Permeabilization of Raft-Containing Lipid Vesicles by δ-Lysin: A Mechanism for Cell Sensitivity to Cytotoxic Peptides†

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ABSTRACT: δ-Lysin is a linear, 26-residue peptide that adopts an α-helical, amphipathic structure upon binding to membranes. δ-Lysin preferentially binds to mammalian cell membranes, the outer leaflets of which are enriched in sphingomyelin, cholesterol, and unsaturated phosphatidylcholine. Mixtures including these lipids have been shown to exhibit separation between liquid-disordered (ℓ_d) and liquid-ordered (ℓ_o) domains. When rich in sphingomyelin and cholesterol, these ordered domains have been called lipid "rafts". We found that δ-lysin binds poorly to the ℓ_o (raft) domains; therefore, in mixed-phase lipid vesicles, δ-lysin preferentially binds to the ℓ_d domains. This leads to the concentration of δ-lysin in ℓ_d domains, enhancing peptide aggregation and, consequently, the rate of peptide-induced dye efflux from lipid vesicles. The efficient lysis of eukaryotic cells by δ-lysin can thus be attributed not to specific δ-lysin−cholesterol or δ-lysin−sphingomyelin interactions but, rather, to the exclusion of δ-lysin from ordered rafts. The degree to which the kinetics of dye efflux are enhanced in mixed-phase vesicles over those observed in pure, unsaturated phosphatidylcholine vesicles directly reflects the amount of ℓ_d phase present in mixed-phase systems. This effect of lipid domains has broader consequences, beyond the hemolytic efficiency of δ-lysin. We discuss the hypothesis that bacterial sensitivity to antimicrobial peptides may be determined by a similar mechanism.

δ-Lysin is a peptide secreted by *Staphylococcus aureus* (*1*) that efficiently lyses eukaryotic cells and does not require binding to cell surface receptors to exert its action. It is soluble in water and associates with phospholipid bilayers as an amphipathic α-helix (*2*, *3*). A few recent studies examined the interaction of this peptide with lipid model membranes (*4*, *5*). Over the past few years, we have studied in detail the kinetics of the interaction of δ-lysin with fluid-phase, lipid bilayer membranes of a single, monounsaturated phosphatidylcholine (*6*, *7*). We found that dye efflux from vesicles of this type is caused by a rapid translocation of a small peptide aggregate, most likely a trimer, across the membrane.

Eukaryotic plasma membranes are, however, complex mixtures of different types of lipids and proteins (*8*), and are not approximated well by single-component lipid vesicles. Typical cell membranes are highly heterogeneous structures with lipid and protein components organized in functional domains. Over the past 10 years, special domains, called "lipid rafts" (*9*, *10*), have been a major focus of studies of eukaryotic membrane structure. Lipid rafts are domains rich in sphingomyelin and cholesterol, with which certain types of membrane proteins are typically associated. Rafts have been implicated in a number of cellular functions, including the facilitation of reactions between proteins and lipids that partition preferentially into the rafts and sorting of components between different cell membranes (*9*). The physical−chemical nature of the interactions of lipid raft components and the biological implications stemming from the existence of these types of domains have been reviewed recently by several authors (*11*−*14*). The predominant lipid components in the outer leaflet of eukaryotic membranes are sphingomyelin (SM),[1] 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and cholesterol (Chol) (*15*). Ternary mixtures of these lipids seem to embody the essential features of the lipid component of raft-containing membranes (*16*−*19*). This system can exhibit a coexistence between liquid-disordered (ℓ_d) and liquid-ordered (ℓ_o) domains, similar to what is found in binary mixtures of phospholipids and cholesterol (*20*−*25*).

In this investigation, we test two mutually exclusive hypotheses for the susceptibility of eukaryotic cells to δ-lysin. The first, more obvious possibility, is that the peptide binds to or interacts favorably with lipid rafts or its main components, SM and Chol. The second hypothesis is that δ-lysin binds preferentially to unsaturated phosphatidylcholine (PC) and is actually excluded from the lipid rafts. To test these hypotheses, we measured the activity of δ-lysin toward lipid bilayer vesicles composed of ternary mixtures of SM, Chol, and POPC, in the ℓ_d−ℓ_o phase coexistence region, as a function of the fractions of the two liquid phases.

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[1] Abbreviations: PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SM, sphingomyelin; Chol, cholesterol; DOPC, dioleoylphosphatidylcholine; $M(IP)_2C$, mannose-(inositol phosphate)$_2$-ceramide; LUV, large unilamellar vesicle; DSM, detergent-soluble membranes; DRM, detergent-resistant membranes; ℓ_d, liquid-disordered; ℓ_o, liquid-ordered; K_D, equilibrium dissociation constant.

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We found that the global membrane structure of the membrane, in particular, its domain organization, appears to be responsible for the susceptibility to δ-lysin, rather than specific interactions between the peptide and individual lipid components. The same may apply to antimicrobial peptides, which share many structural and functional similarities with δ-lysin. Antimicrobial peptides are often cytotoxic to a specific genus of bacteria but may be relatively inactive toward other, sometimes closely related genera (*27*−*29*). On the basis of recent findings reported in the literature, we discuss the hypothesis that the preferential interaction with certain lipid domains may also lie at the basis of the target specificity of antimicrobial peptides.

MATERIALS AND METHODS

Chemicals. POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), in a chloroform solution, and SM [porcine brain sphingomyelin, (2*S*,3*R*,4*E*)-2-acylaminooctadec-4-ene-3-hydroxy-1-phosphocholine], as powder or in a chloroform solution, were purchased from Avanti Polar Lipids, Inc. The fatty acid chain composition of this porcine brain sphingomyelin, specified by the vendor, is as follows: 16:0 (2%), 18:0 (49%), 20:0 (5%), 22:0 (8%), 24:0 (6%), 24:1 (20%), and other chains (10%). Cholesterol, as powder, was obtained from ICN Biochemicals, Inc. Carboxyfluorescein (99%) was purchased from ACROS. Organic solvents (ACS/HPLC) were purchased from Burdick & Jackson. Lipids and probes were tested by TLC and used without further purification.

δ-Lysin. δ-Lysin (formyl-MAGDIISTIGDLVKWIID-TVNKFTKK) was a gift from H. Birkbeck. Its purification was described previously (*6*, *30*). For the stopped-flow fluorescence measurements, δ-lysin was added from a 1 μM solution in 0.10 M KCl (pH 3.0), which imparts the peptide with a positive charge, minimizing aggregation and sticking to glass surfaces prior to mixing, as described previously (*7*).

Preparation of Lipid Vesicles. Large unilamellar vesicles (LUVs) were prepared by mixing lipid solutions in a 4:1 chloroform/methanol mixture in a round-bottom flask. The solvent was then evaporated using a rotary evaporator (Büchi R-3000) at 60−70 °C. The lipid film that was obtained was placed under vacuum for 5−8 h and hydrated by the addition of 20 mM MOPS buffer (pH 7.5) containing 100 mM KCl, 0.01 mM EGTA, and 0.02% NaN_3, which has the same osmolarity as the carboxyfluorescein-containing buffer. For experiments using carboxyfluorescein-encapsulated vesicles, the lipid film was hydrated in 20 mM MOPS (pH 7.5), 0.01 mM EGTA, 0.02% NaN_3, and 50 mM carboxyfluorescein, to give a final lipid concentration of 10 mM. The suspension of multilamellar vesicles was subjected to five freeze−thaw cycles. It was then extruded 10 times through two stacked Nuclepore polycarbonate filters with a pore size of 0.1 μm, using a water-jacketed high-pressure extruder from Lipex Biomembranes Inc., at room temperature for POPC and at 70 °C for the mixtures containing SM and Chol. Following extrusion, carboxyfluorescein-containing LUVs were subjected to gel filtration chromatography through a Sephadex-G25 column to separate the dye in the external buffer from the vesicles. The suspension was diluted in carboxyfluorescein-free buffer to the desired concentration and used for fluorescence measurements. Lipid concentrations were assayed by the Bartlett phosphate method (*31*), modified as previously described (*6*), with the absorbance read at 580 nm.

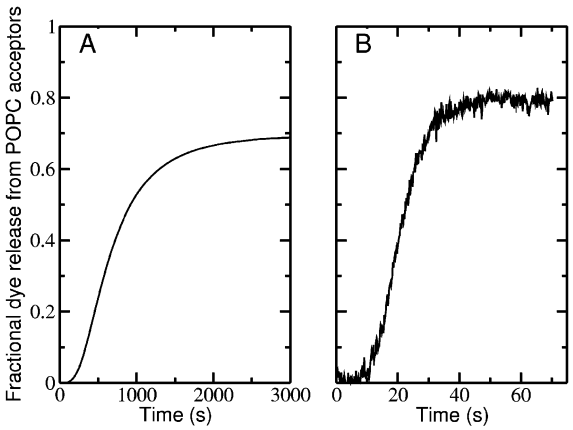

FIGURE 1: Release of carboxyfluorescein from POPC acceptor vesicles induced by δ-lysin in a reverse experiment. Empty vesicles (460 μM) composed of either pure POPC or SM and Chol (1:1) were allowed to equilibrate with 1 μM δ-lysin for 15 min and used as peptide donors. The donor vesicles were then mixed with 40 μM dye-loaded, pure POPC acceptor vesicles, and the dye efflux from these acceptors was monitored as a function of time. The experiments were performed at 22 °C. (A) Donor vesicles are composed of pure POPC (data from ref *7*). (B) Donor vesicles are composed of SM and Chol (1:1).

Carboxyfluorescein Efflux Experiments. The kinetics of carboxyfluorescein efflux were measured using a SLM-Aminco 8100 spectrofluorimeter, adapted with a RX2000 rapid kinetics spectrometer accessory (Applied Photophysics), equipped with a RX pneumatic drive accessory (Applied Photophysics). Carboxyfluorescein efflux was measured by the relief of self-quenching of fluorescence, measured by excitation at 470 nm and emission at 520 nm. The maximum amount of dye release was measured by the addition of Triton X-100 to a final concentration of 1% (w/v).

Calculation of Average Relaxation Times. The curves of carboxyfluorescein release as a function of time (t) were characterized by a mean relaxation time (τ) as follows. Let $F(t)$ be the experimental curve of the (normalized) fluorescence increase as a function of time, which increases as carboxyfluorescein is released, until it essentially reaches a plateau (see Figure 2, for example). Let $f(t)$ be its time derivative

$$f(t) = \frac{\mathrm{d}F(t)}{\mathrm{d}t} \tag{1}$$

This time derivative behaves like the probability density function (*32*, *33*). The mean relaxation time is then obtained by

$$\tau = \frac{\int_0^\infty t f(t)\,\mathrm{d}t}{\int_0^\infty f(t)\,\mathrm{d}t} \tag{2}$$

For example, for a multiexponential decay τ is simply the weighted average of the relaxation times of each exponential function. The calculation of the derivative from eq 1 directly from the data results in a large noise that introduces a significant error. To avoid this, the experimental curves were smoothened by first fitting them with an arbitrary function

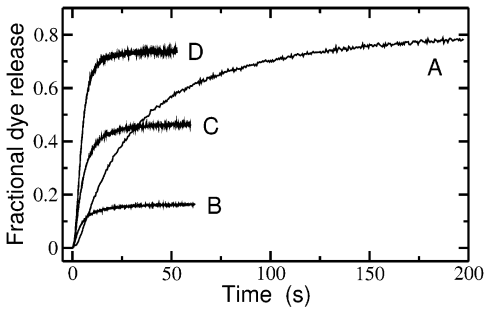

FIGURE 2: Kinetics and fraction of dye released from lipid vesicles of varying composition induced by δ-lysin. The vesicles used were composed of either 100% POPC or mixtures of SM and Chol containing varying amounts POPC. Final lipid and δ-lysin concentrations after mixing were 200 and 0.5 μM, respectively. The experiments were performed at 22 °C: (A) POPC, (B) SM and Chol in a 1:1 ratio, (C) SM, Chol, and POPC in a 4:4:2 ratio, and (D) SM, Chol, and POPC in a 2:2:6 ratio.

to obtain an excellent fit (which was possible in all cases). This smooth line was then differentiated numerically and the mean relaxation time obtained by numerical integration using eq 2.

RESULTS

Binding of δ-Lysin to ℓd and ℓo Phases. A combination of tight binding to POPC vesicles, peptide translocation across the membrane, and changes induced by δ-lysin on the vesicles precludes direct determination of the constants for binding to these vesicles by simple titration. Therefore, the on- and off-rate constants previously obtained from the fits to the kinetic data (*6*, *7*) were used to estimate binding. The apparent equilibrium dissociation constant (K_D) of δ-lysin from pure POPC vesicle (ℓd phase) was estimated to be less than ≈0.1 μM. The K_D from the SM/Chol ℓo phase was estimated to be approximately 40 μM, as follows. Empty POPC vesicles (460 μM) were first incubated with δ-lysin (1 μM) for 15 min and then used as peptide donors to induce carboxyfluorescein leakage from dye-loaded POPC vesicles (acceptors, 40 μM) upon mixing in a stopped-flow unit. After mixing, the peptide and lipid acceptor concentrations are 0.5 and 20 μM, respectively. Under these conditions, the half-time for dye release is approximately 1000 s (Figure 1A) (*6*, *7*). This long time is mainly due to a slow desorption from the donor vesicles. If, instead of POPC, 1:1 SM/Chol vesicles are used as donors and then mixed with POPC acceptor vesicles, the half-time is approximately 20 s (Figure 1B). This indicates that a significant fraction of δ-lysin was not bound to the SM/Chol vesicles. That fraction was estimated by considering that 20 s is the half-time for carboxyfluorescein leakage induced by δ-lysin when the peptide:lipid ratio is ∼1:500 (*7*). Assuming that most of the leakage from the acceptors is caused by peptides that were already in solution at time zero (and did not have to desorb first) and knowing that association is close to the diffusion limit (*7*), that ratio should be approximately the ratio between the peptide remaining in the aqueous solution and the POPC acceptor vesicles (20 μM) at time zero. That is, the concentration of δ-lysin was ≈0.04 μM at time zero in the stopped flow. Thus, prior to the δ-lysin being mixed with acceptor vesicles, the concentration of δ-lysin in solution in equilibrium with the SM/Chol donor vesicles (460 μM) was 0.08 μM (twice the value after mixing), out of 1 μM total δ-lysin. Therefore, from $K_D/[L] = (1 - \theta)/\theta$, where $\theta = 1 - 0.08 = 0.92$ is the fraction of peptide bound and [L] is the lipid concentration (460 μM), the K_D from SM/Chol vesicles is ≈40 μM. The finding that K_D is <0.1 μM for POPC and ≈40 μM for 1:1 SM/Chol vesicles means that δ-lysin binds to the ℓd phase 100−1000 times better than to the ℓo phase. The significance of this result is that in a vesicle with coexisting ℓd (mainly POPC) and ℓo (mainly SM/Chol) phases, δ-lysin will bind almost exclusively to the ℓd phase.

Carboxyfluorescein Efflux from POPC (ℓd phase) and 1:1 SM/Chol (ℓo phase) Vesicles. Under the same conditions, the release of carboxyfluorescein induced by δ-lysin is close to complete (≈80%) from POPC vesicles (Figure 2, curve A), which are in the ℓd phase, but very minor (≈15%) from 1:1 SM/Chol vesicles (Figure 2, curve B), which are in the ℓo phase. Even this small release from the SM/Chol vesicles is probably due to a small amount of short or unsaturated chains present in the porcine brain SM that was used (acyl chain composition given in Materials and Methods). The combination of poor binding to and poor efflux across the ℓo phase suggests that in vesicles with ℓd and ℓo coexistence dye efflux will arise almost exclusively from peptides associated with the ℓd domains. We have previously shown that self-association of δ-lysin in POPC vesicles is necessary for peptide-induced dye efflux (*6*, *7*). The combination of those two facts leads to the prediction that, if the peptide is concentrated in small ℓd domains in a matrix of the ℓo phase, the extent of self-association will increase and the rate of dye efflux will also increase. Thus, although the peptide does not bind or release dye efficiently from SM/Chol vesicles, increasing the SM/Chol content in SM/Chol/POPC vesicles with coexisting ℓd and ℓo phases should increase the apparent rate of carboxyfluorescein efflux. To test this prediction, the concentration of POPC in SM/Chol/POPC vesicles was varied, while maintaining a 1:1 SM:Chol ratio.

Carboxyfluorescein Efflux from SM/Chol/POPC Vesicles as a Function of Composition. The rates of carboxyfluorescein release induced by δ-lysin from SM/Chol/POPC vesicles were measured at 22 °C for several lipid mixtures with an equimolar ratio of SM and Chol, containing different amounts of POPC (SM:Chol:POPC ratios of 50:50:0, 40:40:20, 30:30:40, 25:25:50, 20:20:60, 15:15:70, 10:10:80, and 0:0:100). As the mole percentage of POPC is increased, the final fraction of dye released increases, as shown in Figure 2 for a 40:40:20 SM:Chol:POPC ratio (curve C), and it reaches the level of release found in pure POPC when the concentration of POPC is ≥40 mol % (Figure 2, curve D is for a 20:20:60 SM:Chol:POPC ratio). The average values of the percent carboxyfluorescein released for the complete set of POPC concentrations examined are shown in Figure 3. According to the phase diagram for palmitoyl-SM/Chol/POPC vesicles (*18*), the 4:4:2 SM/Chol/POPC mixture should be in the ℓo phase, but we observe a somewhat higher extent of dye release from these vesicles than from 1:1 SM/Chol vesicles. This could be explained because the SM used here (from porcine brain) is a mixture, so the phase diagrams may be slightly different. It should be noted that the 4:4:2 mixture is very close to the ℓd−ℓo, two-phase boundary in any case (see Figure 7, below, for a scheme of the phase diagram). The other possible explanation stems from the fact that phase diagrams are only strictly valid as descriptions of macroscopic systems. For small systems, which include those

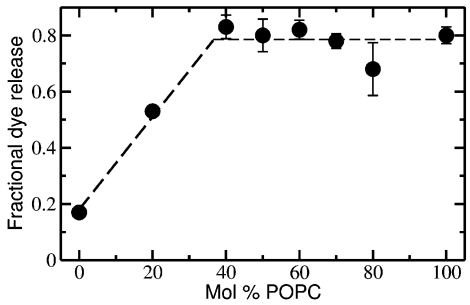

FIGURE 3: Mean fraction of dye released from vesicles composed of ternary mixtures of SM, Chol, and POPC induced by δ-lysin as a function of the amount of POPC contained in the mixture. The vesicles were composed of SM and Chol in equal mole fractions with a varying amount of POPC (1:1:*x* SM:Chol:POPC ratio). The experiments were performed at 22 °C. Each data point represents the average and standard deviation of at least three experiments. Final lipid and δ-lysin concentrations after mixing were 200 and 0.5 μM, respectively, for all experiments.

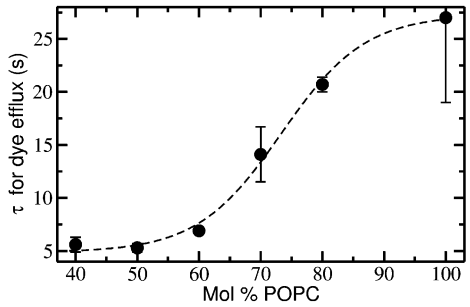

FIGURE 4: Mean relaxation time of carboxyfluorescein efflux (τ) from 1:1:*x* SM/Chol/POPC vesicles induced by δ-lysin as a function of POPC content at 22 °C. Each data point represents the average and standard deviation of three experiments. Final lipid and δ-lysin concentrations after mixing were 200 and 0.5 μM, respectively. The line has been added to guide the eye.

studied here, they are an approximation. The 4:4:2 SM/Chol/POPC mixture may appear to be entirely in the l_o phase but may actually contain microscopic inhomogeneities richer in POPC, which the peptide detects. This is particularly likely for points close to the two-phase boundary, as appears to be the case for the 4:4:2 SM/Chol/POPC mixture, using this phase diagram (Figure 7).

It is also evident from Figure 2 that, for lipid mixtures that support essentially complete carboxyfluorescein release, the apparent time for dye efflux is much shorter if the vesicles contain 60 mol % POPC (Figure 2, curve D) than in pure POPC (curve A). The apparent time of carboxyfluorescein release was quantitatively expressed by calculating the mean relaxation time (τ) for dye efflux, as a model-independent parameter (see Materials and Methods). The complete set, revealing the dependence of τ on POPC content, is shown in Figure 4. It is clear that release is fastest (small τ) when only a small amount of the l_d phase exists (POPC) and a large part of the system is in the l_o phase.

DISCUSSION

δ-Lysin is a strongly hemolytic, amphipathic peptide of bacterial origin (*Staphylococcus aureus*). A plausible hypothesis considered here was that it might interact favorably with lipid rafts or lipid components contained in rafts on the erythrocyte membrane and that this interaction would be part of its specificity determinant. We found this not to be true. Instead, a much more subtle mechanism appears to operate. To understand this process, we examined carboxyfluorescein efflux induced by δ-lysin from SM/Chol/POPC vesicles with an equimolar ratio of SM and Chol, as a function of POPC concentration. The phase diagram for this ternary system with the particular SM that we used is not available. However, the phase diagram for a closely related system (see Figure 7), palmitoyl-SM/Chol/POPC (*18*), indicates that it is safe to assume that the ternary mixtures examined here exist in the l_d, l_o, or as mixtures of l_d and l_o phases, at room temperature. Furthermore, differential scanning calorimetry performed on the mixtures examined here indicates the absence of solid phases (A. Hinderliter, personal communication). We found that δ-lysin binds poorly to the Chol- and SM-rich raft phase. Thus, in a system that is composed of both l_o and l_d phases, the peptide will bind preferentially to the l_d phase and accumulate in these, nonraft, domains. This observation is contrary to the first hypothesis. However, an alternative hypothesis is possible. If a system is primarily composed of the l_d phase, the l_o-phase domains correspond to the rafts and the unsaturated PC provides the "sea" of fluid phase (Figure 5A). With increasing amounts of saturated SM and Chol, the fraction of the l_o phase will increase. At some point, the system will cross a percolation threshold (*26*) and its configuration will be reversed. Eventually, small domains or islands of the l_d phase will become surrounded by a connected l_o phase (Figure 5B). If the process is continued, the system will be entirely in a single, l_o phase (Figure 5C). As long as a sufficient amount of the l_d phase exists (Figure 5A,B), δ-lysin binds well to these membranes, but this does not happen if only the l_o phase is present (Figure 5C). Because peptide self-association on the membrane is a necessary condition for dye efflux from lipid vesicles (*6*, *7*), the kinetics of dye efflux are strongly dependent on the amount of peptide bound per unit area of lipid: if the two-phase system becomes enriched in the l_o phase at the expense of the l_d phase, δ-lysin will accumulate in l_d domains. Enrichment of δ-lysin in the disordered lipid domains favors aggregation, and consequently, dye efflux from lipid vesicles becomes more efficient as the amount of l_d phase decreases. For example, permeabilization, by δ-lysin, of SM/Chol/POPC lipid vesicles containing ≤60% POPC is significantly faster than that observed for pure POPC

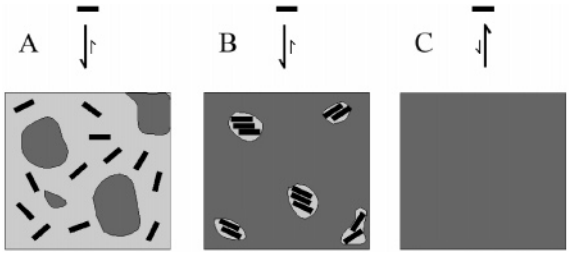

FIGURE 5: Schematic representation of a lipid mixture exhibiting phase separation between an l_d phase (light) and an l_o phase (dark). The peptides are represented as black rods. (A) The peptide binds preferentially to the l_d phase; since the mixture is predominantly in the l_d phase, the peptide is dispersed. (B) With increasing amounts of SM and Chol, the system crosses its percolation threshold and is mostly l_o phase. The peptides become concentrated in the l_d phase domains, which are now the minor phase, and associate. (C) Further increasing the amounts of SM and Chol causes the system to cross a phase boundary. After this point, the lipid mixture is in a pure l_o phase, to which the peptides bind only poorly.

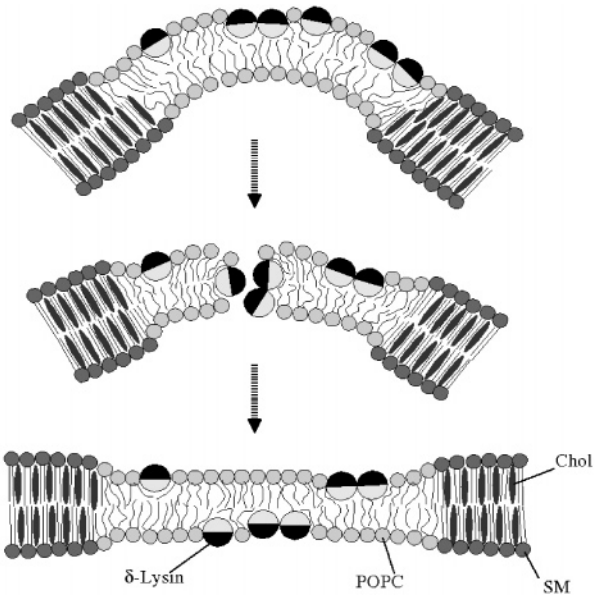

FIGURE 6: Sinking-raft model (*7*) for δ-lysin-induced transient pore formation and peptide translocation across a phase-separated lipid bilayer. The δ-lysin α-helices are shown as cross sections with the dark half-circles representing the hydrophilic faces. The liquid-disordered, ℓ_d, phase is represented by lipids with light gray headgroups and disordered acyl chains. The liquid-ordered, ℓ_o, phase is represented by SM molecules (darker gray headgroups and extended acyl chains) and Chol molecules (dark gray ellipses). δ-Lysin is excluded from the ℓ_o phase which leads to a locally increased peptide concentration in the ℓ_d phase. Preferential binding to the ℓ_d phase leads to a mass imbalance across the bilayer in the ℓ_d regions (top), which is the driving force for peptide translocation. The strain is relieved as the peptides associate, sink into the bilayer, and translocate to the inside (middle), in a process that perturbs the membrane and causes dye efflux. Peptide translocation continues until equilibration across the membrane is established (bottom).

vesicles (Figure 4). It could be argued that defect lines between ℓ_d and ℓ_o domains might also play a role in increasing the rate of dye release. We do not think this is the case for two reasons. First, if defect lines existed, they would be expected to render the mixed vesicles leakier even in the absence of δ-lysin; however, we find that SM/Chol/POPC vesicles are as tight as pure component vesicles, in the absence of peptide. Second, in a previous paper (*34*), we examined the rate of insertion of a small lipid amphiphile into the ℓ_d−ℓ_o coexistence region of dimyristoylphosphatidylcholine/cholesterol vesicles. The kinetics of that process were described exactly by contributions from insertion into ℓ_d and ℓ_o areas, without any increase in the rate due to the presence of interfaces. Therefore, it appears that ℓ_d−ℓ_o interfaces are smooth, not defect, lines.

Thus, the pronounced hemolytic activity of δ-lysin can be attributed not to its preferential interaction with Chol-containing membranes but rather to the exclusion of δ-lysin from Chol-rich, ℓ_o domains. Exclusion of δ-lysin from ℓ_o domains combined with a very small amount of efflux from those areas implies that, in a system with ℓ_d−ℓ_o phase coexistence, dye efflux results mainly from peptides bound to ℓ_d areas. These concepts are illustrated in the model shown in Figure 6, which combines our previous proposal (*6*, *7*) for the mechanism of bilayer permeation with the effect of exclusion from ordered lipid regions of the membrane. The peptides initially bind to the membrane outer leaflet, and the mass imbalance leads to curvature strain (Figure 6, top).

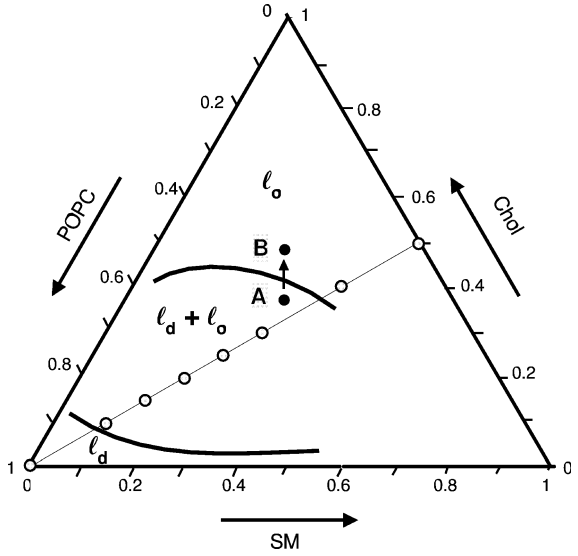

FIGURE 7: Simplified phase diagram for SM/Chol/POPC vesicles at room temperature (23 °C) based on the diagram for palmitoyl-SM/Chol/POPC vesicles (*18*). The boundaries of the ℓ_d−ℓ_o two-phase region are indicated by the thick curves. A mixture of composition and temperature represented by point A is in a two-phase region composed mainly of the ℓ_o phase. At point B, after only a small change in the overall composition, the system lies entirely in the ℓ_o phase. The mixtures examined in this work are indicated by the open circles on the phase diagram, along the line corresponding to mixtures containing equimolar amounts of SM and Chol.

The strain is relieved as the peptides associate, sink into the bilayer, and translocate to the inside of the vesicle (Figure 6, middle), in a process that perturbs the membrane and causes dye efflux. Peptide translocation continues until equilibration across the membrane is established (Figure 6, bottom).

The significance of the mechanism proposed here for the sensitivity of a eukaryotic membrane to the cytolytic peptide can be appreciated with reference to Figure 7, which shows a schematic, simplified version of the phase diagram of palmitoyl-SM/Chol/POPC vesicles (*18*). For lipid mixtures in the ℓ_d−ℓ_o phase coexistence region close to the ℓ_o region (point A in Figure 7), a small change in the mole fraction of cholesterol would move the system into the single-phase region (point B in Figure 7). For the activity of a peptide such as δ-lysin, a dramatic change would occur, from a situation of very high activity to essentially none, because of its very poor binding to the ordered phase. Considering that, in mammalian cells, typically 20−40 mol % of the lipid is Chol, it is plausible that a cell that is very susceptible to δ-lysin (because only a small fraction of its membrane is in an ℓ_d phase) could acquire resistance to the peptide by increasing the Chol content by a few mole percent in its plasma membrane, thereby shifting completely to an ℓ_o phase. This concept corresponds to a transition between the situations represented in panels B and C of Figure 5.

The importance of exclusion from ℓ_o areas as a way of promoting peptide concentration in other membrane areas may be generally important for other antimicrobial and cytolytic peptides, especially if their action on the cell membrane requires peptide self-association. This is because the preferential partitioning of both amphipathic (surface-associated) and hydrophobic (membrane-spanning) peptides

to the ℓ_d rather than to the ℓ_o phase appears to be the rule. This is true, for example, of SNARE proteins that span the membrane with a single α-helix, which prefer the ℓ_d phase (*35*); of the synthetic, transmembrane peptide, LW (acetyl-$K_2W_2L_8AL_8W_2K_2$-amide) (*36*); and of a 36-residue, transmembrane helix from bacteriorhodopsin, which partitions preferentially into the ℓ_d phase [dioleoylphosphatidylcholine (DOPC)] relative to the ℓ_o phase (1:1 SM:Chol ratio) with a partition coefficient (K_P) of approximately 10 (*37*). It was also shown (*38*) that hydrophobic peptides of 23, 29, and 31 residues partitioned preferentially to more disordered lipid membranes [detergent-soluble membranes (DSM)] as opposed to ordered membranes [detergent-resistant membranes (DRM)] with a K_P of ≃6−10 at 4 °C, where the compositions of these two types of membranes are significantly different. [At 4 °C, DSM had an ∼3:1 phospholipid (SM + DOPC): Chol ratio and DRM had an ∼1:1 phospholipid:Chol ratio (*38*).] With respect to amphipathic peptides, the same partitioning behavior was apparent for the 23-residue MPR peptide (presequence of mitochondrial rhodanese) and for melittin (*37*), both of which prefer ℓ_d vesicles of DOPC to ℓ_o vesicles of SM and Chol with a K_P of 10−500, the exact value depending on whether the vesicles used are small unilamellar vesicles (smaller K_P) or LUVs (larger K_P). Finally, it may also be mentioned that addition of Chol to POPC, 1-palmitoyl-2-oleoylphosphatidylglycerol, or 1-palmitoyl-2-oleoylphosphatidylserine dramatically reduces the level of binding of a synthetic gramicidin S analogue (*39*).

Like δ-lysin, many antimicrobial peptides form amphipathic α-helices when bound to membranes and cause membrane perturbation or disruption (*40*). However, different bacteria display very different sensitivities to these antimicrobial peptides. It is thus possible that these varied sensitivities arise from differences in the interaction of peptides with specific lipid domains in the membranes of their target cells. The most obvious mechanism is a preferential interaction of the peptide with a particular lipid domain; however, the exclusion from domains, as demonstrated in this paper, is an alternative possibility. Bacterial membrane composition varies significantly between Gram-positive and Gram-negative bacteria, and, more subtly, among different genera within each class (*41*, *42*). Sensitivity to antimicrobial peptides may thus be linked to heterogeneity in the membrane structure of these bacteria. Recently, this type of heterogeneity has been directly observed (see ref *43* for a recent review). Furthermore, plasma membrane heterogeneity is not limited to phase separations between ordered and disordered phases but also involves clustering of lipids based on charge or hydrogen bonding ability (*44*, *45*).

Recent data definitely link acquisition of resistance to antimicrobial peptides by yeast and fungi to significant changes in the lipid composition of their membranes (*46*). First, a mutation in the *IPT1* gene, causing the absence of the enzyme responsible for the last step in the synthesis of mannose-(inositol phosphate)$_2$-ceramide [M(IP)$_2$C] in *Saccharomyces cerevisiae* and, consequently, the absence of M(IP)$_2$C from the plasma membrane, is responsible for the acquired resistance of the yeast to plant defensin DmAMP1 (*46*, *47*). Second, in the fungus *Neurospora crassa*, changes in the chain composition of glucosylceramide (GlcCer) are correlated with acquired resistance to defensins RsAFP2, DmAMP1, and Hs-AFP1 (*48*). The acyl chain composition of GlcCer changes almost completely from C18:1 in the wild type to C16:0 in plant defensin-resistant mutants. Yeasts and most fungi do not contain cholesterol, but they always contain large amounts of other sterols, most often ergosterol (*49*, *50*), which may play a role similar to that of cholesterol in the formation of ordered liquid domains (*36*, *51*). Furthermore, the role of SM in mammalian membranes, as the major phospholipid in raftlike or ℓ_o domains, may be taken by glycosphingolipids, in yeasts and fungi. Glycosphingolipids are major components of the plasma membrane in yeast (*52*), plants (*53*), and probably fungi, which have similar levels of glycosphingolipids relative to total lipid, but for which data on membrane localization are not available (*53*). However, as with plants and mammals, it is likely that glycosphingolipids reside mainly in the outer leaflet of the plasma membrane, where their fraction could therefore be even larger. This leads to the possibility that the coexistence of raftlike domains (composed of glycosphingolipids and sterols) and liquid disordered phases (unsaturated PC) may occur in these organisms, and changes in domain structure may lie at the root of the acquired resistance to antimicrobial and cytotoxic peptides.

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