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Lysylated phospholipids stabilize models of bacterial lipid bilayers and protect against antimicrobial peptides ☆

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ABSTRACT

Aminoacylated phosphatidylglycerols are common lipids in bacterial cytoplasmic membranes. Their presence in *Staphylococcus aureus* has been linked to increased resistance to a number of antibacterial agents, including antimicrobial peptides. Most commonly, the phosphatidylglycerol headgroup is esterified to lysine, which converts anionic phosphatidylglycerol into a cationic lipid with a considerably increased headgroup size. In the present work, we investigated the interactions of two well-studied antimicrobial peptides, cecropin A and mastoparan X, with lipid vesicles composed of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG), containing varying fractions of an aminoacylated phosphatidylethanolamine, a stable analog of the corresponding phosphatidylglycerol-derivative. To differentiate between the effects of headgroup size and charge on peptide–lipid interactions, we synthesized two different derivatives. In one, the headgroup was modified by the addition of lysine, and in the other, by glutamine. The modification by glutamine results in a phospholipid with a headgroup size comparable to that of the lysylated version. However, whereas lysyl-phosphatidylethanolamine (Lys-PE) is cationic, glutaminyl-phosphatidylethanolamine (Gln-PE) is zwitterionic. We found that binding of mastoparan X and cecropin A was not significantly altered if the content of aminoacylated phosphatidylethanolamines did not exceed 20 mol.%, which is the concentration found in bacterial membranes. However, a lysyl-phosphatidylethanolamine content of 20 mol% significantly inhibits dye release from lipid vesicles, to a degree that depends on the peptide. In the case of mastoparan X, dye release is essentially abolished at 20 mol.% lysyl-phosphatidylethanolamine, whereas cecropin A is less sensitive to the presence of lysyl-phosphatidylethanolamine. These observations are understood through the complex interplay between peptide binding and membrane stabilization as a function of the aminoacylated lipid content. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

1. Introduction

Interactions between antimicrobial peptides and their target membranes have been studied using mostly mixtures of unsaturated phospholipids, with headgroup compositions that reflect those of the major lipid classes found in bacterial cytoplasmic membranes. However, bacterial lipid species are more diverse and bacterial cytoplasmic membranes more complex than suggested by these simple model systems. For instance, in many Gram-positive bacteria, including the genus *Staphylococcus*, saturated but terminally branched fatty acids functionally replace the unsaturated chains found in other organisms [1]. Moreover, the formation of aminoacylated phospholipids is a common secondary modification of bacterial membrane lipids and occurs in a number of pathogenic bacteria, both Gram-positive and Gram-negative. In *Staphylococcus aureus*, 20–30% of the phosphatidylglycerol (PG) headgroups are modified by lysine, or, less commonly, by alanine and ornithine [1]. The aminoacylation results in the formation of a net-cationic (lysine and ornithine) or zwitterionic phospholipid, which appears to inhibit binding and cell entry of cationic antibiotics and antimicrobial peptides [2–5].

While investigating the impact of branched fatty acid-containing phospholipids on peptide–bilayer interaction, we made an unexpected observation, which prompted the research presented here. Synthetic lipids with a branched acyl chain composition characteristic of that found in the *S. aureus* cytoplasmic membrane produced lipid vesicles that were unstable and prone to release encapsulated dye even in the absence of a membrane-active peptide. By contrast, lipid vesicles made from purified staphylococcal lipid extracts were stable for days and considerably less susceptible to attack by the cytolytic peptide δ-lysin than those made from the synthetic, branched lipids (Fig. 1). The staphylococcal lipid extract was later shown to contain a significant fraction of lysine-modified PG (Lys-PG), which had co-eluted with unmodified PG during purification. Based on these experimental observations, we hypothesized that the presence of aminoacylated phosholipids is required for the stabilization of PG-rich membranes, especially if these are composed of branched lipids. Improved lipid

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packing and stabilization of the lipid bilayer are likely to also protect the membrane against perturbations by membrane-active peptides. If correct, the degree of protection afforded by the inclusion of aminoacylated PG is likely to also depend on the type of membrane perturbation induced by a peptide and, thus, on the type of dye release from lipid vesicles. Amphipathic peptides can be broadly grouped into two categories according to the type of dye release: those that cause release of content in an all-or-none fashion and those that cause graded release [6–8]. We chose to examine one peptide of each type.

The two peptides used to probe the lipid model systems were Mastoparan X (MasX, graded release) and Cecropin A (CecA, all-or-none release), both of which are extensively characterized cationic antimicrobial peptides [9–11]. The two peptides were tested against lipid vesicles composed of a 70:30 mixture of POPG and 1-palmitoyl-2-oleoyl-phosphatidylcholine. To test the effect of aminoacylated lipids, a fraction of the POPG was replaced by an equivalent amount of aminoacylated lipids. The ester linkage in naturally occurring Lys-PG is susceptible to hydrolysis [12] and commercially available unsaturated Lys-PG is derived from dioleoyl-phosphatidylglycerol (DOPG). To avoid hydrolysis and secondary effects due to the different acyl chain composition [13], we synthesized stable aminoacylated lipids derived from POPE rather than POPG. In order to differentiate between the effect of net lipid charge and headgroup size on peptide–lipid interactions, POPE was derivatized with either lysine or glutamine. Lysylation of PE results in the formation of a net-cationic lipid (Lys-PE); glutaminylation, in a zwitterionic one (Gln-PE). The relative effects of membrane surface charge and lipid headgroup size are differentiated based on the following idea. Inclusion of either Lys-PE or Gln-PE in PG lipid mixtures will reduce the negative surface charge density, but Lys-PE should have a larger effect on peptide–lipid interactions, due to its net positive charge. Moreover, with regard to charge, the effect of Gln-PE on peptide binding and dye release should be comparable to that of additional POPC in the mixtures, since both Gln-PE and POPC are zwitterionic lipids. The headgroup sizes of Lys-PE and Gln-PE are, however, very similar. Thus, if membrane stabilization is simply a consequence of increased headgroup size, Lys-PE and Gln-PE should have a similar effect on peptide–lipid interactions.

We found that membrane binding of both peptides was only significantly impaired if the content of aminoacylated lipid was greater than ~35 mol.%. In the case of MasX, however, the extent of peptide-induced dye release from dye-encapsulated lipid vesicles was drastically reduced in the presence of as little as 10 mol.% Lys-PE. Gln-PE had a much less pronounced effect on dye-release caused by MasX. In the case of cecropin A, the extent of dye-release was an exponential function of the fraction of peptide bound at the membrane–water interface.

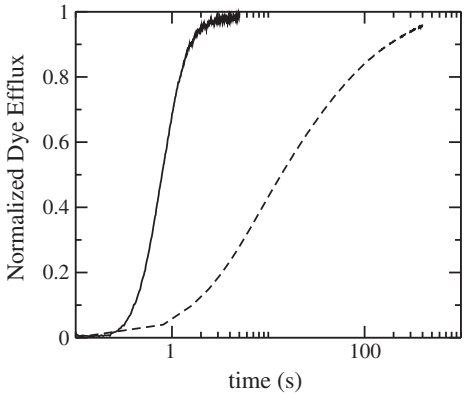

Fig. 1. Carboxyfluorescein efflux from lipid vesicles induced by the cytolytic peptide δ-lysin. The kinetics of dye efflux from vesicles composed of synthetic branched phosphatidylcholine (16:0-ai15:0PC) is faster by a factor of ~20 (solid line) compared with dye efflux from vesicles composed of a *S. aureus* membrane lipid extract (dashed line). Peptide concentration was 0.5 μM and lipid concentration, 200 μM.

These results indicate that the incorporation of physiologically relevant amounts of aminoacylated phospholipids in lipid bilayers leads to a moderate electrostatic repulsion of cationic antimicrobial peptides and a significant stabilization of the lipid bilayer to peptide-induced perturbations. However, the results also suggest that the protective effect of aminoacylated phospholipids depends on the peptide and appears more pronounced for peptides that cause graded release.

2. Methods

2.1. Chemicals

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-cho-line (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phos-pho-(1′-rac-glycerol) (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phos-pho-ethanol-amine (POPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Carboxyfluorescein (99% pure, lot A015252901) was purchased from ACROS (Morris Plains, NJ, USA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phos-pho-ethanol-amine-N-(7-meth-oxy-cou-ma-rin) (7MC-POPE), POPE labeled with 7MC through an amide bond to the amino group of the ethanolamine headgroup, was synthesized in our lab as previously described [14]. Lipids and probes were tested by TLC and used without further purification. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Novabiochem (Darmstadt, Germany), *t*-BOC protected amino acids from CreoSalus (Louisville, KY, USA), analytical thin layer chromatography (TLC) plates from Analtech (Newark, DE, USA), flash grade silica (32–63 μ) from Dynamic Adsorbents (Norcross, GA, USA), and Molecular sieves 3A from Sigma-Aldrich (St. Louis, MO, USA). Organic solvents (high performance liquid chromatography/American Chemical Society grade) were purchased from Burdick & Jackson (Muskegon, MI, USA). BBL Mueller Hinton (MH) Broth was purchased from Becton, Dickinson and Company (Sparks, MD, USA), and celite 545 from Fisher Scientific (Fair Lawn, NJ, USA). Mastoparan X (MasX, Ile-Asn-Trp-Lys-Gly-Ile-Ala-Ala-Met-Ala-Lys-Lys-Leu-Leu-NH_2) and cecropin A (CecA, Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Glu-Lys-Val-Gly-Gln-Asn-Ile-Arg-Asp-Gly-Ile-Ile-Lys-Ala-Gly-Pro-Ala-Val-Ala-Val-Val-Gly-Gln-Ala-Thr-Gln-Ile-Ala-Lys-NH_2) were purchased from American Peptide Co. (Sunnyvale, CA, USA). δ-Lysin was a gift from Dr. Birkbeck, University of Glasgow, UK. Stock solutions of MasX and CecA were prepared by dissolving lyophilized peptide in deionized water/ethyl alcohol, 1:1 (*v*/*v*) (AAPER Alcohol and Chemical Co., Shelbyville, KY). Stock solutions of δ-lysin were prepared in deionized water, pH ~3. Peptide stock solutions (~200 μM) were stored at −80 °C and kept on ice during experiments. Peptide concentration of the stock solutions was determined precisely by measuring the absorbance at 280 nm, and using a molar extinction coefficient of tryptophan of 5600 M^{-1} cm^{-1}.

2.2. Synthesis of aminoacylated phosphatidylethanolamines

Details of the methods used to synthesize aminoacylated phospholipids are described in detail elsewhere [15]. Briefly, equimolar amounts of *t*-BOC protected amino acids, N-α, N-ε-bis(*tert*-butoxycarbonyl)-L-lysine or N-α-*tert*-butoxycarbonyl-l-glutamine and HBTU were combined in DMF to form the activated benzotriazolyl esters of the N-protected amino acids. The activated amino acids were reacted with POPE to form Boc-Gln-POPE or Boc-Lys(Boc)-POPE. The reaction product was purified by flash column chromatography on silica and deprotected by the addition of trifluoroacetic acid. The final product, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(N-glutaminyl)-ethanolamine (Gln-PE) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(N-lysyl)-ethanolamine (Lys-PE), was purified by flash column chromatography on silica gel. The identity of the final product was confirmed by LC–MS and NMR.

2.3. Isolation of staphylococcal membrane lipids

Bacterial cultures were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *S. aureus* subsp. *aureus* Rosenbach ATCC 12600 was cultured in 2 L MH broth for 24 h at 37 °C. Cells were harvested by centrifugation and resuspended in methanol. CH_2Cl_2 was added to the suspension to a final ratio of 1:2 CH_2Cl_2:methanol. The mixture was sonicated for 5 min. in a water bath sonicator and stored for 12–24 h. The suspension was filtered into a round bottom flask through a celite pad in a 60 mL, medium-porosity Büchner funnel. The filtrate was reduced to dryness in a rotary evaporator, dried under high vacuum, and redissolved in 2:1 CH_2Cl_2/methanol. This crude lipid extract was reduced to approximately 2 mL in a rotary evaporator and separated into its constituent lipid classes by flash column chromatography on silica using a CH_2Cl_2/methanol/water gradient (85:15:1, 80:20:2, 70:30:3, 100% methanol). Fractions were analyzed by TLC and phospholipids were visualized with a modified Dittmer-Lester reagent [16,17]. Pure phosphatidylglycerol fractions were combined, reduced to dryness in a rotary evaporator and stored in $CHCl_3$ at −20 °C. Lipid concentrations were determined by the Bartlett phosphate method [18], modified as previously described [19].

2.4. Preparation of large unilamellar vesicles

Large unilamellar vesicles (LUVs) were prepared by mixing the lipids in chloroform in a round-bottom flask. For vesicles containing 7MC-POPE, the probes were added to the lipid in chloroform solution at a final probe concentration of 2 mol%. The solvent was rapidly evaporated using a rotary evaporator (Büchi R-3000, Flawil, Switzerland) at 60 °C. The lipid film was then placed under vacuum for 4 h and hydrated by the addition of buffer containing 20 mM MOPS, pH 7.5, 0.1 mM EGTA, 0.02% NaN_3, and 100 mM KCl or appropriately modified as indicated below. The final concentration of the lipid suspension was approximately 5 mM. The suspension of multilamellar vesicles was subjected to five freeze–thaw cycles and extruded 10× through two stacked polycarbonate filters of 0.1 μm pore size (Nuclepore, Whatman, Florham, NJ, USA), using a water-jacketed high pressure extruder (Lipex Biomembranes, Inc., Vancouver, Canada) at room temperature. Lipid concentrations were assayed as previously described [19].

2.5. Kinetics of peptide binding to and dissociation from lipid vesicles

The kinetics of peptide association with LUVs were recorded on an Applied Photophysics SX.18MV stopped-flow fluorimeter (Leatherhead, Surrey, UK). Fluorescence resonance energy transfer (FRET) between a Trp residue located in the peptide sequence and 7MC-POPE incorporated in the lipid membrane was used to monitor peptide binding and dissociation from LUVs. The Trp residue was excited at 280 nm and transferred energy to 7MC-POPE, which absorbs maximally at 348 nm. The emission from 7MC, with a maximum at 396 nm, was measured using a GG-385 cut-off filter (Edmund Industrial Optics, Barrington, NJ, USA). After mixing, the concentration of peptide was 0.5 μM.

2.6. Peptide-induced dye release from lipid vesicles

LUVs for carboxyfluorescein (CF) efflux kinetics measurements were prepared by hydrating the dried lipid film with CF-containing buffer (20 mM MOPS pH 7.5, 0.1 mM EGTA, and 0.02% NaN_3, 50 mM CF) to give a final lipid concentration of 5 mM. Following extrusion, CF-containing LUVs were passed through a Sephadex-G25 column to separate the dye in the external buffer from the vesicles. The suspension was diluted in buffer to the desired lipid concentration and used for fluorescence measurements. The buffer used was 20 mM MOPS pH 7.5, containing 100 mM KCl, 0.1 mM EGTA, and 0.02% NaN_3, which has the same osmolarity as the CF-containing buffer. The kinetics of carboxyfluorescein efflux were recorded in an Applied Photophysics SX.18MV stopped-flow fluorimeter. CF was excited at 470 nm and the emission was recorded through a GG 530 long-pass filter (Edmund Industrial Optics, Barrington, NJ, USA). The final fraction of CF release was determined by comparison of the fluorescence with that obtained by addition of the detergent Triton-X-100 (1%).

3. Results & discussion

Lipid vesicles constructed from a *S. aureus* PG extract are more resistant to perturbations induced by the cytolytic peptide δ-lysin than those made from synthetic lipids closely resembling those found in the cytoplasmic membrane of *S. aureus* (Fig. 1). We found that lysyl-PG had coeluted with the unmodified PG during purification, as confirmed by a positive ninhydrin reaction. This led us to hypothesize that aminoacylation of the phospholipid headgroup would stabilize lipid bilayers and render them more resistant to attack by membrane-active peptides.

We set out to test this hypothesis by asking two specific questions. Does the inclusion of aminoacylated phospholipids in PG-rich membranes protect lipid vesicles against peptide-induced perturbations? And, if such a protective effect can be confirmed in model lipid systems, is it a consequence of the charge or to the headgroup size of the modified lipids? To address these questions, we measured lipid binding and peptide-induced dye release from lipid vesicles containing Lys-PE, a net cationic lipid, or Gln-PE, a zwitterionic lipid with approximately the same headgroup size as Lys-PE. The results were then compared to those obtained from lipid vesicles in which a fraction of aminoacylated lipid was replaced by the same amount of POPC. Two well characterized antimicrobial peptides, CecA and MasX, were used to probe vesicles containing headgroup-modified lipids. The two peptides were chosen to test if the type of dye release, all-or-none versus graded, had any influence on binding or dye release.

We synthesized two analogs to the naturally-occurring aminoacylated PG, lysyl-POPE (Lys-PE) and glutaminyl-POPE (Gln-PE) (Fig. 2). Linkage of

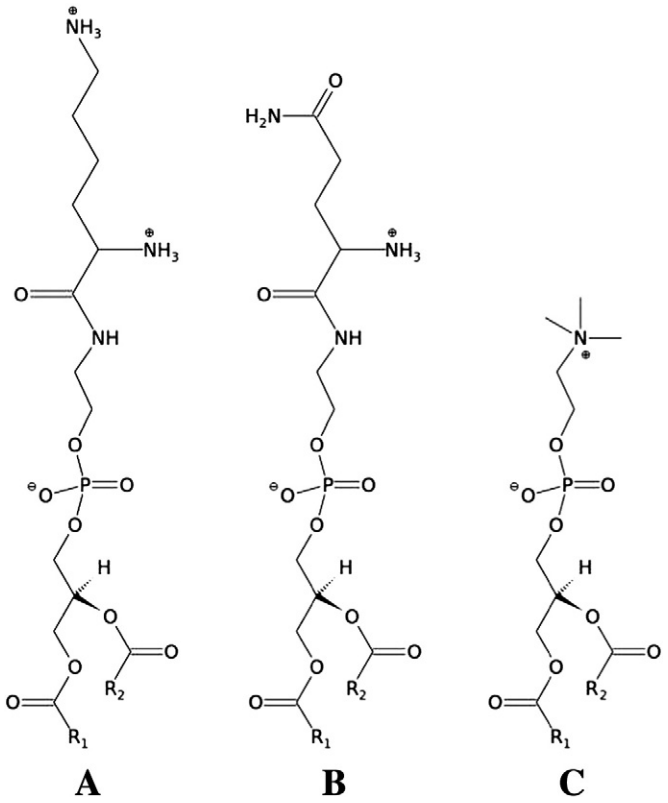

Fig. 2. Structures of aminoacylated lipids and POPC. A, addition of lysine to POPE leads to the formation of Lys-PE, and the addition of glutamine, B, to Gln-PE. C, POPC. R_1, 16:0 and R_2, 18:1.

the lysine or glutamine carboxyl-group to the PE headgroup through an amide bond results in an aminoacylated phospholipid that is very similar in size to the corresponding PG derivatives but stable to hydrolysis in aqueous solution. Increasing the fraction of either Lys-PE or Gln-PE in the lipid mixtures will lower the negative surface charge of the membrane; however, Lys-PE can be expected to do so more efficiently than Gln-PE since it carries a net-positive charge. Moreover, Gln-PE and POPC are both zwitterionic lipids and if charge effects were determining peptide–membrane interactions, the relative effects of POPC and Gln-PE should be similar. With respect to headgroup size, however, Lys-PE and Gln-PE are very similar. Thus, if membrane stabilization is simply a consequence of increased headgroup size, both should affect peptide–lipid interactions in similar ways.

3.1. Membrane binding

Peptide binding was tested in lipid vesicles composed of a base mixture of 70 mol% POPG and 30 mol% POPC. A minimum of 30 mol% POPC was included to minimize vesicle fusion. To test the effect of headgroup-modified lipids on peptide binding, a fraction of the POPG was replaced by an equivalent amount of aminoacylated lipids. Peptide binding to lipid vesicles was measured by the increase in FRET from the intrinsic Trp residue of the peptides to a fluorescent lipid probe, 7MC-POPE, incorporated in the membrane [20]. The time course of peptide binding is characterized by an apparent rate constant, k_{app}, which is a function of the molecular on- and off-rate constants, $k_{app} = k_{on}[V] + k_{off}$, where $[V]$ is the vesicle concentration. The slope and y-intercept of a linear regression to k_{app} as a function of vesicle concentration for each lipid composition yield k_{on} and k_{off} [20,6]. The dissociation constant, K_D, is given by the ratio of k_{off} and k_{on}, $K_D = k_{toff}/k_{on}$.

In the case of CecA, K_D increases in proportion to the fraction of aminoacylated lipid in the mixture, with Lys-PE and POPC having a more pronounced effect than Gln-PE on binding (Fig. 3 A). MasX appears to not be very sensitive to the net charge of the added lipid and binds with an affinity that parallels the fraction of PG remaining (Fig. 3 D). Notice also the ordinate scales.

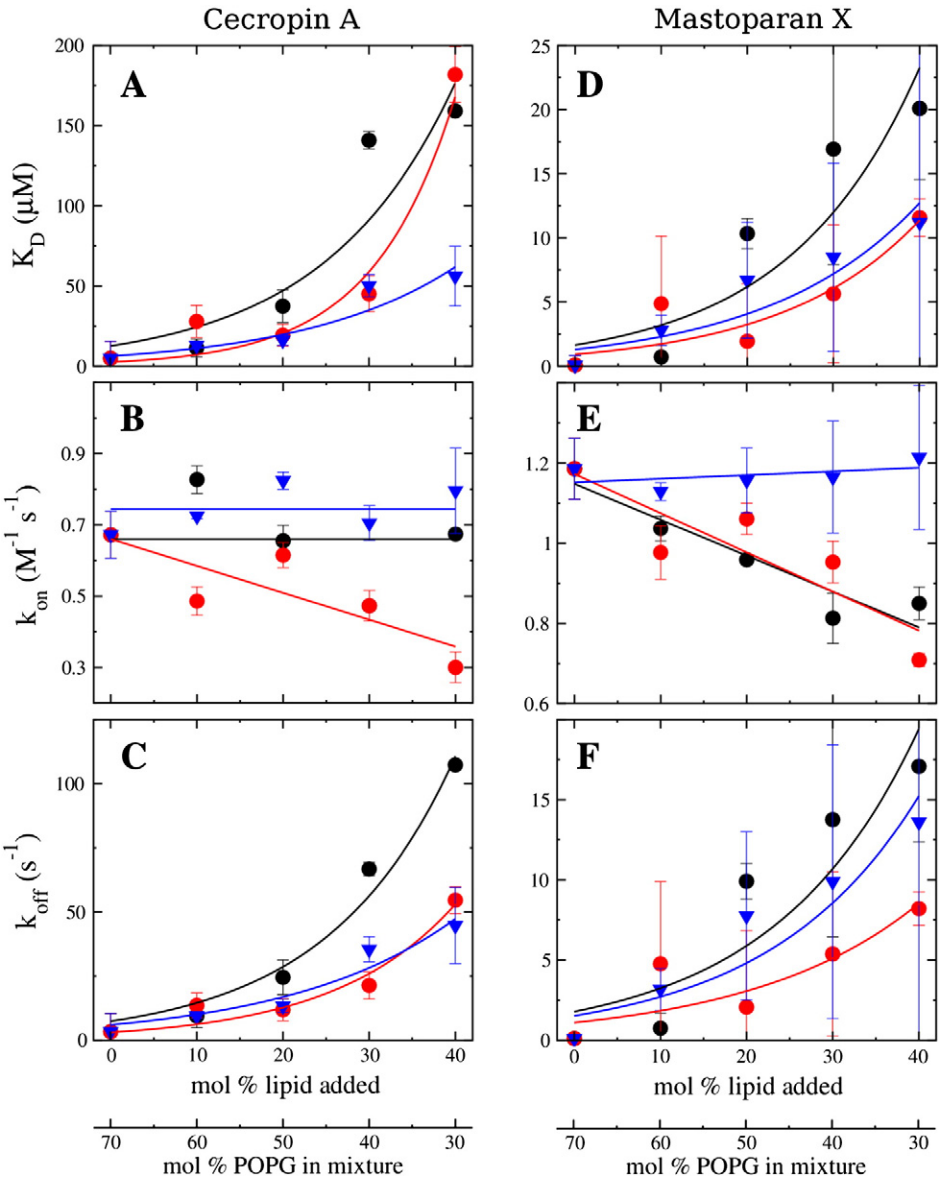

Fig. 3. Dissociation constants and molecular rate constants for binding and desorption as a function of lipid composition for CecA and MasX. A, dissociation constants, K_D, as a function of lipid composition for CecA and D, for MasX. B, rate constants for binding, k_{on}, for CecA, and E, MasX. C, rate constants for desorption, k_{off} for CecA, and F, MasX. The x-axes show the mol% of lipid used to replace POPG in the mixture (upper axis) and the total amount of POPG present in the lipid vesicles (lower axis). Colors: black, POPC; red, Lys-PE; blue, Gln-PE. The error bars represent the standard deviations of 2–4, but usually 3 or more sets of experiments.

A closer look at the rate constants for binding and desorption shows that the effects on K_D are largely due to changes in k_{off}, which increases with the content of aminoacylated lipid or POPC for both CecA and MasX (Fig. 3, C, F). However, the nature of the added lipid, POPC, Lys-PE, or Gln-PE, appears to play a minor role. In general, k_{off} for CecA changes more with the fraction of added lipid than k_{off} for MasX, which we ascribe to the higher net charge on CecA (+7) compared with that of MasX (+4). The effects on k_{on} are smaller, with k_{on} being either not influenced at all or reduced by maximally a factor of 2 (Fig. 3, B andE).

We conclude that, at physiologically relevant levels of aminoacylated lipids (20 mol%), the effect on peptide binding is clearly discernible but moderate for both peptides. Within experimental error, peptide binding occurs in proportion to the fraction of anionic lipid in the membrane and is not strongly dependent on the nature of the lipid that is used to replace PG. A similar conclusion was reached for the binding of RP-1, an antimicrobial peptide modeling the C-terminal microbicidal domain of the mammalian platelet factor-4, to lipid vesicles as a function of lysyl-PG [21].

3.2. Peptide-induced dye release

To obtain information on how aminoacylated lipids influence peptide-induced membrane perturbation, we measured the extent and kinetics of dye release from vesicles as a function of Lys-PE, Gln-PE, and POPC content. We will discuss the results for CecA first. Fractional dye release as a function of time and Lys-PE content are shown in Fig. 4 A, those as a function of Gln-PE content in Fig. 4 B, and those as a function of additional POPC are shown in Fig. 4 C. In all cases, dye release becomes progressively slower as the fraction of POPG decreases in the lipid mixtures, but the effect is much less pronounced for Gln-PE.

This observation may reflect increased resistance of the bilayer to peptide-induced perturbations or simply fewer bound peptides as more POPG is replaced by Lys-PE, Gln-PE, or POPC in the vesicles. To examine the cause for the reduced efflux, we plotted the extent of dye release at 500 s as as a function of the fraction of bound peptide as a measure for efficient membrane permeabilization. The result for Lys-PE, Gln-PE, and additional POPC are shown in Fig. 5. In all cases, the degree of dye release is a function of the fraction of peptide bound to a degree that depends on the identity of the lipid replacing POPG. Lys-PE is most efficient in blocking dye efflux, which is closely followed by POPC. The addition ofGln-PE is the least efficient in inhibiting CecA-induced dye efflux. It is noted that we consistently observed faster and more complete dye release from vesicles that contained 10 mol% Gln-PE than from those containing no Gln-PE (Fig. 4 B).

In general, we expect the extent of dye efflux, E_c, to scale exponentially with the fraction of peptide bound due to the distortion of the lipid matrix at high peptide-to-lipid ratios. If we assume that dye efflux occurs through membrane defects created by bound peptides, E_c should scale with the area covered by bound peptides. At low concentrations, each bound peptide occupies a circular area, A, with a diameter equal to the peptide length. Thus, $A = r^2\pi$, where r is 1/2 the length of a helical peptide. The number of defects, n, is then $n = f_b P_t/L$, where f_b is the fraction of bound peptide, and P_t/L is the ratio of the total peptide to lipid concentration. The total area occupied by peptides is $1 - exp(-An)$, which accounts for the overlap of peptide-occupied areas at high fractions of bound peptide [22]. Thus, $E_c = E_0 exp(A_0(1 - exp(-An)))$, where E_0 is the extent of dye efflux extrapolated to zero bound peptide. A_0 is a constant that reflects the susceptibility of lipid vesicles to peptide-induced dye release as a function of bound peptide for the three added lipid species (Lys-PE, Gln-PE, POPC). This equation describes the experimental data well, as shown by the fits in Fig. 5 (solid lines). The data indicate that for each lipid series, the efficiency of dye release is a function of the fraction of bound peptide. A_0, however, depends on the identity of the lipid used to replace POPG, with Lys-PE being the most effective in inhibiting dye efflux by CecA. The values for E_0 and A_0 obtained from the fits were 0.014 and 30 for Lys-PE, 0.4 and 6.7 for Gln-PE, and 0.09 and 17 for POPC. A_0 for Lys-PE is larger by a factor of 2 than that obtained for POPC, which makes Lys-PE twice as effective in reducing dye release. We attribute this finding to a more efficient reduction of electrostatic headgroup repulsion in PG-rich bilayers by Lys-PE compared to POPC because of its net positive charge.

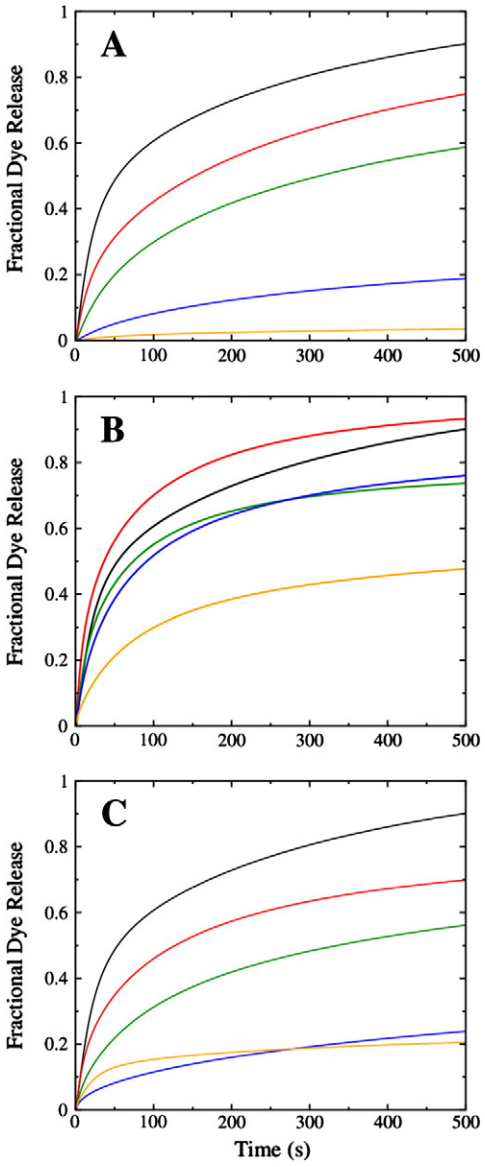

Fig. 4. CecA-induced dye efflux from lipid vesicles at various mol% of Lys-PE, Gln-PE and POPC. A, dye-efflux as a function of Lys-PE content. B, dye efflux as a function of Gln-PE content. C, dye efflux as a function of additional POPC in the lipid mixtures. Colors: black, 70:30 POPG:POPC; red, 60:30:10 POPG:POPC:added lipid; green, 50:30:20 POPG:POPC:added lipid; blue, 40:30:30 POPG:POPC:added lipid; brown, 30:30:40 POPG:POPC:added lipid. With the exception of the kinetics obtained for 30 and 40 mol% Lys-PE, the dye release curves are averages of 2–4, but usually 3 or more independent experiments. Lipid and peptide concentrations were 100 and 0.5 μM, respectively.

Electrostatic repulsion has been shown to destabilize PG-rich bilayers by introducing lateral tension in the lipid headgroup region [23]. If subjected to additional mechanical tension, PG-rich bilayers thus show a substantiallyreduced rupture tension compared to pure PC bilayers [23]. The tension required to rupture vesicles composed of PG:PC mixtures is an approximately linear function of the PG content and dramatically reduced in mixtures that contain ≥40 mol% PG citeshoemaker02. Peptide binding at the bilayer–water interface has been shown to contribute mechanical tension [24] that can give rise to transient pore formation [25]. Mechanical and electrostatic tensions in

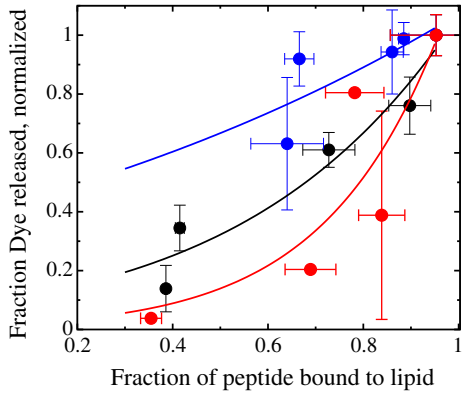

Fig. 5. Extent of dye efflux at 500 s induced by CecA as a function of the fraction of bound peptide. The amount of dye released is normalized to that released from 70:30 POPG:POPC vesicles. The fraction of bound peptide has been calculated from K_D and the lipid concentration. Red, Lys-PE; blue, Gln-PE series; black, POPC series. Solid lines are fits to the data using the equation $E_c = E_0 exp(A_0(1-exp(-\frac{1}{2}An)))$. Values for E_0 and A_0, respectively, obtained from the fits: Lys-PE, 0.014 and 30; Gln-PE, 0.4 and 6.7; POPC, 0.09 and 17. The error bars represent the standard deviations of a minimum of 2–4, but usually 3 or more independent experiments. The concentration of lipid was 100 μM and the total peptide concentration, 0.5 μM.

the lipid headgroup region are additive [23]. Thus, reducing the fraction of anionic lipid in the lipid mixtures used here will have two effects. One, peptide binding is reduced in the presence of aminoacylated lipids, and two, the lipid bilayer is stabilized due to reduced electrostatic repulsion in the lipid headgroup region.

The kinetics and extent of dye release induced by MasX are shown in Fig. 6. Qualitatively, the effects seen with MasX show the same trends as those seen for CecA but there are important quantitative differences. Increasing the fraction of Lys-PE has a dramatic effect on the efficiency of dye release (Fig. 6 A). At a Lys-PE fraction of 20 mol%, which corresponds to the amount of lysylated PG typically found in bacterial membranes, dye-release is essentially abolished. The plot of extent of dye release versus the fraction of lipid-bound peptide in Fig. 7 shows that 95% of MasX is bound to vesicles containing 20 mol% Lys-PE. Thus, weak peptide binding is clearly not responsible for the observed effect. However, POPC has a very similar effect to Lys-PE: increasing the POPC fraction by 10% from 30 to 40 mol% leads to significantly reduced dye release and is comparable to the effect produced by including 10 mol% Lys-PE (Fig. 6 C). MasX-induced dye release as a function of Gln-PE, on the other hand, is markedly different (Fig. 6 B). Inclusion of 10 mol% Gln-PE was again observed to increase the efficiency of dye-release, just as we had observed for CecA. Higher Gln-PE fraction render dye release at 500 s less complete but the initial rate of release is significantly faster than that observed for vesicles containing no Gln-PE at all.

In its membrane-bound, α-helical conformation, MasX is approximately half as long as a fully helical CecA monomer. Thus, if MasX behaved similarly to CecA, MasX-induced dye efflux, E_m, should scale as $E_m = E_0 exp(A_0(1-exp(-\frac{1}{2}An)))$, where the factor $\frac{1}{2}$ accounts for the length of MasX relative to CecA (Fig. 7, dashed lines, using the same values for E_0 and A_0 as for CecA). Clearly, the equation fails to describe the pronounced decrease in the extent of dye release that occurs at low fractions of Lys-PE and additional POPC, where the fraction of bound peptide is still close to 100%. MasX and CecA obviously perturb the lipid bilayer in different ways and the observed manner of dye release, all-or-none in the case of CecA and graded for MasX, are likely a consequence of these different behaviors. It is tempting to speculate that the action of MasX requires a more significant distortion of the lipid matrix, which is critically sensitive to the presence of lipids with large headgroups.

Thus, in contrast to peptide binding, the effect of the headgroup-modified lipids on peptide-induced dye release is significant, but depends on both the identity of the peptide and the nature of the lipid

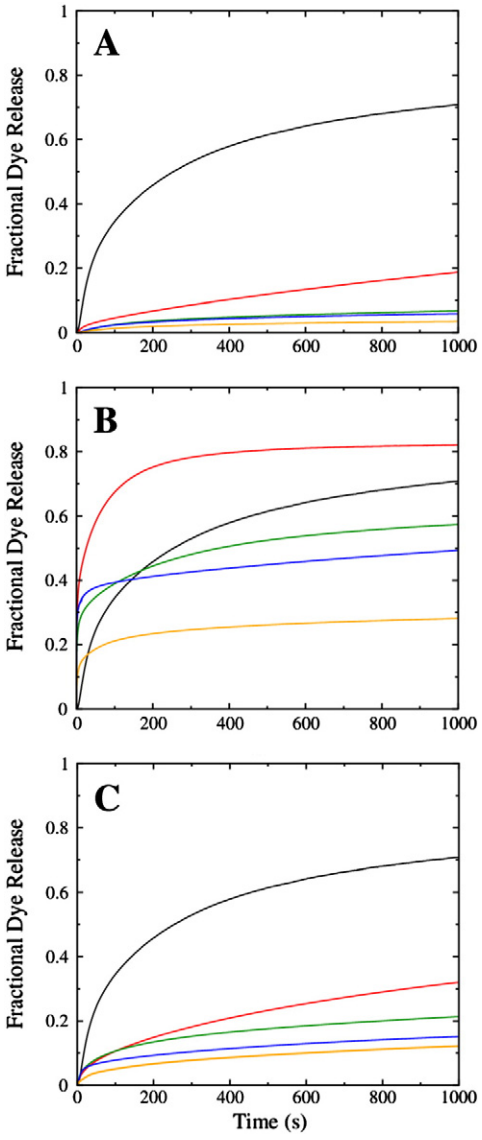

Fig. 6. MasX-induced dye efflux from lipid vesicles at various mol% of Lys-PE, Gln-PE and POPC. Dye-efflux as a function of Lys-PE content, A; dye efflux as a function of Gln-PE content, B; dye efflux as a function of additional POPC in the lipid mixtures, C. 70:30 POPG:POPC, black; 60:30:10 POPG:POPC:added lipid, red; 50:30:20 POPG:POPC:added lipid, green; 40:30:30 POPG:POPC:added lipid, blue; 30:30:40 POPG:POPC:added lipid, light brown. With the exception of the kinetics obtained for 30 and 40 mol% Lys-PE, the dye release curves are averages of a minimum of two, usually three or more independent experiments. Lipid and peptide concentrations were 50 and 0.5 μM, respectively.

headgroup. As little as 10 mol% of Lys-PE effectively protect lipid vesicles against permeabilization by MasX and reduce dye release by more than 90% relative to the vesicles containing no Lys-PE (Fig. 6).

The results also indicate that the protection afforded by Lys-PE against membrane permeabilization by MasX is not unique to Lys-PE since POPC has a comparable effect. RP-1, a peptide modeled on mammalian platelet factor-4 [21], has also been shown to be markedly less effective in lipid vesicles containing lysylated PG. However, in that study, lysylated DOPG, a symmetric lipid, was used in mixtures with POPG. Symmetric, unsaturated lipids have been shown to behave differently from asymmetric lipids in peptide-induced due efflux experiments and are likely to have influenced those results beyond the headgroup modification [13].

In conclusion, the effects of Lys-PE, Gln-PE and POPC in mixtures with POPG result from a reduction in peptide binding, and a general membrane stabilization, presumably due to a lower headgroup repulsion in POPG-rich bilayers. Thus, lysylation is a modification well suited

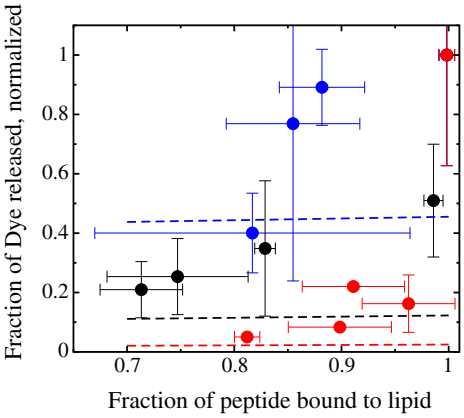

Fig. 7. Extent of dye efflux at 500 s induced by MasX as a function of the fraction of bound peptide. The amount of dye released is normalized to that released from 70:30 POPG:POPC vesicles. The fraction of bound peptide has been calculated from K_D and the lipid concentration. Red, Lys-PE; blue, Gln-PE series; black, POPC series. Dashed lines represent curves calculated from the equation $E_m = E_0 exp(A_0(1-exp(-\frac{1}{2}An)))$. The values for E_0 and A_0 were the same as those obtained from the fits to the data in Fig. 5, but with A adjusted for the length of a MasX monomer. The error bars represent the standard deviations of a minimum of 2–4, but usually 3 or more independent experiments. The concentration of lipid was 50 μM and the total peptide concentration, 0.5 μM.

to stabilize anionic bilayers. Lysylated lipids are, in that sense, a good analog of phosphatidylcholine, which is rare in bacteria and altogether absent in the *staphylococci*. Of the three lipids investigated, Gln-PE is the least effective in preventing peptide-induced dye efflux. The effects of Gln-PE parallel neither those of POPC (same net charge) nor those of Lys-PE (similar size), which indicates that the presence of the the terminal amide group in Gln-PE leads to behavior that is very different from that observed for Lys-PE. Thus, Gln-PE is a poor analog of Lys-PE and size effects alone cannot account for the observed experimental results. The reasons underlying the effects of Gln-PE in POPG-rich bilayers are not entirely clear but poor mixing of Gln-PE with POPG resulting in the formation of POPG-rich and Gln-PE-rich domains would provide a simple explanation for the observed results.

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