligomerization of the toxin in liposomes was dependent upon temperature. It is known that the fluidity of the membrane increases in a temperature-dependent manner (*25*). Therefore, these results show that an increase in membrane fluidity promotes the binding of the toxin to liposomes and the assembly process of the toxin, supporting the argument that an increase in membrane fluidity results in insertion of the toxin in the bilayer of the membrane and oligomerization of the toxin in the membrane. It has been reported that liposomes composed of various PC species permit the insertion of *S. aureus* α-hemolysin and *Escherichia coli* α-hemolysin into membranes above the respective transition temperatures of their PCs (*25*, *26*). The

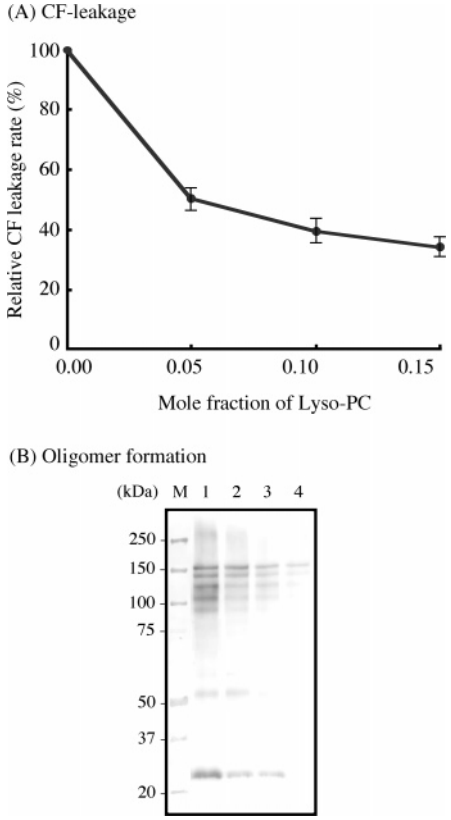

FIGURE 6: Effect of lyso-PC on ϵ-toxin-induced leakage of CF. Various proportions of lyso-PC were added to preformed CF-loaded DPOPC−liposomes. Percent changes in the rate of ϵ-toxin (10 μg/mL) induced leakage of CF are shown. The values are the mean ± SEM ($n = 5$). ϵ-Toxin (10 μg/mL) was incubated with liposomes composed of DPOPC containing various proportions of lyso-PC at 37 °C for 60 min (B). The liposomes were washed and subjected to 4−20% gradient SDS−PAGE and Western blotting using anti-ϵ-toxin antibody. The molar ratio of lyso-PC/DPOPC was 0 (lane 1), 0.05 (lane 2), 0.1 (lane 3), or 0.15 (lane 4). M = prestained molecular-weight markers.

findings support that membrane fluidity plays an important role in inducing the activity of ϵ-toxin.

The integration of ϵ-toxin into liposomes was assessed using a photoactivatable radiolabeled probe, [^{125}I]TID, incorporated into the target lipid bilayer. This photocross-linking approach has been successfully used to indicate the membrane-inserted regions of several bacterial toxins, such as the botulinum and tetanus toxin (*27*), diphtheria toxin (*28*), and *E. coli* hemolysin (*21*). The transmembrane region of ϵ-toxin was labeled by photoactivatable [^{125}I]TID in the hydrophobic phase in the bilayers of the liposomes composed of DPOPC, but labeled oligomer was rarely detected in liposomes composed of DSPC, suggesting that the oligomer can insert into fatty acyl residues of DPOPC in the bilayer of liposomes but not to fatty acyl residues of DSPC. It therefore appears that the insertion of the oligomer into the bilayer of the membrane is dependent upon membrane fluidity.

The biological significance of nonbilayer lipids in natural membranes is recognized. Thus, it is thought that the hexagonal phase or "inverted cone"-shaped lipids provide the flexibility for local rearrangements induced by biological events (*23*, *24*). It was reported that hexagonal phase lipids increase the rate at which aerolysin forms channels in the bilayer (*22*). The present study showed that lipids inducing a negative curvature (DAG and PE), i.e., favoring inverted nonlamellar phases, increased ϵ-toxin-induced CF leakage from liposomes, and oligomerization of the toxin, and that lyso-PC, a lipid inducing a positive curvature, counteracted the effects of DAG or PE and reduced the toxin-induced leakage of CF and the formation of the oligomer. It has been reported that inverted cone-shaped lipids impart a "negative" curvature to bilayer leaflets, suggesting that they lower the surface pressure at the interface (*23*, *24*). It therefore appears that the condition could favor the access of ϵ-toxin to the hydrophobic matrix. Therefore, it is suggested that ϵ-toxin has a high affinity for a hydrophobic region in membranes.

On the basis of our findings and previous reports, a mechanism for the functional oligomerization of ϵ-toxin is proposed as follows: ϵ-toxin binds to liposomes as a monomer and assembles, and the toxin inserts into the hydrophobic region in the bilayer.

Petit et al. (*19*) had reported that ϵ-toxin was able to form channels in an artificial lipid bilayer and no lipid specificity for the channel formation was detected in the bilayer utilized. We also showed that ϵ-toxin formed an oligomer in the bilayer of liposomes. Therefore, the findings show that no specific receptor is necessary for the binding of ϵ-toxin to artificial lipid membranes. However, it is speculated that a specific receptor of the toxin is required to permit ϵ-toxin to exert an effect on cells, because MDCK cells are highly susceptible to ϵ-toxin. α-Toxin from *Staphylococcus aureus* (*25*) and aerolysin from *A. hydrophila* (*22*) form channels in the bilayer of liposomes without requiring receptors, whereas they all need a receptor for biological activity and require higher concentrations for the destruction of liposomes than susceptible cells. The sensitivity of ϵ-toxin to liposomes is about 100 times lower than that to MDCK cells. Unresolved factors that mediate the interactions between the toxin and the cells and/or enhance the sensitivity of the toxin to the cells may be required. A structural study showed that ϵ-toxin is a pore-forming toxin belonging to the aerolysin family, known as β-pore-forming toxins (*14*). ϵ-Toxin acted with a hydrophobic region of the lipid bilayer. Therefore, it is possible that the membrane-spanning region of ϵ-toxin formed by the β-strand can insert without the help of receptors in liposomes, although there may be receptors that promote the interaction between the toxin and biological membranes. This result concerning oligomerization of the toxin provides a clue to the formation of the heptameric oligomer in biological membranes.

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