Lipids Favoring Inverted Phase Enhance the Ability of Aerolysin To Permeabilize Liposome Bilayers†

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ABSTRACT: Channel formation by the bacterial toxin aerolysin follows oligomerization of the protein to produce heptamers that are capable of inserting into lipid bilayers. How insertion occurs is not understood, not only for aerolysin but also for other proteins that can penetrate membranes. We have studied aerolysin channel formation by measuring dye leakage from large unilamellar egg phosphatidylcholine vesicles containing varying amounts of other lipids. The rate of leakage was enhanced in a dose-dependent manner by the presence of phosphatidylethanolamine, diacylglycerol, cholesterol, or hexadecane, all of which are known to favor a lamellar-to-inverted hexagonal (L−H) phase transition. Phosphatidylethanolamine molecular species with low L−H transition temperatures had the largest effects on aerolysin activity. In contrast, the presence in the egg phosphatidylcholine liposomes of lipids that are known to stabilize the lamellar phase, such as sphingomyelin and saturated phosphatidylcholines, reduced the rate of channel formation, as did the presence of lysophosphatidylcholine, which favors positive membrane curvature. When two different lipids that favor hexagonal phase were present with egg PC in the liposomes, their stimulatory effects were additive. Phosphatidylethanolamine and lysophosphatidylcholine canceled each other's effect on channel formation.

Aerolysin is a 47 kDa channel-forming protein that contributes to the pathogenicity of *Aeromonas hydrophila*, a bacterium associated with intestinal and deep wound infections. The toxin is secreted in the form of an inactive precursor called proaerolysin that can be proteolytically processed to aerolysin by a number of proteases including trypsin and furin. The active form of the toxin is a water-soluble molecule that can spontaneously oligomerize, producing heptamers that may then insert into lipid bilayers, giving rise to discrete hydrophilic channels (see ref *1* for a recent review of aerolysin). On the basis of the crystal structure of proaerolysin, which reveals extensive β-structure (*2*), it seems likely that the oligomeric form of the toxin contains an amphipathic β-barrel analogous to that observed in the heptamer of *Staphylococcus aureus* α toxin (*3*). The steps leading to channel formation in target cell membranes and in artificial lipid bilayers are shown in Figure 1. Sensitive cells display specific high-affinity receptors for both aerolysin and proaerolysin (K_d approximately 10^{-9} M; 4). These receptors have been shown to be glycosylphosphatidylinositol- (GPI)[1] anchored proteins, and aerolysin and proaerolysin have been shown to bind to the anchor itself (*5*−*7*). Once bound, proaerolysin can be converted to aerolysin by cell-associated proteases such as furin (*8*). The primary function of the receptor appears to be to concentrate the toxin on the cell surface, promoting oligomerization. As a result, cells displaying aerolysin receptors are sensitive to toxin concentrations as low as 10^{-11} M (*6*).

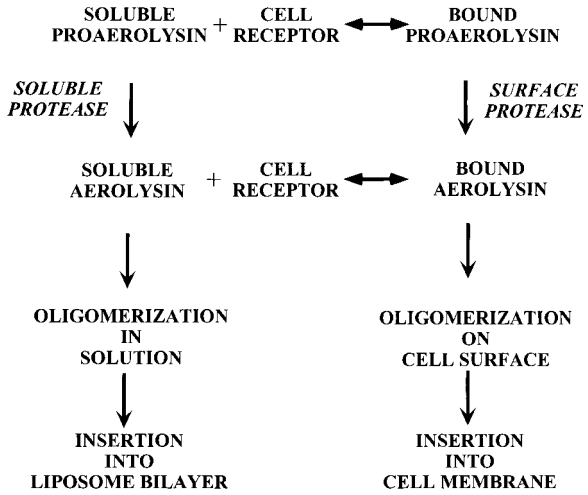

FIGURE 1: Steps in channel formation by aerolysin. A separate binding step may precede oligomer insertion into liposomes.

Large unilamellar liposomes (LUV) and planar lipid bilayers are much less sensitive to aerolysin than mammalian cells, not only because they lack specific glycoprotein

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[1] Abbreviations: 6-CF, 6-carboxyfluorescein; Ch, cholesterol; DAG, diacylglycerol; FLAER, fluorescently labeled proaerolysin; GPI, glycosylphosphatidylinositol; H_{II}, inverted hexagonal phase; L, lamellar phase; L−H, lamellar to inverted hexagonal; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; QELS, quasi-elastic light scattering; Q_{II}, inverted cubic phase; T_h, lamellar-to-inverted hexagonal transition temperature.

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receptors but also because aerolysin has no apparent affinity for lipids until it has oligomerized (*9*−*11*). Nevertheless, when the toxin concentration is high enough so that appreciable oligomerization can occur spontaneously in solution (micromolar range), channels do form in the absence of receptor (Figure 1). Aerolysin does not form a prepore on the surface of the bilayer, in apparent contrast to *S. aureus* α toxin (*12*) and anthrax toxin protective antigen (*13*). Instead it appears that the oligomer can insert directly from solution. In the absence of bilayers, aggregation occurs, implying that the oligomer contains an exposed hydrophobic surface, presumably the outside of the amphipathic barrel. How such a large structure as the barrel penetrates a lipid bilayer is a largely unexplored puzzle. On a real cell such as a T-lymphocyte the receptor may help to orient the oligomer or facilitate its entry into the bilayer, although there is no direct evidence for either role. As it approaches the surface of an artificial lipid bilayer, the oligomer will first encounter the polar interfacial region comprising the polar headgroups and water of hydration. Some peptides that can form helical structures are apparently able to partition into the interfacial region as a first step in membrane insertion, then entering the hydrophobic core (*14*). It is hard to imagine how a large rigid structure like the aerolysin barrel could do the same, even if it entered at an oblique angle, as the interfacial region is only about 15 Å thick (*15*). On the other hand, the barrel also seems too large to enter the hydrocarbon region directly by slipping between the headgroups on the bilayer surface.

The aerolysin oligomer may overcome the barrier of the polar interfacial region by destabilizing the bilayer locally, causing the formation of nonbilayer structures. The interaction of lipids with the aerolysin oligomer may be analogous to the interaction that has been proposed between influenza hemagglutinin and bilayer lipids during membrane fusion (*16*). The viral protein appears to promote the formation of a bent lipid-containing stalk intermediate during the fusion process. Inverted cone-shaped lipids such as phosphatidylethanolamine and diacylglycerol, which favor negative membrane curvature or hexagonal phase, promote viral fusion, while cone-shaped lipids such as lysophosphatidycholine, which favors positive membrane curvature or micelles, inhibit viral fusion (*17*). Insertion of the oligomer into pure lipid bilayers may also rely on the transient appearance of hydrophobic patches on the bilayer surface, which might provide access points. The occurrence of these patches may be promoted by the presence of lipids in the bilayer that promote negative curvature, which results in decreased lateral pressure between the headgroups (*18*). The ability of diacylglycerol to promote binding to apolipophorin III has been attributed to hydrophobic patches (*19*).

In the present paper we have studied the ability of aerolysin to form channels in large unilamellar egg phosphatidylcholine vesicles (LUV) entrapping the self-quenching, water-soluble fluorophore 6-carboxyfluorescein (6-CF). Heptamer insertion results in efflux of the fluorophore, which can be measured as an increase in fluorescence. We found that the inclusion of lipids that facilitate the lamellar-to-inverted hexagonal (L−H) phase transition enhanced channel formation, whereas the opposite occurred when lipids that stabilize the lamellar phase or the cone-shaped lipid lysophosphatidylcholine were present.

MATERIALS AND METHODS

Materials. Proaerolysin was purified as described previously (*20*). Alexa 488 labeled proaerolysin (FLAER; *21*) was from Protox Biotech (Victoria, BC, Canada). Pure lipids were obtained from Avanti (Alabaster, AL). Molecular Probes (Eugene, OR) supplied 6-carboxyfluorescein. Trypsin from bovine pancreas was supplied by Sigma (St. Louis, MO)

Liposome Preparation and Characterization. Organic solvents were evaporated from lipid mixtures under nitrogen. The resulting films were kept under vacuum for at least 2 h to remove traces of solvent. The lipids were then hydrated with 100 mM 6-CF in 20 mM Hepes and 100 mM NaCl, pH 7.4, subjected to 10 cycles of freezing and thawing, and extruded through polycarbonate filters of 0.2 μm pore diameter to obtain LUV (*22*). Nonentrapped 6-CF was removed by gel filtration on Sephadex G-25 columns.

After phosphorus determination (*23*), the LUV were tested for 6-CF entrapment. This was performed in a PTI (London, ON) spectrofluorometer, with monochromators set at 477 nm excitation and 520 nm emission, a Schott 515 cutoff filter, and slits at 3 nm. The sample (1 μM lipid in a total volume of 2.5 mL) was transferred to 3-mL quartz cuvettes, with continuous stirring, at room temperature. The intensity of fluorescent emission was measured before and after addition of Triton X-100 [0.1% (w/v) final concentration]. From these readings, the extent of 6-CF fluorescence quenching inside the liposomes could be estimated (*24*). Liposome preparations with 6-CF quenching higher than 85% were used in our experiments.

The size of the extruded LUVs was measured by quasi-elastic light scattering in a Nicomp Model 370 submicrometer particle sizer. Average diameters were largely independent of lipid composition for the lipid mixtures used in our experiments, ranging from 170 to 200 nm. The vesicle size remained the same after aerolysin treatment, an indication that channel formation did not lead to major structural changes or to vesicle fusion, consistent with planar bilayer evidence that aerolysin forms small well-defined channels (*10, 11*).

Measurement of Proaerolysin Binding to Liposomes. The binding of proaerolysin to lipid bilayers was studied with Alexa-labeled proaerolysin. Pure PC LUV (0.3−1 mM lipid) were incubated with 0.5 μM labeled proaerolysin for 30 min at room temperature. The liposomes were then sedimented in a Beckman TLA 100.2 rotor at 75 000 rpm for 30 min at 4 °C, and the pellets and supernatants were assayed for protein (fluorescence) and lipid (phosphorus assay).

Aerolysin-Induced Leakage Assay. LUV (1 μM lipid) in 2.5 mL of buffer (20 mM Hepes and 150 mM NaCl, pH 7.4) were transferred to 3-mL quartz cuvettes in the spectrofluorometer. A fluorescence intensity baseline was recorded for 5 min to ensure that there was no spontaneous leakage. Proaerolysin (0.5 μM final concentration) was then added, and the mixture was left to equilibrate for another 5 min. Trypsin (6 μL of a 1 mg/mL solution in 20 mM Tris-HCl and 20 mM $CaCl_2$, pH 7.4) was then added to the cuvette, to convert proaerolysin into active aerolysin. After 40 min, when apparent equilibrium had been reached under most conditions, Triton X-100 [0.1% (w/v) final concentration] was added and the resulting maximum fluorescence of the sample was recorded. Experimental values were normal-

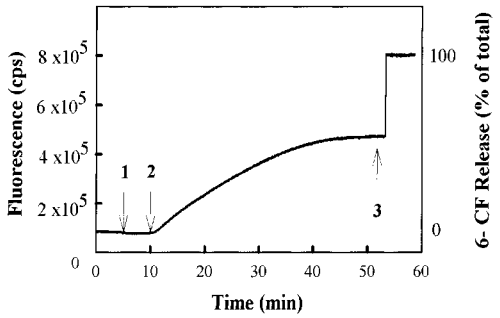

FIGURE 2: Assay of channel formation in LUV by aerolysin. A representative time-course measurement of release of 6-carboxyfluorescein from egg PC LUV is shown. Additions (to a liposome suspension 1 μM in lipid): 1, 0.5 μM proaerolysin; 2, trypsin; 3, Triton X-100. The *y*-axis (right-hand side) shows the normalized 0−100% scale, while the figures on the left-hand *y*-axis correspond to direct readings from the spectrofluorometer. The slope corresponding to the initial leakage rate had a value of 0.040% leakage•s^{-1} in this case.

ized by setting the fluorescence values before trypsin and after Triton addition as 0 and 100%, respectively. Corrections were made for dilutions due to addition of protein and detergent. Initial leakage rates were measured from the slope of the fluorescence intensity vs time plots. Mathematical operations on the spectral signals were performed with FELIX (PTI, London, ON) and SigmaPlot (Chicago, IL) software.

RESULTS

Channel Formation in PC Liposomes. Results of a typical experiment with egg PC LUV and 0.5 μM aerolysin, produced by adding trypsin to a mixture of the liposomes and proaerolysin, are shown in Figure 2, together with the normalization procedure. It may be seen that dye leakage started very quickly after trypsin addition and continued for approximately 40 min. Leakage rates were estimated from the maximum slopes of the fluorescence vs time plots. The maximum slopes were not always the initial slopes, since a lag period was sometimes observed, particularly under conditions favoring low leakage rates. Pure PC LUVs were routinely used in control experiments throughout this work. The average observed leakage rate was 0.036 ± 0.0035% leakage•s^{-1} (mean ± SEM, $n = 19$). Liposomes of different lipid compositions were normally compared to freshly prepared egg PC LUV and leakage rates are expressed as a percentage of rates obtained with egg PC liposomes. We were unable to detect any binding of fluorescently labeled proaerolysin variant FLAER (which, like wild-type proaerolysin, binds to cells containing GPI-anchored proteins; *21*) to the liposomes, under conditions otherwise similar to those used in the leakage experiments. This confirms that proaerolysin has little or no affinity for lipid bilayers in the absence of receptors. The overall structure of the liposomes was not changed by aerolysin insertion, as deduced from QELS measurements of PC LUV before and after treatment. The following are data from the Gaussian analysis of vesicles extruded through 0.2 μM polycarbonate filters. Control vesicles: mean diameter, 169 nm; coefficient of variance, 0.30; standard deviation, 51.3; χ^2, 1.87. Vesicles after 1 h of incubation with aerolysin, conditions as in the leakage assay: mean diameter, 170 nm; coefficient of variance, 0.37; standard deviation, 62.0; χ^2, 1.82.

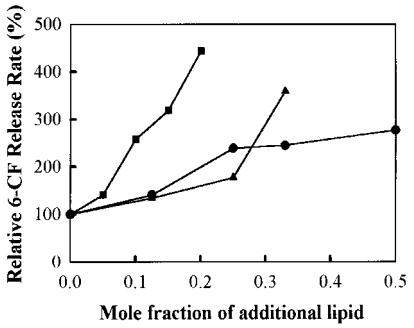

FIGURE 3: Effect of lipids that favor the L−H phase transition on channel formation. The LUV were composed of egg PC, to which DAG (■), PE (▲), or cholesterol (●) was added in varying proportions. The 100% leakage rate was obtained with pure egg PC LUV.

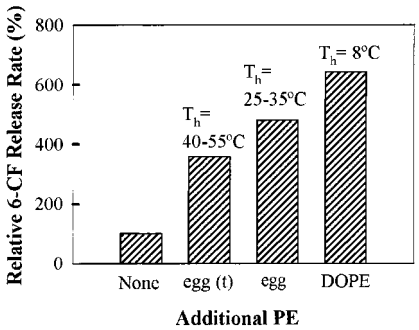

FIGURE 4: Relationship between T_h of various phosphatidylethanolamines and their ability to enhance channel formation. The LUV consisted of either pure PC (control) or PC/PE (2:1 mole ratio) mixtures, in which the source of the PE was, respectively, PE transphosphatidylated from egg PC (egg t), natural egg PE, or dioleoyl-PE. Percent changes in rate of aerolysin-induced leakage of 6-CF are given for each LUV composition.

Lipids That Favor the L−H Transition Enhance Aerolysin Permeabilization. LUV were prepared from binary mixtures of egg PC and cholesterol (Ch) or diacylglycerol (DAG). These latter two lipids are known to facilitate a transition from lamellar to inverted hexagonal phases (see Table 2 and references in ref *25*). Figure 3 shows that both lipids enhanced aerolysin-induced leakage rate in a dose-dependent fashion. Diacylglycerol, which is more effective than cholesterol in promoting hexagonal phase (*26*−*29*), had a larger effect on channel formation.

Phosphatidylethanolamine (PE) is another inverted cone-shaped lipid that can promote hexagonal phase formation when added to lamellar PC (*30*−*32*). As seen in Figure 3, PE from bovine liver also increased the susceptibility of PC bilayers to aerolysin permeabilization in a dose-dependent way.

The phase behavior of PE molecular species in water is largely determined by their fatty acid compositions: unsaturation generally increases the tendency to form H_{II} phases. The L−H transition can be thermotropically driven for experimental purposes. Three commonly used PEs, dioleoyl-PE, egg PE, and PE obtained by transphosphatidylation of egg PC (producing a PE with the same fatty acid composition as egg PC), exhibit the L−H transition at T_h temperatures of 8 °C, 25−35 °C, and 40−55 °C, respectively. These three PEs were tested in PC/PE mixtures (2:1 mole ratio). The results in Figure 4 show that the higher their propensity to form H_{II} phases (that is, the lower their T_h), the more effective they were in promoting vesicle leakage.

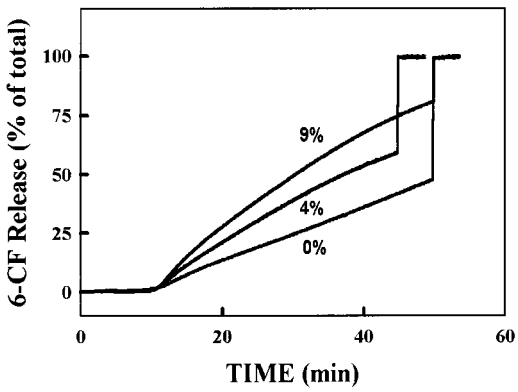

FIGURE 5: Enhancing effect of small proportions of hexadecane on channel formation. Curves correspond to the leakage experiment for LUV composed of pure egg PC (control), PC/hexadecane (96:4 mole ratio), and PC/hexadecane (91:9 mole ratio). The initial slopes are 0.0248% leakage•s^{-1}, or 100% rate (0% curve); 0.0415% leakage•s^{-1}, or 177% rate (4% curve); and 0.0587% leakage•s^{-1}, or 248% rate (9% curve).

Table 1: Effect of Lipid Composition on Channel Formation in Egg Phosphatidylcholine LUV by Aerolysin

bilayer composition	leakage rate[a]
pure egg PC	100
PC/lyso-PC (95:5)[b]	67
PC/lyso-PC (95:5)[c]	58
PC/lyso-PC (90:10)[b]	44
PC/lyso-PC (90:10)[c]	40
PC/sphingomyelin (90:10)[b]	44
PC/sphingomyelin (80:20)[b]	34
PC/DPPC (80:20)[b]	36

[a] Expressed as percent of leakage from egg PC LUV. A parallel egg PC control was run for each entry for comparison. Average values of at least two experiments performed on different liposome preparations are shown. [b] Lipids were mixed in organic solvent prior to LUV preparation. [c] Lyso-PC was added in aqueous suspension to preformed PC liposomes.

Small amounts (<5 mol %) of alkanes such as hexadecane facilitate inverted phase formation by filling in the voids between the H_{II}-phase hydrocarbon cylinders (*33*−*36*). As seen in Figure 5, low amounts of hexadecane also promoted aerolysin-dependent leakage of LUV aqueous contents.

Lipids That Stabilize the Lamellar Phase Decrease Aerolysin Effects. Certain lipids are known to hinder the formation of inverted hexagonal or cubic phases. This is the case for the cone-shaped lipid lyso-PC (*35, 37, 38*), which favors micellar structures (structures with positive curvature), and for other lipids, such as sphingomyelin (*39*) or dipalmitoylphosphatidylcholine (DPPC; *30*), which form very stable lamellar phases. When present in egg PC bilayers, each of these lipids lowered the rate of aerolysin-dependent leakage. The results are summarized in Table 1.

Lyso-PC, unlike most other lipids tested in this study, can be prepared in micellar suspensions in aqueous media. When it is added to preformed lipid bilayers, it will tend to partition into the outer leaflet (*40*). We tested the effect of LPC incorporated into liposomes in this way on channel formation by aerolysin. For this purpose, LUV were transferred to the spectrofluorometer cuvette as in the standard procedure, and fluorescence intensity was recorded for 5 min. Then lyso-PC (in 10% aqueous methanol) was added in a small volume (always less than 10 μL). It was independently checked that the same amount of methanol did not, by itself, alter LUV permeability. After addition of lyso-PC, fluorescence was recorded for an additional 5-min period. Under our conditions, addition of lyso-PC in methanol−water did not elicit any significant leakage of 6-CF. Then proaerolysin followed by trypsin was added according to the standard protocol. The results, presented in Figure 6, indicate a dose-dependent inhibition of channel formation by lyso-PC added in this way.

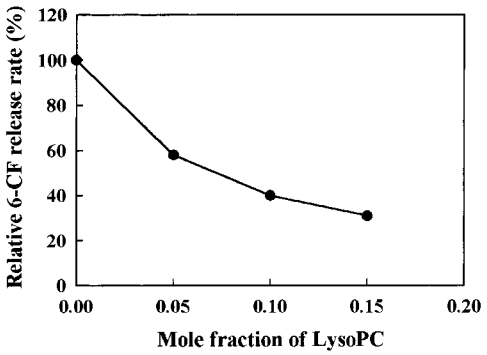

FIGURE 6: Inhibitory effect of lyso-PC on aerolysin permeabilization. Lyso-PC was added in a small volume of 10% (v/v) aqueous methanol to preformed LUV of egg PC. Percent changes in rate of aerolysin-induced leakage of 6-CF are shown. The 100% leakage rate corresponds to pure egg PC vesicles.

Although in these experiments the concentration of free aqueous lyso-PC was probably negligible (*40*), the possibility of a direct inhibitory effect of lyso-PC on proaerolysin or on trypsin was considered. For this purpose, before being added to the liposome suspension, proaerolysin (or trypsin) was incubated for 10 min with 0.17 μM lyso-PC, equivalent to the amount of this lipid for a PC:lyso-PC mole ratio of 85:15. When the lyso-PC-treated proaerolysin or trypsin was added to the cuvette, the lyso-PC was diluted out by about 30-fold. Neither proaerolysin nor trypsin was affected by preincubation with lyso-PC (data not shown).

It is unlikely that lyso-PC incorporated into preformed lipid bilayers from the outer aqueous phase undergoes significant transmembrane movement. Hence the vesicles in experiments such as that shown in Figure 6 are probably asymmetric, with most of the lyso-PC located in the outer monolayer. Interestingly, the results in Table 1 indicate that lyso-PC exerts its inhibitory action as a function of total mole fraction in the bilayer. Whether it is symmetrically or asymmetrically distributed in the bilayer is less important.

Lipid Effects Are Additive. The results in Figure 7 show that when two lipids that promote channel formation by aerolysin were present together in the PC LUV, their effects were additive. Conversely, the effects of mixtures of stimulatory and inhibitory lipids were subtractive. The series of additions (1) PC, PC + PE, PC + PE + Ch, and PC + PE + Ch + DAG or (2) PC, PC + PE, and PC + PE + hexadecane are good examples of additive enhancement. The series (3) PC, PC + PE, and PC + PE + lyso-PC or (4) PC, PC-DAG, and PC + DAG + lyso-PC provide examples of lipids with opposing properties on aerolysin permeabilization.

DISCUSSION

The biological significance of nonbilayer lipids in natural membranes is increasingly recognized (*41, 42*). Thus hexagonal phase or "inverted cone"-shaped lipids are thought to provide the flexibility for local rearrangements necessary

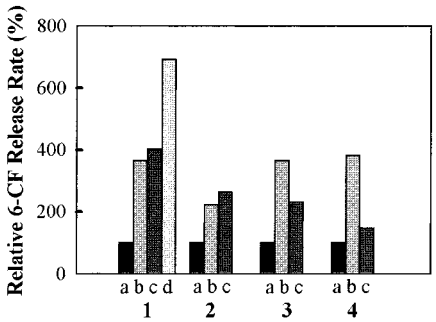

FIGURE 7: Additive effects of lipids on aerolysin permeabilization. Percent changes in rate of aerolysin-induced leakage of 6-CF are given, relative to pure PC. Results corresponding to four different series of experiments are shown, with the respective bilayer compositions being as follows: 1a−d, PC, PC/PE (2:1), PC/PE/Ch (2:1:1), and PC/PE/Ch:DAG (2:1:1:0.2); 2a−c, PC, PC/Ch (2:1), and PC/Ch/hexadecane (2:1:0.12); 3a−c, PC, PC/PE (2:1), and PC/PE/lyso-PC (2:1:0.75); 4a−c, PC, PC/DAG (4:1), and PC/DAG/lyso-PC (4:1:0.75).

for normal functions such as fusion events, division, and budding, and their presence somehow increases the activity of a variety of enzymes including protein kinase C (*43*), mitochondrial ubiquinol−cytochrome *c* reductase (*44*), phospholipases A_2 and C (*35*, *45*), and cytochrome P450SCC (*46*).

Hexagonal phase lipids also increased the apparent rate of channel formation by aerolysin. This was almost certainly not due to bulk formation of a nonbilayer phase in the liposomal membranes, as this would have required vesicle aggregation ("trans-monolayer contact"; *47*), which did not occur in our system. The size of the vesicles was preserved after aerolysin treatment (not shown here), and evidence from planar bilayers indicates that solute efflux only occurs through discrete pores.

The effects on channel formation of most of the lipids we studied might be at least partly due to the way they affect packing at the lipid−water interface. Inverted cone-shaped lipids impart a "negative" curvature to bilayer leaflets and as a result they lower the surface pressure at the interface (*48*, *49*), which could favor access of the aerolysin heptamer to the hydrophobic matrix. A similar explanation has been used to account for the increased membrane binding of protein kinase C (*50*), CTP:phosphocholine cytidyltransferase (*51*), and apolipophorin III in the presence of hexagonal phase lipids (*19*). However, changes in membrane curvature cannot completely explain how hexadecane increased the rate of channel formation, as this molecule does not appear to alter membrane curvature. Instead it is thought to promote the L−H transition by filling voids between lipid molecules (*35*, *36*).

Hexagonal phase lipids may promote channel formation by aerolysin by simply facilitating the localized and transitory generation of nonbilayer structures as the large oligomeric amphipathic barrel enters the membrane. The oligomer could first bind to a hydrophobic patch on the membrane surface and a transient structure with the geometry of an inverted phase could evolve from the contact point, enveloping the barrel. A detailed understanding of the permeabilization process will require further experimentation and modeling.

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