Coupling of Cholesterol and Cone-shaped Lipids in Bilayers
Augments Membrane Permeabilization by the Cholesterol-specific
Toxins Streptolysin O and Vibrio cholerae Cytolysin*

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Vibrio cholerae cytolysin (VCC) forms oligomeric pores in lipid bilayers containing cholesterol. Membrane permeabilization is inefficient if the sterol is embedded within bilayers prepared from phosphatidycholine only but is greatly enhanced if the target membrane also contains ceramide. Although the enhancement of VCC action is stereospecific with respect to cholesterol, we show here that no such specificity applies to the two stereocenters in ceramide; all four stereoisomers of ceramide enhanced VCC activity in cholesterol-containing bilayers. A wide variety of ceramide analogs were as effective as d-erythro-ceramide, as was diacylglycerol, suggesting that the effect of ceramide exemplifies a general trend of lipids with a small headgroup to augment the activity of VCC. Incorporation of these cone-shaped lipids into cholesterol-containing bilayers also gave similar effects with streptolysin O, another cholesterol-specific but structurally unrelated cytolyisin. In contrast, the activity of staphylococcal α-hemolysin, which does not share with the other toxins the requirement for cholesterol, was far less affected by the presence of lipids with a conical shape. The collective data indicate that sphingolipids and glycerolipids do not interact with the cytolyssins specifically. Instead, lipids that have a conical molecular shape appear to effect a change in the energetic state of membrane cholesterol that in turn augments the interaction of the sterol with the cholesterol-specific cytolyssins.

To bacterial pore-forming cytolyssins, cholesterol is a logical choice as a target molecule, because it confers specificity for animal as opposed to bacterial cell membranes. The specificity for cholesterol is shared between Vibrio cholerae cytolysin (VCC)† (1) and streptolysin O (SLO) (2). Otherwise, these toxins are not related, and the oligomeric pores they form are very different in size and morphology (1, 3). Although with SLO the sterol is already required in the initial event of membrane binding of the monomeric toxin (4), it only comes into play at the stage of oligomerization in the case of VCC (5, 6). When the sterol is incorporated into phosphatidycholine (PC) bilayers at physiologically realistic concentrations (i.e. up to 40% by mol), these membranes do not become significantly sensitive to VCC. However, it was previously found that membrane susceptibility toward the cytolyisin was greatly enhanced by inclusion of ceramide; free ceramide and monohexosyl ceramides proved similarly effective (7). A combined specificity for cholesterol and sphingolipids has previously been shown for the fusion protein of Semliki Forest virus. In that instance, the interaction with ceramide proved to be highly stereoselective (8–10). Accordingly, we have examined the structural properties of the ceramide molecule responsible for the sensitization of membranes to VCC. To our surprise, no dependence on stereospecific features of ceramide could be detected. Membrane sensitization was readily achieved with a variety of synthetic ceramides and even with 1,2-diacyl-sn-glycerol, which is not closely related in structure to ceramide. However, with both sphingo- and glycerolipids, the presence of a phosphocholine headgroup led to a decrease in membrane susceptibility toward VCC. In glycerolipids other than PC and diacylglycerol, headgroups smaller in size than phosphocholine were associated with higher membrane susceptibility to the cytolyisin. Among lipid species sharing the same headgroup, a complementary trend was generally apparent in which acyl chains having a large cross-section were associated with higher VCC activity, although there were significant exceptions to this correlation. Remarkably, despite its lack of a structural homology with VCC, SLO was affected in its activity toward cholesterol-containing bilayers in a very similar way by the incorporation of sphingo- and glycerolipids into the bilayer. We propose that the effects of sphingo- and glycerolipids on membrane susceptibility to VCC and SLO do not arise because of any direct, specific effect upon the toxins but instead are mediated by their ability to enhance the interaction of cholesterol with the toxins.

MATERIALS AND METHODS

Purification of VCC, SLO, and α-Hemolysin—VCC and α-hemolysin were purified from bacterial culture supernatants as described previously (5, 11). SLO was expressed recombinantly in Escherichia coli as a yolk PC; EYPC, egg yolk PG; LUV, large unilamellar vesicles; RD50 (RD25) (RD50)

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Maltose-binding protein fusion protein and purified from bacterial cell lysates (12).

Lipids—1,16-Indolyl-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (1,16-bromo-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine) were kindly supplied by Dr. John Silivus, McGill University. The synthesis of these compounds was provided by the phospholipid synthesis laboratory of the National Research Council of Canada. The synthesis of the ceramide analogs described previously (13) and characterized as the N-biphenylycarboxylic acid derivatives of sphingosine by high pressure liquid chromatography (14). \( \text{t-d-erythro-Triple bond-C6-Cer} \) (see Fig. 1) was synthesized as described previously (15). \( \text{t-d-erythro-Aryl-C4-Cer} \) (see Fig. 1) was synthesized as described recently (16). The other ceramide analogs shown in Fig. 1 were prepared by using the synthetic sequences outlined below. EYPC, EYPG, bovine brain \( \beta \)-galactosidase, bovine brain non-hydroxy- \( \alpha \)-hydroxy fatty acyl ceramides, and cholesterol acetate were obtained from Sigma. All other lipids were obtained from Avanti Polar Lipids (Alabaster, AL).

\((2S,3R,4S,5S)-2\)-N-Capryloylamino-4,5-cyclopropyl-1,3-dihydroxyoctadecane (t-d-erythro-CS-Cer)—Into a round-bottomed flask were combined 25 mg (0.08 mmol) of t-d-erythro-sphingosine, 24 mg (0.09 mmol) of \( \beta \)-naphthol caprylate, and 5 ml of anhydrous tetrahydrofuran. The reaction mixture was stirred at room temperature for 24 h, at which time TLC analysis indicated the consumption of sphingosine (silica gel-coated aluminum plates, eluted with ethyl acetate (\( \text{Rf} = 0.73 \)) and visualized with 10% sulfuric acid in methanol). The reaction mixture was concentrated and precipitated by using the solvent sequence of ethyl acetate (R = 0.73) and hexanes, which was loaded onto a silica gel column (100 mm × 10 mm). The fractions containing the product were eluted with ethyl acetate (R = 0.73) and hexanes as a clear yellow oil. A 28-mg portion of the sulfonyl chloride was loaded onto silica gel column (100 mm × 10 mm) and eluted with ethyl acetate (R = 0.73) and hexanes as a colorless powder. \( \text{1H NMR (400 MHz, CDCl}_3 \) with 200 ml of ethyl acetate. The fractions containing the product were pooled, and the ethyl acetate was removed under reduced pressure to provide a colorless amorphous powder. \( \text{D-erythro-Cyclopropyl-C8-Cer} \) and Its Mono-O-methyl Analog—

\( \text{t-d-erythro-Sulfonamido-Cer} \)—22.1, 16.2, 14.14, 14.08, 9.9. 31.7, 29.71 (4), 29.68, 29.55, 29.51, 29.4 (2), 29.2, 29.0, 25.7, 22.7, 22.6, 22.1, 16.2, 14.14, 14.08, 9.9.

The residue was dissolved in 1 ml of a 25% solution of ethyl acetate in hexanes, which was loaded onto a silica gel column (10 × 100 mm). The column was eluted with 100 ml of 25% ethyl acetate in hexane and then with 200 ml of methanol. The fractions containing the product were collected and concentrated to afford 33 mg (97%) of the product as a colorless solid. The resulting mixture was lyophilized from 5 ml of anhydrous benzene to afford a colorless powder. \( \text{1H NMR (400 MHz, CDCl}_3 \) with 200 ml of ethyl acetate. The fractions containing the product were pooled, and the ethyl acetate was removed under reduced pressure to provide a colorless amorphous powder. \( \text{1H NMR (400 MHz, CDCl}_3 \) with 200 ml of ethyl acetate. The fractions containing the product were pooled, and the ethyl acetate was removed under reduced pressure to provide a colorless amorphous powder.

Control of Lipid Phosphorylation—

Liposomes were prepared by using the following procedure. The EtOH solution of each lipid was used in a 1:4 ratio by molar content, 30% cholesterol, and 20% EYPC (making up the remainder to 100 mol %). From these liposomes, the quenching lipid (16-Br-palmitoyl)-oleoyl-PC instead of \( \text{D-erythro-Cyclopropyl-C8-Cer} \) was added to 20 mol % to give a uniform background mixture of \( \text{CS}_{\text{d}} \) and \( \text{C}_{\text{d}} \) ceramide, respectively. Denaturation was then monitored by using the sedimentation equilibrium method of synthetic ceramide derivatives. Fig. 1 shows the structures of the synthetic ceramide analogs we used. The ceramides were added to 20 mol % to give a uniform background mixture of lipids comprising cholesterol (35 mol %), EYPG (2 mol %), and EYPC (making up the remainder to 100 mol %).

**Preparation of LUV**—

The lipids were dissolved in chloroform and mixed at the molar ratios indicated under “Results” (total amount, 5 mg) and dried with nitrogen in a round-bottom flask to form a thin film. The lipids were resuspended in 10 mg HEPES/100 mM NaCl/50 mM calcine (pH 7.5) by warming, if necessary, and bath sonication. The suspensions were frozen and thawed and then repeatedly extruded through polycarbonate membranes (Nuclepore; pore size 100 nm) using a 10-ml thermostellar extruder (Lipex, Vancouver, Canada). The non-entrapped calcine was removed by gel filtration on Sephadex G50 in 10 mM HEPES/10 mM NaCl (pH 7.5). The lipid concentration of the final liposome suspension was quantified by using a commercial enzymatic assay for cellular Biochrom (Hamburg, Germany).

Calcine Release Assay—From a solution of the cytolyisin in question (200 mg/ml in HEPES/NaCl with 0.1% bovine serum albumin), 2-fold serial dilutions were prepared. To each dilution, an equal volume of the respective preparation of calcine liposomes (total lipid content, 0.2 mg/ml) was added. Following incubation for 10 min at 37°C, the samples were diluted into 30 volumes of HEPES/NaCl, and the calcine fluorescence was assayed in a SPEX Fluorimax fluorometer (excitation wavelength, 488 nm; emission, 520 nm). The extent of calcine release was calculated from the relative increase of fluorescence intensity over a sample of untreated liposomes, whereby the fluorescence intensity corresponding to 100% permeabilization was assayed on a sample lysed with sodium deoxycholate (final concentration, 6 mol%). The amounts of released calcine were expressed as intensity of the undissociated sample. The decrease in fluorescence intensity by the quenching lipid with \( \text{C}_{\text{d}} \)- and \( \text{C}_{\text{d}} \)-ceramide, respectively, was determined from the normalized values. The assay was performed likewise with dipamitoyl-PE and diarlauryl-PE in place of \( \text{C}_{\text{d}} \)- and \( \text{C}_{\text{d}} \)-ceramide, respectively.

Sphingomyelinase Treatment and Hemolysis of Sheep Erythrocytes—

Sheep erythrocytes were made up to 20% (by volume) with 10 mM HEPES, 140 mM NaCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), pH 7.0. They were incubated for 40 min at 30°C with 2.5, or 10 milliliters/ml sphingomyelinase (Bacillus cereus; EC 3.1.4.12, Sigma). They were then washed three times with the former buffer (without CaCl\(_2\) and MgCl\(_2\)) by centrifugation. After resuspension to 2%, FCC was added to 1.5 mg/ml. Hemolysis was followed over time by cell turbidity (\( \text{A}_{540} \)).

**RESULTS**

Lack of Effect of Chiral and Structural Modifications in Ceramide on VCC Activity in LUV—In a previous study, it was found that addition of ceramide to PC bilayers containing cholesterol (35% by mol) enhanced VCC-induced permeabilization (7). To determine the structural features of the ceramide molecule responsible for this sensitizing action, we tested a variety of synthetic ceramide derivatives. Fig. 1 shows the structures of the synthetic ceramide analogs we used. The ceramides were added to 20 mol % to give a uniform background mixture of lipids comprising cholesterol (35 mol %), EYPG (2 mol %), and EYPC (making up the remainder to 100 mol %).

molecular content, 30%), cholesterol (35%) EYPC (32%), and EYPG (2%), and the fluorescently labeled phospholipid (16-indolyl-palmitoyl)-oleoyl-PC (1%; total amount of lipids, 200 nmol). Parallel samples contained the quenching lipid (16-Br-palmitoyl)-oleoyl-PC instead of \( \text{D-erythro-Cyclopropyl-C8-Cer} \) was added to 20 mol % to give a uniform background mixture of \( \text{CS}_{\text{d}} \) and \( \text{C}_{\text{d}} \) ceramide, respectively. Denaturation was then monitored by using the sedimentation equilibrium method of synthetic ceramide derivatives.
lipid mixtures liposomes loaded with the aqueous fluorescent marker calcein were prepared as described previously (7), and the release of calcein was determined as a function of VCC concentration. Fig. 2A shows that replacement of the ε-erythro-ceramide (which represents the physiological configuration) by the l-erythro, d-threo, or l-threo isomer does not cause a major change in liposome membrane susceptibility, indicating that there is no stereospecific interaction between ceramide and VCC.

The dosages of VCC required to induce the release of calcein to 50% (RD_{50}) or 25% (RD_{25}) were calculated to provide a concise estimate of the effect of various lipids on membrane susceptibility to VCC. Fig. 2B gives the RD_{50} values obtained with further molecular modifications in ceramide structure. It is evident that changing the stereochemistry of the C4=C5 double bond of the sphingoid chain from trans to cis or its replacement by a triple bond, a cyclopropyl group, or even a benzene ring does not significantly change the activity of VCC. This finding is in clear contrast to the known role of the double bond of the sphingosine moiety (7). The finding that diacylglycerol is more effective than ceramide in sensitizing membranes to VCC, suggesting an inhibitory role of the phosphocholine headgroup. In ceramides, the headgroup size is expected to be a key feature. Fig. 3 shows a clear relationship between phospholipid headgroup volume and cytolysin dosage (i.e., an inverse relationship of headgroup volume and membrane susceptibility), which is consistent with the umbrella concept.

Another way to increase the "umbrella-shielded space" available to the sterol would be to decrease the cross-sectional area of the fatty acyl chains. The variation of membrane susceptibility to VCC on changing the acyl chain cross-section was evaluated with various synthetic PEs. The results are shown in Fig. 3B. Clearly, dilauroyl-PE and dimyristoyl-PE have a lower susceptibility to VCC, suggesting an inhibitory role of the phosphocholine headgroup (7). The finding that diacylglycerol is more effective than PC indicates that the phosphocholine moiety is inhibitory not only in sphingolipids but also in glycerolipids.

In mixed lipid bilayers with cholesterol, the headgroups of phospholipids are thought to shield the sterol ring system of cholesterol from water (21). Cholesterol shielded from water might then also be shielded from VCC, which may account for the inhibitory effect of the phosphocholine headgroup. In comparisons of cholesterol solubility in PC and PE bilayers, it was proposed that PC, because of its larger headgroup, would make a more efficient "umbrella" for the sterol than PE (22). The effect of phospholipid headgroup size on the activity of VCC was examined with the dioleoyl species of the following glycerophospholipids: phosphatidic acid, phosphatidylserine, phosphatidyglycerol, PE, N-methyl-PE, and N,N'-dimethyl-PE. A
membrane susceptibility to VCC decreased. A regular relationship between the fatty acyl chain length and membrane susceptibility can only be expected if homogeneous mixing is assumed to take place for all of the lipid species present in the membrane.

However, both glycerolipids (23, 24) and ceramides (25, 26) may undergo lateral phase separation. Lipid molecules bearing long, highly saturated acyl chains are particularly prone to undergo segregation within the bilayer, which might explain the decrease of membrane susceptibility with ceramide acyl chain length increasing beyond 12 carbons. Liposomes containing 30 mol % of C_{12}-ceramide or C_{20}-ceramide were examined using a fluorescence-quenching assay of lipid phase separation. These liposomes also included two labeled PC species, the fluorescent compound (16-indolyl-palmitoyl)-oleoyl-PC, and the fluorescence-quenching species (16-bromo-palmitoyl)-oleoyl-PC. In similar experiments involving cerebrosides, this pair of probes was found to display enhanced fluorescence quenching on phase separation, which was ascribed to their cosegregation into the cerebroside-depleted phase (27). The fluorescence intensity was indeed more strongly quenched with C_{20}-ceramide, the relative intensity being 70% with respect to C_{12}-ceramide. This suggests that C_{20}-ceramide does not mix ideally in the bilayer and segregates more readily than C_{12}-ceramide. Segregation of C_{20}-ceramide into ceramide-rich regions would lower the effective concentration of this lipid that is available for interactions with other membrane components and may therefore account for the lower degree of membrane sensitization effected by C_{20}-ceramide. Similarly, dipalmitoyl-PE appeared to undergo phase separation more readily than dilauroyl-PE (the relative fluorescence being 0.75 in this case), which may likewise account for the lower membrane susceptibility observed with dipalmitoyl-PE.

Lateral segregation of a lipid sparingly soluble in the bulk phase of the membrane is less likely to occur when its concentration is decreased. Fig. 3C shows that, indeed, C_{16}- and C_{20}-ceramides are slightly more active than C_{12}-ceramide at 5 mol %. The finding of significant membrane susceptibility to VCC with such low levels of ceramide is quite remarkable in its own right; similar observations were made with the most effective of the glycerolipids tested, dioleoylglycerol (data not shown). The data support the notion that the molecular shape of the lipid influences the activity of VCC on the target membrane. Lipids that have a conical molecular shape, i.e. those whose hydrophobic moiety occupies a larger cross-section than does the polar headgroup, enhance membrane susceptibility to VCC. That this effect may be significant not only with synthetic but also with natural membranes is exemplified in Fig. 3D. With sheep erythrocytes, enzymatic conversion of sphingomyelin to ceramide strongly enhances VCC-mediated hemolysis.

Effect of Phospho- and Glycerolipid Molecular Shape on the Activities of Streptolysin O and Staphylococcal α-Hemolysin—We propose that the putative mode of action of ceramides and glycerolipids is an indirect one; they change the state of the lipid influences the activity of VCC on the target membrane. Lipids that have a conical molecular shape, i.e. those whose hydrophobic moiety occupies a larger cross-section than does the polar headgroup, enhance membrane susceptibility to VCC. That this effect may be significant not only with synthetic but also with natural membranes is exemplified in Fig. 3D. With sheep erythrocytes, enzymatic conversion of sphingomyelin to ceramide strongly enhances VCC-mediated hemolysis. However, both glycerolipids (23, 24) and ceramides (25, 26) may undergo lateral phase separation. Lipid molecules bearing long, highly saturated acyl chains are particularly prone to undergo segregation within the bilayer, which might explain the decrease of membrane susceptibility with ceramide acyl chain length increasing beyond 12 carbons. Liposomes containing 30 mol % of C_{12}-ceramide or C_{20}-ceramide were examined using a fluorescence-quenching assay of lipid phase separation. These liposomes also included two labeled PC species, the fluorescent compound (16-indolyl-palmitoyl)-oleoyl-PC, and the fluorescence-quenching species (16-bromo-palmitoyl)-oleoyl-PC. In similar experiments involving cerebrosides, this pair of probes was found to display enhanced fluorescence quenching on phase separation, which was ascribed to their cosegregation into the cerebroside-depleted phase (27). The fluorescence intensity was indeed more strongly quenched with C_{20}-ceramide, the relative intensity being 70% with respect to C_{12}-ceramide. This suggests that C_{20}-ceramide does not mix ideally in the bilayer and segregates more readily than C_{12}-ceramide. Segregation of C_{20}-ceramide into ceramide-rich regions would lower the effective concentration of this lipid that is available for interactions with other membrane components and may therefore account for the lower degree of membrane sensitization effected by C_{20}-ceramide. Similarly, dipalmitoyl-PE appeared to undergo phase separation more readily than dilauroyl-PE (the relative fluorescence being 0.75 in this case), which may likewise account for the lower membrane susceptibility observed with dipalmitoyl-PE.

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shape is not an important determinant of the activity of staphylococcal α-hemolysin.

**Effect of Enhanced Cholesterol Content and Augmentation of Membrane Susceptibility to Toxins by Cholesteryl Acetate**—In the absence of any ceramides or glycerolipids, both VCC and SLO are still highly active when the concentration of membrane cholesterol is raised from 35 to 55 mol % (data not shown). A very similar effect can be obtained by supplementation of 35 mol % cholesterol with 20 mol % cholesteryl acetate. However, cholesteryl acetate is a very poor substitute of cholesterol as a specific ligand for SLO or VCC (data not shown), because the 3-β-hydroxy group is an important determinant of stereospecificity with both VCC (1) and SLO (2, 28). This suggests that the sensitizing effect of supplementation of the cholesterol content does not involve stereospecific interaction between the toxins and the additional sterol.

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**DISCUSSION**

The present results do not confirm our previous proposal that VCC displays a dual specificity for cholesterol and ceramide (7). In marked contrast to the fusion protein of Semliki Forest virus, which truly displays such dual specificity (8, 29), VCC is not significantly affected even by major structural alterations in the ceramide molecule. Nevertheless, ceramides and some glycerolipids strongly augment the activity of VCC, up to several hundred times, in the presence of modest membrane concentrations of cholesterol.

Lee et al. (30) studied the impact of various headgroups on bilayer stability in binary mixtures of PE and the respective phospholipid in question; the mixed membranes were monitored for hexagonal-II phase transition. It is instructive to compare their results with the effects of the same lipids on cytolysin activity (Fig. 5). With all cytolysins examined, there is a correlation between the ability of lipids to promote the hexagonal-II phase and cytolysin activity. Because α-hemolysin is not strictly dependent on cholesterol, there may be a non-cholesterol-specific component in the effect of bilayer stability on pore-forming toxins. Such a component would be in agreement with recent findings on aerolysin, which, like α-hemolysin, does not strictly require membrane cholesterol, although those results are not directly comparable in quantitative terms (31). However, the correlation between the phase transition temperature and toxin activity is much steeper and much more consistent with two cholesterol-specific toxins than with α-hemolysin (Fig. 5). This finding strongly suggests that the sterol participate in the observed modulation of toxin activity.

How, then, is cholesterol linked to bilayer stability? Like all of the activating lipids, cholesterol is a cone-shaped lipid, i.e. it claims space in the hydrophobic layer but does not provide for adequate headgroup coverage, which it must borrow from adjacent lipids. Thus, cholesterol molecules within a bilayer compete with all other cone-shaped molecules for headgroup coverage, no matter whether these are glycerolipids, sphingolipids, different sterols, or other cholesterol molecules. The latter case occurs in membranes with high cholesterol content. Shortage of headgroup coverage will expose the sterol to an energetically unfavorable state; as a consequence, the free energy of cholesterol is likely to rise steeply with its membrane concentration (21). Eventually, cholesterol will dissociate from the bilayer and precipitate, which takes place more readily with PE than with PC bilayers (22), in accordance with the notion that PC makes a better umbrella for the sterol (21). The binding sites of cytolysins may offer another opportunity for
cholesterol molecules to seek shelter from the unfavorable contact with water. To the extent that the sterol is closely embraced by the cytolysin, cholesterol will in effect leave the bilayer and hence get rid of the associated chemical potential. Thus, the tighter the interaction between the sterol and the protein, the more sensitive the protein becomes to the cholesterol free energy, and, in turn, to the thermodynamic stability of the bilayer.

The proposed model rationalizes the effects of a wide variety of lipids and of variations in the cholesterol concentration (32) under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle. It may also accommodate the previous finding that, at constant lipid composition, VCC is under a single common principle.

In conclusion, the present study shows that the activity of cholesterol-specific bacterial cytolysins on mixed bilayers may be strongly enhanced by lipids other than cholesterol. It provides evidence that these accessory lipids do not act upon the toxins specifically but instead modulate the interaction of the toxins with cholesterol. The proposed mode of coupling between cholesterol and cone-shaped lipids within mixed bilayers may be of broad interest, because it could similarly apply to the regulation of cellular membrane proteins endowed with cholesterol binding sites, e.g. hormone or neurotransmitter receptors.

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