The influence of cholesterol on melittin lipidation in neutral membranes

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Abstract:

The effects of cholesterol on the process of intrinsic lipidation, whereby an acyl chain is transferred from a lipid as donor to a membrane-associated acceptor molecule, have been explored using melittin as the acceptor. Membranes comprising lipids with saturated acyl chains (1,2-dipalmitoyl sn-glycero-3- phosphocholine, DPPC; 1,2-dimyristoyl sn-glycero-3-phosphocholine, DMPC) yielded no acyl transfer, whereas membranes composed of lipids with unsaturated acyl chains (1,2-dioleoyl sn-glycero-3- phosphocholine, DOPC; 1-palmitoyl-2-oleoyl sn-glycero-3-phosphocholine, POPC) produced detectable lipidation activity. For all lipids, inclusion of cholesterol led to a significant increase in lipidation activity, with the greatest effect observed for 20 mol% cholesterol in POPC. In the case of membranes composed of POPC, the inclusion of cholesterol also produced small changes in the selectivity for transfer from the sn-1 vs. sn-2 positions of the lipid. Qualitatively, for fluid membranes, the trend in lipidation activity exhibits a positive correlation with the bending modulus of the bilayer and is accounted for in terms of the penetration depth of the peptide. Access of water to reactive intermediates also has the potential to influence lipidation rates.

Introduction:

The activity of peptides in membranes has been the subject of intense study over a number of years. Melittin (H-GIGAVLKVLT TGLPALISWIKRKRQQ-NH2), a 23-residue peptide from the venom of the bee Apis mellifera, 1 is one of the most studied. In aqueous solution, melittin, with three lysine and two arginine residues and no acidic residues, has a net positive charge and adopts a random coil conformation at low concentrations, forming helical tetramers at higher concentrations (typically 4104 M in 100 mM NaCl at pH 7).2 Tetramer formation is favoured by increasing pH and ionic strength.3–5 The X-ray crystal structure of melittin is characterised by the presence of two helical stretches either side of the central proline (Pro17).6 Following binding to membranes, the peptide similarly adopts a largely helical structure, although the degree of helicity is lower than that in the crystal form.6,7 The helical structure of melittin has a high degree of amphiphilicity,8,9 which, together with its cationic nature, accounts for its ability to bind to both neutral phosphocholine (PC) membranes and membranes with a negative electrostatic potential. Binding to the latter generally occurs with higher affinity than to neutral membranes.10,11 There is some evidence that above a critical peptide to lipid ratio (P : L*), melittin exists in equilibrium between a surface bound (S) orientation with the helix axis parallel to the membrane surface and an inserted (I) orientation with the helix parallel to the membrane normal. It is proposed that the I state constitutes pores that are toroidal in nature,12,13 with P : L* varying in the range to 1/30, with values of 1/62 and 1/99 for POPC and DOPC respectively,14 although the onset of reorientation in DOPC has also been noted at the higher P : L of 1/24.7 From a range of studies, it is apparent that melittin alignment in membranes is sensitive to membrane properties and experimental conditions such as temperature, with surface-only15 and partial insertion also being noted.16

It has recently become apparent that a number of peptides exhibit reactivity with membrane lipids. These reactive peptides undergo an intrinsic lipidation process involving acyl transfer from the lipid to the peptide (Scheme 1).17–19 Lipidation sites include amino groups (N-

terminus and Lys), hydroxyl groups (Ser) and imidazolyl groups (His) and is favoured when these sites are close to the hydrophobic surface of amphiphilic structures. Of the peptides investigated to date, melittin has been found to be the most reactive in this regard.18 Reactivity has been detected at peptide to lipid ratios above and below P : L* in both pure neutral PC membranes and mixtures of PCs with PG, PE or PS. In general, the intrinsic lipidation activity of melittin is favoured in membranes with a negative surface potential (PC/PS or PC/PG), possibly as a consequence of a higher melittin binding affinity for these membranes. The acyl transfer process has been found to exhibit little selectivity for the sn-1 vs. sn-2 position of the lipid, and occurs from both lipid components in binary mixtures. Selectivity is observed, however, for the reactive sites on the peptide, with lipidation favoured at the N-terminal amino group 4 K23 amino group 4 K7 amino group c S8 hydroxyl group B K21 amino group.17,18 Inclusion of PE into binary PE/PC membrane mixtures was also found to yield higher intrinsic lipidation activity than single component PC membranes, indicating a potential role for the intrinsic curvature of the membrane in influencing activity. On the basis of these observations we hypothesised that the rate and selectivity of intrinsic lipidation would depend on the membrane binding affinity of melittin and the disposition of the peptide in the membrane-associated state. These two parameters will in turn be influenced by the membrane packing density (area per lipid), and the area compressibility (KA) and bending (kc) elastic moduli. In order to test this hypothesis, in the work reported here, experiments have been conducted at physiological temperature on single component membranes in fluid phases composed of 1,2-dioleoyl sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl sn-glycero-3-phosphocholine (POPC), or 1,2- dimyristoyl sn-glycero-3-phosphocholine (DMPC) and gel phases composed of 1,2-dipalmitoyl sn-glycero-3-phosphocholine (DPPC). This work is extended to 2- and 3-component mixtures composed of these lipids with cholesterol (chol) that exhibit liquid-ordered (Lo) phases (for 2-component PC/chol mixtures) or a combination of Lo and liquid disordered (Ld) phases (for 3-component PC/chol mixtures). The effect of membrane curvature is also probed by incubating peptides with small unilamellar vesicles (SUVs).

Methods

2.1 Materials

Melittin (synthetic, Alexis brand) was purchased from Enzo Life Sciences, Exeter, UK. Lipids were purchased as dry powders from Avanti Polar Lipids via Instruchemie B.V., The Netherlands. All other materials were obtained from SigmaAldrich, Dorset, UK. A stock solution of melittin in water was prepared at an approximate concentration 1 mg ml1 . Multiple (at least 4) 5 ml aliquots of the peptide stock solution were added to water (1 ml) and the absorbance measured following each addition. Absorbance measurements were between 200 nm and 400 nm at 21 1C using a CARY100 UV-visible spectrophotometer with Cary WinUV Scan Software 3.00(182). Measurements were made in a quartz cuvette (1 mm path length). After correction for dilution, the melittin concentration was calculated using the Beer–Lambert law with a calculated extinction coefficient at 280 nm of 5570 M1 cm1. 17 Concentration measurements were made in triplicate. The stock melittin solution was portioned in to 20 ml aliquots that were stored at 20 1C. Once thawed, any aliquot was used once before disposal and not refrozen. Stock solutions of phospholipid in CHCl3 were prepared at a concentration of 10 mg ml1 and stored at 20 1C. A stock solution of cholesterol at 10 mg ml1 in CHCl3 was stored at 20 1C.

2.2 Liposome preparation

Phospholipid and cholesterol stock solutions in chloroform (100 ml) were added to a round bottomed flask in the appropriate ratio, and the solvent removed in vacuo to form a lipid film. Following desiccation overnight in vacuo, the phospholipid film was hydrated with buffer solution (90 mM NaCl, 10 mM NaHCO3, pH 7.4) and agitated using a vortex mixer. For extruded large unilamellar vesicles (LUVs), the dispersion was subjected to 5 freeze–thaw cycles between 196 1C and 30 1C before being extruded 10 through a 100 nm laser-etched polycarbonate filter (Whatman Nucleopore) at 50 1C using a Lipext thermobarrel extruder (Northern Lipids, Burnaby, Canada) under a positive pressure of nitrogen. For the preparation of small unilamellar vesicles (SUVs), the dispersion was subjected to sonication using a Soniprep 150 sonication probe (MSE, Lower Sydenham, UK) at 15 micron amplitude placed in the dispersion for 25 minutes.

2.3 Lipidation experiments

A portion of the stock melittin solution (at a typical concentration of B0.2 mM) was transferred by pipette into a 0.5 ml Eppendorf tube along with sufficient freshly prepared liposome dispersion to give a molar peptide : lipid ratio of 1 : 10 and a final lipid concentration of 0.26 mM after making the mixture up to 100 ml with buffer (90 mM NaCl and 10 mM NaHCO3 in water at pH 7.4). The mixture was agitated to ensure homogeneity before incubation at either 37 1C in a heating block or room temperature (21 1C).

2.4 Sample analysis

A sample of the reaction mixture (10 ml) was removed and diluted with water in a 1 : 10 ratio. 5 ml of this solution was then injected onto an Xbridge C18 3.5 mm 2.1 100 mm column (Waters Ltd, UK) for separation by reverse phase liquid chromatography. A gradient of 0.1% formic acid in water and 0.1% formic acid in MeCN was run at 0.2 ml/minute over 20 minutes. Electrospray ionisation (ESI) was used in positive mode, and the mass analyser used was a LQT-FT (Thermo-Finnigan, UK). The following positive ion electrospray setting were used: the nitrogen sheath gas was kept between 8–10 arbitrary units, the auxiliary gas and sweep gas were set between 2–4 arbitrary units, as per the manufacturer's software, the capillary was heated to 250 1C and the spray voltage was held at 4.0 kV. The tube lens voltage was varied to deliver the optimal ion intensity. All full scan MS data were measured in the Fourier transform-ion cyclotron resonance mass analyser surrounded by a 7 Tesla superconducting magnet. MSMS was carried out on the same equipment and conditions, using CID for ion activation and running alternating MS and MSMS scans. Collision-induced dissociation (CID) experiments were performed entirely within the linear ion trap with a fixed isolation window of 4 m/z, using helium as a collision gas and an optimised normalised collision energy level of 25%. MS Data were recorded using the acquisition software Xcalibur version 2.0.7 (Thermo-Finnigan Corp, Bremen, Germany) and processed using the embedded program Qual Browser. Further processing and preparation of figures was done using the XCMS package20 in the R Statistical Programming Environment.21 Assignment of the lipidation sites has been described previously.17,18

Measurements of particle size distribution were carried out using a Malvern Zetasizer mV Version 6.34. LUVs or SUVs in water (50 ml) were added to a Sarstedt ZEN0117 100 ml disposable cuvette, and 5 runs of 10 second duration were carried out to obtain a size distribution. Data were processed using the instrument software.

3. Results

3.1 Lipidation of melittin in DPPC and DPPC/chol membranes

Membranes composed of DPPC, with a gel-to-liquid crystal phase transition at 41.4 1C,22 are in the rippled gel phase at the temperature used for this work (37 1C), whereas mixtures of chol with DPPC at this temperature yield pure Lo phases at chol concentrations 420 mol% and mixed phases at 5–10 mol% chol.23–25 Incubation of melittin with DPPC and DPPC/chol membranes therefore enables the effects on the lipidation process of membranes presented in a relatively condensed phase (relative to the La phase) to be evaluated. Incubation of melittin with DPPC membranes yielded no evidence of lipidated peptide formation (Fig. 1). An additional experiment was conducted using DMPC LUVs in order to attempt to separate the effects of chain saturation and lipid phase on lipidation activity. DMPC, like DPPC is a fully saturated lipid, but unlike DPPC is in the fluid La phase at 37 1C22 and may therefore be expected to bind melittin more favourably, increasing the probability of reaction. It is striking therefore, that no evidence for reactivity with DMPC could be obtained after 24 h (Fig. S1). Inclusion of 20 mol% chol into the DPPC membrane led to significantly increased levels of lipidation activity after 24 h, with approximately 30% of the total peptide peak area accounted for by the lipidated peptide. When compared with earlier studies in fluid membranes that have used predominantly POPC or DOPC as the major PC component,17,18 a marked shift in selectivity in favour of lipidation at K21 was notable in all cases involving DPPC/chol mixtures. Increasing the cholesterol content further to 50 mol% led to a decrease in activity relative to 20 mol% chol/DPPC. Previous work to address the effects of melittin on the morphology and integrity of membranes composed of DPPC and DPPC/chol have found that in the case of DPPC, addition of melittin to pre-formed membranes at temperatures oTm has little effect on morphology. However, heating the sample to temperatures above Tm and then cooling to temperatures below Tm promotes the formation of discoidal particles of diameter 20–50 nm and a thickness that corresponds to a single bilayer. These particles are stabilised by the peptide and co-exist with large vesicles that are formed by their fusion.26–28 In our case, as with direct addition of melittin to DPPC liposomes, such a temperature cycling treatment did not yield any evidence of lipidation activity (Fig. S2b). The addition of melittin to DPPC in the form of SUVs also failed to yield lipidation (Fig. S2c).

Addition of melittin to DPPC membranes containing 30 mol% cholesterol has also been found to yield the formation of discoidal particles,29 in this case without the large vesicles generated by membrane fusion. However, the extent of particle formation is lower than that for melittin addition to pure DPPC, although the particles have a higher stability due to the incorporation of cholesterol.28 At lower cholesterol levels, intermediate behaviour is observed, with discoidal particles below Tm and large vesicles above Tm, but with the transition between then occurring over a broader temperature range.29 Our data illustrate a significant increase in lipidation activity in DPPC/chol membranes at moderate cholesterol levels (20 mol%) which then reduces as the cholesterol content is increased further. If melittin is more reactive in the discoidal particle form of the melittin–lipid adduct, increased reactivity would be expected at higher cholesterol levels. Therefore we may conclude that the predominant reactive form is not found in the discoidal particle. As the affinity of melittin for DPPC/cholesterol membranes is lower than for DPPC,30 the lipidation data also cannot simply be explained by the relative affinity of the peptide for both types of membrane. The phase diagram for DPPC/chol indicates that at the temperature of the experiments, the system is close to the phase boundary between liquid ordered and gel phases. Indeed, phase co-existence has been noted at similar temperature and composition (42 1C and 20% cholesterol).29 It may be hypothesised that in such a biphasic

mixture, the presence of defects is more likely and may therefore promote melittin binding in an orientation favourable for lipidation.

3.2 Lipidation of melittin in DOPC and DOPC/chol membranes

Melittin lipidation was found to occur in DOPC membranes (Fig. 2) in the absence of cholesterol, in contrast to the observations for DPPC. After 24 h, the extent of conversion in DOPC membranes was comparable to that of DPPC/chol, 4 : 1. Inclusion of cholesterol into DOPC membranes led to an increase in lipidation activity. This effect was apparent at 20 mol% cholesterol and exhibited saturation behaviour, with no significant increase in net activity at higher cholesterol levels. Higher cholesterol levels, however, did induce small changes in product distribution, with the proportion of acylation at Lys23 increasing. It was also notable that the distribution between acylation at the N-terminus and the side chain of Lys23 at cholesterol levels r25 mol% cholesterol was similar to that observed previously in other membrane mixtures,17,18 with acylation at the N-terminus significantly favoured.

Melittin is known to bind to DOPC bilayers with a greater affinity than DPPC bilayers. This has been attributed to the greater area per lipid and lower area compressibility modulus of DOPC bilayers.31 A simple change in binding affinity, however, does not account for the increased lipidation activity in mixtures of DOPC with cholesterol, to which melittin associates with reduced affinity compared to DOPC.32

3.3 Addition of melittin to DOPC/DPPC/chol membranes

Given that both DOPC/chol and DPPC/chol mixtures yielded significant lipidation, it was of some interest to determine the lipidation activity of melittin in membranes composed of the ternary mixture DOPC/DPPC/chol. When mixed in the molar ratio 1 : 1 : 1, this mixture exists as a single phase at 37 1C and exhibits liquid–liquid phase separation below B30 1C to form liquid-ordered (Lo) and liquid-disordered (Ld) phases.33,34 On the basis of literature precedents from a 1 : 3 : 1 ternary mixture35 and other ternary mixtures,36,37 we anticipated that at room temperature at a P : L of 1 : 10, melittin would preferentially loc in the Ld domains and induce coalescence of these domains followed by budding of peptide- and DOPC-rich particles, leading to a subsequent preference for oleoyl transfer to melittin.

At 37 1C, due to the homogeneous lipid mixing there would be less selectivity for the lipid from which the acyl group is transferred. In fact, to our surprise addition of melittin to these membranes did not lead to any detectable lipidated products at either temperature (Fig. S3). In the case of the higher temperature incubation, the signal intensity for unreacted melittin was significantly lower than other samples, raising the possibility that some material had been lost as an insoluble precipitate. Such as loss would not be unreasonable given the high levels of perturbation induced by melittin in other ternary mixtures.36,37 Potential explanations for a lack of reactivity in these systems include a different membrane penetration depth in ternary mixtures and changes in the equilibria between surface bound and inserted forms of the peptide.

3.4 Lipidation of melittin in POPC and POPC/chol membranes

The intrinisic lipidation activity of melittin in POPC and POPC/ chol membranes was greater than either of the DPPC or DOPC membranes with comparable cholesterol content (Fig. 3). The activity in POPC containing 20 mol% cholesterol is particularly striking, as greater than 50% of the peptide is consumed within 24 h of addition to these membranes. In contrast to

DOPC/ cholesterol mixtures, the lipidation activity in POPC/cholesterol mixtures at 50 mol% cholesterol is lower than that at 20 mol%. The product distribution between lipidation at Lys23 and the N-terminus is influenced by the cholesterol content of the membrane, changing from predominant lipidation at the N-terminus in the absence of cholesterol to predominant lipidation at Lys23 at 50 mol% cholesterol. The phase diagram for POPC/cholesterol gives a possible explanation for the high activity in membranes containing 20 mol% cholesterol. At the temperature of these experiments, this membrane composition lies close to a phase boundary between La and La + Lo phase coexistence, whereas POPC + 50 mol% cholesterol lies close to the boundary between Lo and La + Lo phase coexistence.22,38 As with DPPC + 20 mol% cholesterol, the proximity to the phase boundary may result in the presence of bilayer defects to which melittin is able to associate in an orientation favourable for lipidation. However, this still does not resolve the question of why lipidation is more favourable in POPC/chol membranes than either DOPC/chol or DPPC/chol.

4. Discussion

The collected data for all of the lipid systems investigated in this work are summarised in Table 1. Melittin has been shown to have a binding affinity for membranes that is sensitive to temperature, ionic strength and the composition of the membrane. Generally, with neutral membranes, the water– membrane partition coefficient for melittin increases with increasing ionic strength of the medium and decreases with increasing temperature.39 In terms of lipid composition, partition coefficients in the fluid phase (above Tm) decrease in the order DPPC 4 DMPC c DOPC 4 POPC 4 DOPC/chol 4 POPC/chol, with values ranging from Kp = 105 M1 for DPPC to Kp = 103 M1 (at 50 1C) for POPC/chol.32,39 It is clear by examination of the data in Fig. 2–4 and Table 1 that there is no simple correlation between lipidation activity and membrane binding affinity.

Properties of lipid membranes that do have the potential to influence lipidation activity include fluidity (in terms of diffusion coefficients), packing density (area per lipid), and the arcompressibility (KA) and bending (kc) elastic moduli. The latter two will affect both the overall membrane affinity of melittin and the conformational behaviour of the membrane associated form of the peptide. Of these parameters, bending elastic moduli are especially useful as data are available for a broader range of systems. Furthermore, according to classical elasticity theory kc and KA are related parameters, with kc/KA varying quadratically with hydrocarbon thickness, dH (kc/KA = dH 2 /24)40 although this relationship is not as robust for membranes containing cholesterol.41

Interestingly, on scrutinising the lipidation data in the context of published kc data for peptide-free membranes, some correlation is apparent between the two (Fig. 4). This correlation with kc comes with some caveats. Many of the kc data available in the literature for model systems are not obtained at ionic strengths comparable to those used for our work. Generally, increasing sodium chloride concentrations ‘soften’ POPC bilayers,46 and melittin association to SOPC membranes has also been shown to induce a modest softening.47 In our conditions, which are conducted at modest salt levels and ratios of bound peptide to lipid (Pb/L) in the region 0.004–0.08, the kc values for our model systems are likely to be lower than those used for Fig. 4 by factors of between B0.95 (Pb/L = 0.004) and B0.2 (Pb/L = 0.08). In addition, few experiments to determine kc have been conducted at the precise temperature of our experiments. Although little variation of kc is expected for single component lipids well above Tm, 41,48 it is unclear whether the same is true in the presence of cholesterol. Finally, published kc data for equivalent lipid systems often reveal considerable variation according to the method used. We have

therefore used combined data obtained by X-ray scattering methods only to give the widest coverage by a single method of the membrane compositions that we have used. A full table of the values used is presented in the ESI (Table 2). Notwithstanding these limitations, as our goal was not to obtain a quantitative predictive model, but rather a qualitative understanding of factors that affect lipidation activity, correlations between kc and lipidation activity still have value. Fig. 4 shows that there does appear to be some correlation between kc and intrinsic lipidation activity for these systems. What is surprising is that this is a positive correlation, i.e. lipidation is promoted in membranes for which more energy is required to produce membrane deformations.

At present, it is only possible to speculate as to whether there are underlying reasons for this trend. In considering reactivity, both the membrane disposition of melittin and local structural effects in the bilayer have the potential to affect reactivity. It is logical that for intrinsic lipidation to occur, in accordance with collision theory,49 the reactive group on the peptide and the carbonyl group of the lipid must approach each other in a conformation favourable for reaction. Furthermore, amines will only be reactive in the neutral amino form, with the consequence that the pKa of the corresponding ammonium form will influence reactivity. It is no surprise that melittin lipidation occurs extensively at the N-terminal amino group, as experiments to determine the pKa of this group by 15N NMR in 1-myristoyl-2-lyso-PC micelles give a value of 7.9,50 with the consequence that a significant proportion of this group will be in the amino form at pH 7.4. Measured pKa values for the ammonium forms the Lys side chains range from 8.5 to 10.2.2,51,52

Under our conditions of pH, ionic strength and temperature, melittin is expected to be monomeric in aqueous solution and close to or above P : L* when membrane-associated. For practical (chromatographic) reasons, a value for the total peptide to lipid ratio (P : L) of 1 : 10 was used for all experiments. At the absolute peptide and lipid concentrations used and considering available water–membrane partition coefficient (Kp) data,39 the highest experimental value for Pb/L was predicted to be 0.08 for DMPC. At this Pb/L a significant proportion of melittin bound to DMPC LUVs was expected to be in the I state. The absence of reactivity in DMPC LUVs may therefore reflect a low reactivity of the I state. However, a consequence of this assertion would be that lipidation activity across all systems would correlate positively with Kp below P : L* and be relatively invariant above P : L*, which is not the case. Added to this, previous studies have shown that lipidation activity is essentially the same at Pt : L =1 : 100 (with the corresponding Pb/L = 0.001, below P : L*) and Pt : L =1 : 10 following melittin addition to POPC membranes,18 suggesting that either the I and S states have similar reactivity, or that their interconversion is not rate limiting. For the purposes of the rest of this discussion, the reactive species in the membrane is assumed to be the monomer in the S state. Below P : L*, monomeric membrane-associated melittin is predominantly helical, with the helix axis aligned in the plane of the membrane.7,8,53 The last four residues at the C-terminus (KRQQ), however, are less structured8,54 and the C-terminal section as a whole has greater plasticity,55 but nevertheless this section of the peptide still appears to associate in the plane of the membrane.7 The depth of penetration of monomeric melittin has been established in some detail by X-ray diffraction to be in the interfacial region, with centre of the scattering profile for melittin almost exactly corresponding to that of the glycerol part of the lipid.7 This, then, places melittin in an appropriate interfacial location to favour the lipidation process. However, subtle changes in binding are likely to influence the process. On the basis of our data, it is reasonable to suggest that the disposition of melittin in rigid membranes is more suitable for intrinsic lipidation than more fluid membranes. As deep penetration of melittin into the membrane will generate significant curvature strain, the depth of melittin binding is likely

to be shallower in rigid membranes. Support for a reduced penetration of the membrane by melittin in the presence of cholesterol comes from kinetics studies in which single component membranes yield data that are best modelled as two sequential processes. The first process is rapid and corresponds to formation of about 65% of the secondary structure formation and 30–50% of the total hypsochromic shift in the tryptophan fluorescence emission wavelength. The second process is slower and is absent for melittin association with cholesterol containing membranes, which can therefore be modelled as a single exponential process.56 Furthermore, quenching of melittin tryptophan fluorescence by quenchers located in the hydrocarbon core of the membrane is reduced when cholesterol is included in the membrane, consistent with a melittin penetration depth that is further from the bilayer center than the corresponding membranes without cholesterol,16 although some of this extra distance can be accounted for by increases in hydrocarbon thickness in the presence of cholesterol combined with errors in the parallax method for modelling the quenching data.57 Together, these binding data support an assertion that high intrinsic lipidation activity correlates with a reduced depth of penetration by melittin into the membrane interface in two-component membranes containing cholesterol.

The study of melittin analogues labelled at the N-terminus or Lys7 with an NBD group provides evidence that these residues experience different membrane environments. In DOPC membranes, the N-terminal NBD group experiences no red edge excitation shift (REES), whilst the Lys7 NBD group experiences a significant shift. The inclusion of cholesterol produces REES effects at both positions, but the effect is still greater for Lys7.16 Analysis of the penetration depth of the label at each position using fluorescence quenching suggests that the N-terminal NBD group is closer to the bilayer center in DOPC than that of Lys7, but this situation is reversed in the presence of 20 mol% cholesterol. These analyses rely on an assumption that the REES effects result from changes in water penetration into the interface, an assumption that has been challenged.58 Regardless of the origin of the effect, the data point to a change in orientation for the N-terminal section of the peptide in the presence of cholesterol. Higher lipidation activity appears to correlate with a shallower penetration depth for the N-terminal section of the peptide. Considering the sensitivity of the fluorescence of both Trp19 and labels in the N-terminal region of the peptide to the presence of cholesterol, it is apparent that the membrane dispositions of both sections of the peptide are influenced the presence of cholesterol. A generally shallower penetration depth in the presence of cholesterol is consistent with a general increase in lipidation activity. A change in disposition of the N-terminal helix would support the observed shift in the selectivity in favour of lipidation at Lys23, most notable for POPC + 20 mol% cholesterol.

Although it is clear that membrane thinning is induced by melittin association with single component membranes,59 it is harder to establish whether this thinning also occurs when cholesterol is present in the membrane. However, studies on other amphiphilic peptides, such as FP-2360,61 and the CRAC motif of gp41,62 indicate that changes in kc induced by peptide binding to cholesterol rich membranes are comparable in scale to those of cholesterol-free membranes. Membrane thinning effects in these peptide systems are less pronounced, in both absolute and relative terms, for bilayers containing cholesterol. It is therefore possible that for membranes containing cholesterol, for which thinning effects are less pronounced but the changes in kc are similar to those of cholesterol-free membranes, the depth of penetration into the bilayer is significantly influenced by the reduction in curvature modulus. The relationship between kc and the depth of penetration has been considered theoretically and illustrates that kc is not expected to be invariant as a function of depth, but instead to increase as the penetration depth approaches the pivotal plane.63 It is therefore reasonable to suggest that correlation between lipidation activity and curvature modulus reflects a shallower melittin

penetration depth in stiffer membranes, accounting for a correlation with kc. At the upper extreme, such as with DPPC in the gel phase, penetration depth may well be sufficiently low that there is no reactivity.

Interestingly, X-ray diffraction data for a ternary mixture of DOPC/DSPC/chol (0.42 : 0.37 : 0.21) have revealed kc values of $4.4 \pm 1 \times 10^{20}$ J and $12 \pm 2 \times 10^{20}$ J respectively for the Ld and Lo phases.64 The lack of observed lipidation in the DOPC/DPPC/ chol mixture at room temperature may therefore be accounted for by a low kc of the Ld phase, into which the peptide is expected to preferentially localize.

Other considerations

Given that melittin is located in the membrane proximal to the lipid carbonyl and glycerol regions of the interface in DOPC bilayers,7 minor adjustments of average position in response to changes in membrane rigidity can account for increased reaction rate, but only if the rate determining step for lipidation is the formation of an encounter complex between the peptide and the lipid, or if a shallower location facilitates the breakdown of key reaction intermediates. Aminolysis of esters has been well studied and for alkyl esters in solution the rate determining step is breakdown of the initially formed tetrahedral intermediate T± (Fig. 5) via either the neutral intermediate T0 at high pH (with rate k4), or the formation of the negatively charged intermediate T in a second order process (with rate k2[amine]).65 In the case of a peptide such as melittin adsorbed in the membrane interface, the formation of T± as the rate limiting step can be ruled out easily, as the lipidation rate is faster in membranes containing cholesterol, for which lateral diffusion rates are slower than cholesterol-free membranes.66 During melittin lipidation at pH 7.4, the breakdown of T± to T- could in principle occur via either route. Both the formation of T0 from T± and the formation of T- from T0 are solvent mediated processes and therefore should be influenced by changes in interfacial water activity. The direct formation of T from T± involving general base catalysis by the amine is feasible given the relatively low pKa of the N-terminal ammonium discussed above. As the observed rate of lipidation does not change systematically with membrane affinity, and by inference with the concentration of amine, the solvent mediated pathway via T0 appears to predominate.

ESR studies using spin labeled amphiphiles67,68 and small angle neutron scattering measurements69 indicate that cholesterol facilitates water penetration into the interfacial region of the bilayer, to the depth of the carbonyl groups. The quenching of melittin (Trp19) fluorescence by water soluble quenchers when cholesterol is added to PC membranes also suggests increased solvent and solute access to the interface.16 The lipidation intermediates themselves may induce localised perturbations to the structure of the bilayer to enhance water penetration of the interface; however, as these intermediates are transient, their effects are hard to characterize. Changes to bilayer properties following the incorporation of monomeric melittin into DOPC liposomes are modest7 and unlikely to significantly affect interfacial water activity. High salt concentrations would be expected to change interfacial water activity and modify peptide–membrane binding interactions.70 Indeed, previous work with melittin has found a small increase in lipidation in neutral membranes at high salt,18 although the magnitudes of these salt effects are small compared to those of cholesterol inclusion. Overall, water accessibility to the zwitterionic intermediate T± is likely to be a significant factor in controlling reaction rate.

Other possibilities for catalysis, such as electrophilic catalysis involving the choline ammonium group, are likely to be insignificant.71 Bifunctional anions, such as bicarbonate

and phosphate, can potentially act as catalysts for the process by facilitating simultaneous proton removal from, and donation to, the neutral intermediate T± to form T0 . 72 Bicarbonate was used as a buffer in our analyses due to its volatility during sample analysis by mass spectrometry. Previous work, however, has shown that the effect of bicarbonate on reaction progress with POPC membranes is negligible.18

DPPC did not yield evidence of lipidation. DPPC was the only composition used where the bilayer was in the gel phase at 37 1C, under which conditions the water–lipid partition coefficient is significantly lower than the fluid phase26 and is likely to significantly hinder both penetration of the peptide and water to the depth of the carbonyl groups.

Overall, the rate enhancements to melittin lipidation observed in the presence of cholesterol are best accounted for by increased penetration of water into the bilayer interface, which facilitates conversion of the lipidation intermediates into products. This penetration arises due to the inherent solvation properties of bilayers containing cholesterol combined with a slightly more superficial location of the peptide. Changes to the preferred site of lipidation occur as a consequence of changes to the interfacial disposition of the peptide, with concomitant modification of the solvation of the corresponding intermediates and their rate of conversion to products.

5. Conclusions

We have shown that the rate of intrinsic lipidation of melittin is influenced by the composition of neutral bilayers composed of diacyl phosphatidylcholines and cholesterol. Lipidation activity correlates with the curvature modulus of the membrane and is promoted in more rigid membranes. This has been accounted for in terms of the depth of penetration of the peptide into the Fig. 5 Intermediates in aminolysis. bilayer interface in the S state and the accessibility of water to the intermediate species formed during lipidation. Changes in peptide orientation in response to cholesterol content are reflected by changes to the lipidation rates at different sites on the peptide.

Conflicts of interest

There are no conflicts to declare.

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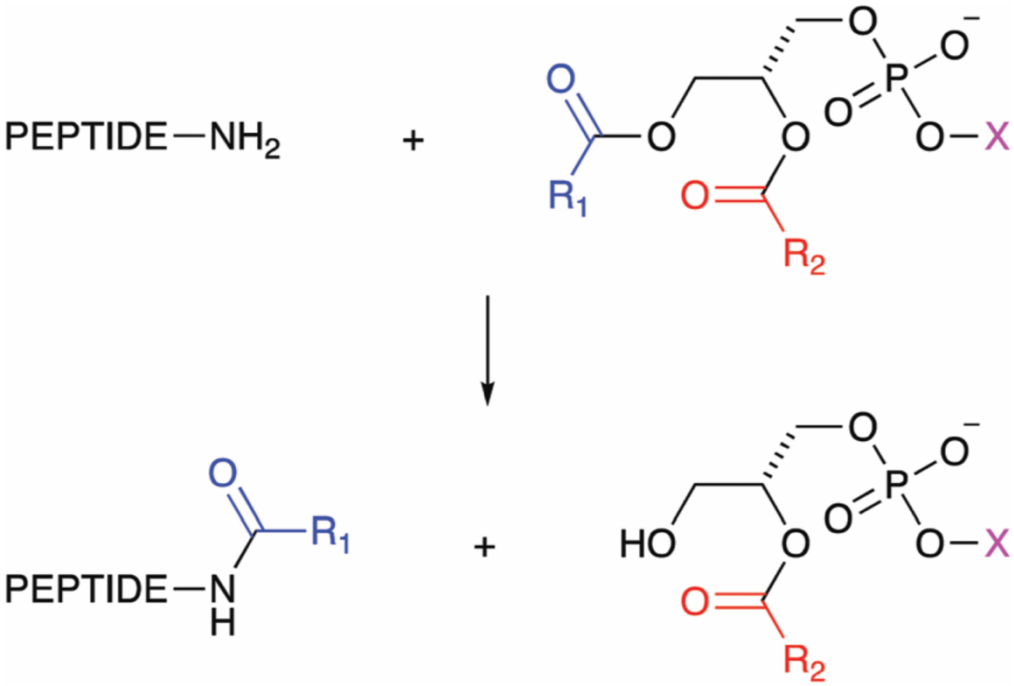
Notes and references

1 E. Habermann, Science, 1972, 177, 314–322.
2 W. Wilcox and D. Eisenberg, Protein Sci., 1992, 1, 641–653.
3 L. R. Brown, J. Lauterwein and K. Wuthrich, Biochim. Biophys. Acta, 1980, 622, 231–244.
4 J. F. Faucon, J. Dufourcq and C. Lussan, FEBS Lett., 1979, 102, 187–190.
5 S. C. Quay and C. C. Condie, Biochemistry, 1983, 22, 695–700.
6 C. E. Dempsey, Biochim. Biophys. Acta, 1990, 1031, 143–161.
7 K. Hristova, C. E. Dempsey and S. H. White, Biophys. J., 2001, 80, 801–811.
8 C. E. Dempsey and G. S. Butler, Biochemistry, 1992, 31, 11973–11977.
9 H. Vogel, FEBS Lett., 1981, 134, 37–42.
10 A. M. Batenburg, J. H. van Esch, J. Leunissen-Bijvelt, A. J. Verkleij and B. de Kruijff, FEBS Lett., 1987, 223, 148–154.
11 A. K. Ghosh, R. Rukmini and A. Chattopadhyay, Biochemistry, 1997, 36, 14291–14305.

12 M.-T. Lee, W.-C. Hung, F.-Y. Chen and H. W. Huang, Proc. Natl. Acad. Sci. U. S. A., 2008, 105, 5087–5092.
13 L. Yang, T. A. Harroun, T. M. Weiss, L. Ding and H. W. Huang, Biophys. J., 2001, 81, 1475–1485.
14 M. T. Lee, F. Y. Chen and H. W. Huang, Biochemistry, 2004, 43, 3590–3599.
15 F. R. Svensson, P. Lincoln, B. Norde´n and E. K. Esbjo¨rner, Biochim. Biophys. Acta, 2011, 1808, 219–228.
16 H. Raghuraman and A. Chattopadhyay, Biophys. J., 2007, 92, 1271–1283.
17 R. H. Dods, J. A. Mosely and J. M. Sanderson, Org. Biomol. Chem., 2012, 10, 5371–5378.
18 R. H. Dods, B. Bechinger, J. A. Mosely and J. M. Sanderson, J. Mol. Biol., 2013, 425, 4379–4387.
19 C. J. Pridmore, J. A. Mosely, A. Rodger and J. M. Sanderson, Chem. Commun., 2011, 47, 1422–1424.
20 C. A. Smith E. J. Want, G. O'Maille, R. Abagyan and G. Siuzdak, Anal. Chem., 2006, 78, 779–787.
21 R Core Team, R: A Language and Environment For Statistical Computing, 2017.
22 D. Marsh, Handbook of Lipid Bilayers, CRC Press, Boca Raton, 2013.
23 P. F. F. Almeida, Biochim. Biophys. Acta, 2009, 1788, 72–85.
24 J. Hjort Ipsen, G. Karlstro¨m, O. G. Mourtisen, H. Wennerstro¨m and M. J. Zuckermann, Biochim. Biophys. Acta, 1987, 905, 162–172.
25 M. R. Vist and J. H. Davis, Biochemistry, 1990, 29, 451–464.
26 J. Dufourcq and J. F. Faucon, Biochim. Biophys. Acta, 1977, 467, 1–11.
27 J. Dufourcq, J. F. Faucon, G. Fourche, J. L. Dasseux, M. Le Maire and T. Gulik-Krzywicki, Biochim. Biophys. Acta, 1986, 859, 33–48.
28 T. Pott and E.-J. Dufourc, Biophys. J., 1995, 68, 965–977.
29 M. Monette, M. R. Van Calsteren and M. Lafleur, Biochim. Biophys. Acta, 1993, 1149, 319–328.
30 T. Benachir, M. Monette, J. Grenier and M. Lafleur, Eur. Biophys. J., 1997, 25, 201–210.
31 J. F. Popplewell, M. J. Swann, N. J. Freeman, C. McDonnell and R. C. Ford, Biochim. Biophys. Acta, 2007, 1768, 13–20.
32 P. Wessman, A. A. Stro¨mstedt, M. Malmsten and K. Edwards, Biophys. J., 2008, 95, 4324–4336.
33 S. L. Veatch and S. L. Keller, Biophys. J., 2003, 85, 3074–3083.
34 S. L. Veatch and S. L. Keller, Phys. Rev. Lett., 2002, 89, 268101.
35 Y. Yu, J. A. Vroman, S. C. Bae and S. Granick, J. Am. Chem. Soc., 2010, 132, 195–201.
36 J. Pan and N. K. Khadka, J. Phys. Chem. B, 2016, 120, 4625–4634.
37 J. E. Shaw, R. F. Epand, J. C. Y. Hsu, G. C. H. Mo, R. M. Epand and C. M. Yip, J. Struct. Biol., 2008, 162, 121–138.
38 D. Marsh, Biochim. Biophys. Acta, 2010, 1798, 688–699.
39 F. Torrens, G. Castellano, A. Campos and C. Abad, J. Mol. Struct., 2007, 834-836, 216–228.
40 W. Rawicz, K. C. Olbrich, T. McIntosh, D. Needham and E. Evans, Biophys. J., 2000, 79, 328–339.
41 J. F. Nagle, Faraday Discuss., 2013, 161, 11–29.
42 M. Rappolt and G. Pabst, in Structure and Dynamics of Membranous Interfaces, ed. K. Nag, John Wiley & Sons, Hoboken, 2008, pp. 45–81.
43 D. Marsh, Chem. Phys. Lipids, 2006, 144, 146–159.
44 J. Pan, S. Tristram-Nagle and J. Nagle, Phys. Rev. E: Stat., Nonlinear, Soft Matter Phys., 2009, 80, 021931.

45 J. Pan, T. T. Mills, S. Tristram-Nagle and J. F. Nagle, Phys. Rev. Lett., 2008, 100, 198103.
46 R. Dimova, Adv. Colloid Interface Sci., 2014, 208, 225–234.
47 T. Pott, C. Gerbeaud, N. Barbier and P. Meleard, Chem. Phys. Lipids, 2015, 185, 99–108.
48 D. Harries and U. Raviv, in Liposomes, Lipid Bilayers and Model Membranes: From Basic Research To Application, ed. G. Pabst, N. Kucˇerka, M.-P. Nieh and J. Katsaras, CRC Press, Boca Raton, 2014, pp. 3–30.
49 E. Melo and J. Martins, Biophys. Chem., 2006, 123, 77–94.
50 P. Yuan, P. J. Fisher, F. G. Prendergast and M. D. Kemple, Biophys. J., 1996, 70, 2223–2238.
51 J. Lauterwein, L. R. Brown and K. Wuthrich, Biochim. Biophys. Acta, 1980, 622, 219–230.
52 L. Zhu, M. D. Kemple, P. Yuan and F. G. Prendergast, Biochemistry, 1995, 34, 13196–13202.
53 S. Frey and L. K. Tamm, Biophys. J., 1991, 60, 922–930.
54 A. Okada, K. Wakamatsu, T. Miyazawa and T. Higashijima, Biochemistry, 1994, 33, 9438–9446.
55 Y. H. Lam, S. R. Wassall, C. J. Morton, R. Smith and F. Separovic, Biophys. J., 2001, 81, 2752–2761.
56 I. Constantinescu and M. Lafleur, Biochim. Biophys. Acta, 2004, 1667, 26–37.
57 M. X. Fernandes, J. Garcıa de la Torre and M. A. Castanho, Anal. Biochem., 2002, 307, 1–12.
58 M. Amaro, H. A. Filipe, J. P. Prates Ramalho, M. Hof and L. M. Loura, Phys. Chem. Chem. Phys., 2016, 18, 7042–7054.
59 M.-T. Lee, W.-C. Hung, F.-Y. Chen and H. W. Huang, Biophys. J., 2005, 89, 4006–4016.
60 P. Shchelokovskyy, S. Tristram-Nagle and R. Dimova, New J. Phys., 2011, 13, 25004.
61 S. Tristram-Nagle and J. F. Nagle, Biophys. J., 2007, 93, 2048–2055.
62 A. I. Greenwood, J. Pan, T. T. Mills, J. F. Nagle, R. M. Epand and S. Tristram-Nagle, Biochim. Biophys. Acta, 2008, 1778, 1120–1130.
63 A. Zemel, A. Ben-Shaul and S. May, J. Phys. Chem. B, 2008, 112, 6988–6996.
64 B. Kollmitzer, P. Heftberger, R. Podgornik, J. F. Nagle and G. Pabst, Biophys. J., 2015, 108, 2833–2842.
65 A. C. Satterthwait and W. P. Jencks, J. Am. Chem. Soc., 1974, 96, 7018–7031.
66 J. M. Sanderson, Mol. Membr. Biol., 2012, 29, 118–143.
67 D. Marsh, Proc. Natl. Acad. Sci. U. S. A., 2001, 98, 7777–7782.
68 W. K. Subczynski, A. Wisniewska, J. J. Yin, J. S. Hyde and A. Kusumi, Biochemistry, 1994, 33, 7670–7681.
69 N. Kucerka, J. Pencer, M. P. Nieh and J. Katsaras, Eur. Phys. J. E: Soft Matter Biol. Phys., 2007, 23, 247–254.
70 S. K. Kandasamy and R. G. Larson, Biochim. Biophys. Acta, 2006, 1758, 1274–1284.
71 A. Pillersdorf and J. Katzhendler, J. Org. Chem., 1979, 44, 549–554.
72 C. C. Yang and W. P. Jencks, J. Am. Chem. Soc., 1988, 110, 2972–2973.



Scheme 1: The intrinsic lipidation process involving acyl transfer from a membrane phospholipid to an acceptor molecule such as a peptide (shown for transfer from the sn-1 position of the lipid; transfer from sn-2 also occurs). 'X' is the lipid headgroup.

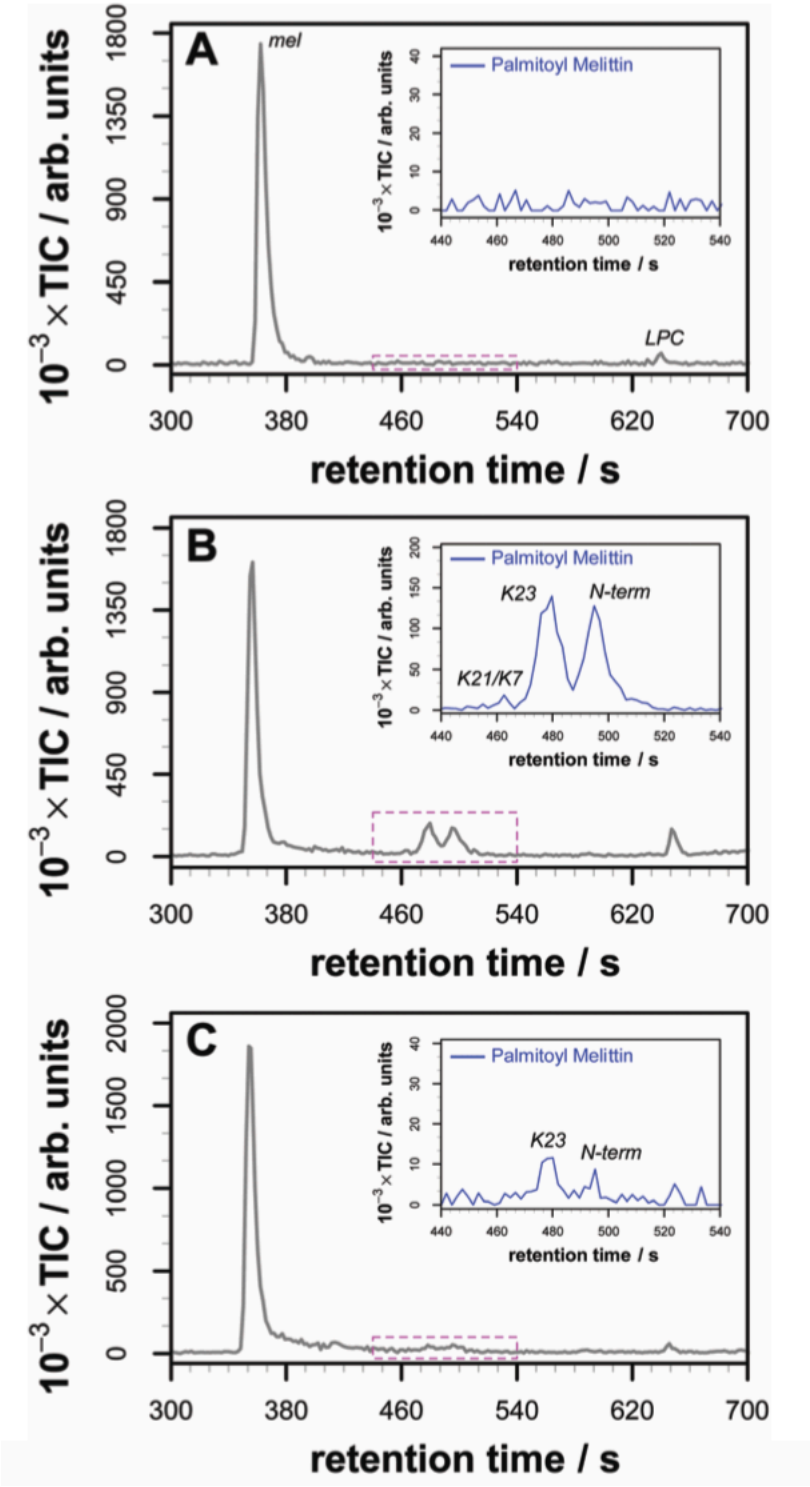


Figure 1: LC-MS analysis (total ion currents, TICs) of melittin/DPPC liposome mixtures after 24 h incubation. Conditions: [melittin], 26 mM; [DPPC + chol], 0.26 mM (P : L, 1 : 10); [NaCl], 90 mM; [NaHCO3], 10 mM; pH 7.4, 37 °C. (A) DPPC; (B) DPPC/chol, 4 : 1; (C) DPPC/chol, 1 : 1. Insets show extracted ion chromatograms for lipidated melittin in the region indicated by the dashed rectangular box. Chromatographic peaks are annotated to indicate the main site of peptide modification responsible for the peak.[17,18]

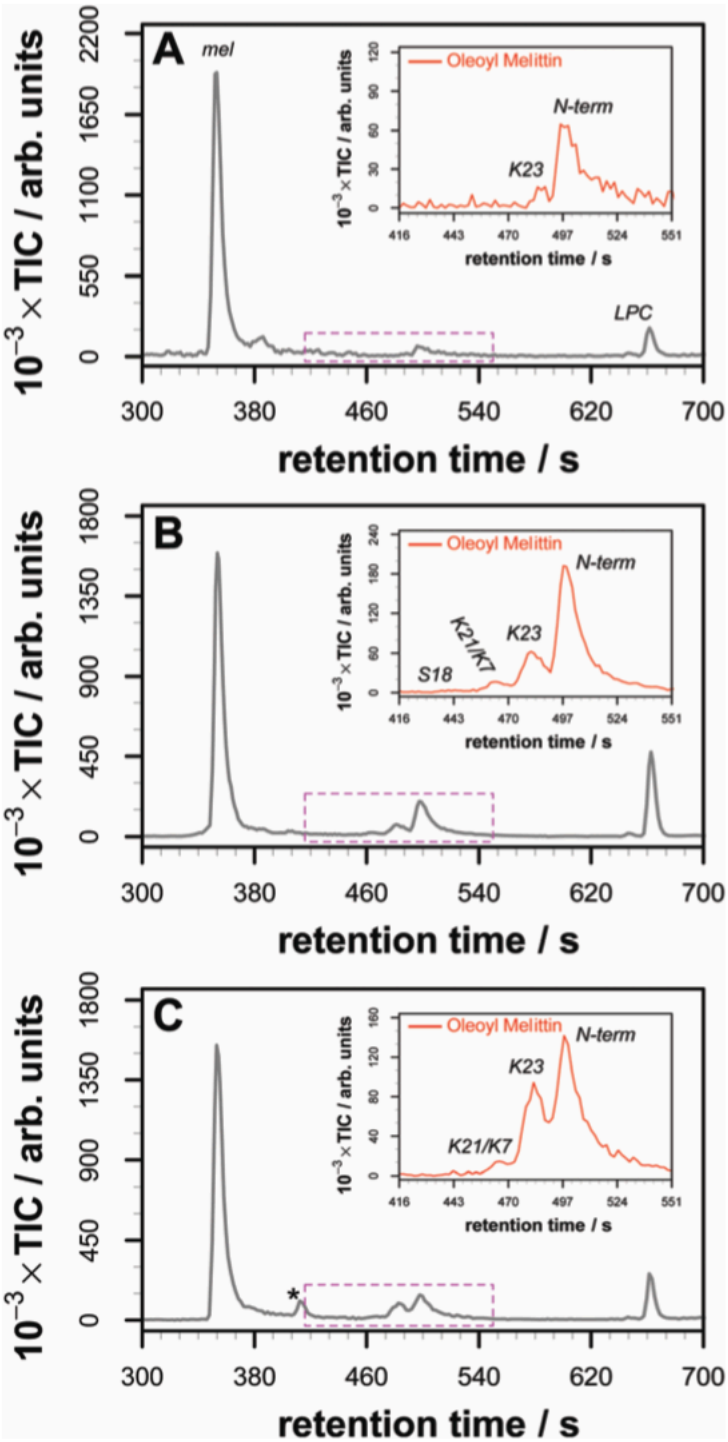


Figure 2: LC-MS analysis (total ion currents, TICs) of melittin/liposome mixtures after 24 h incubation. Conditions: [melittin], 26 mM; [DOPC + chol], 0.26 mM (P:L, 1:10); [NaCl], 90 mM; [NaHCO3], 10 mM; pH 7.4, 37 1C. (A) DOPC; (B) DOPC/chol, 4 : 1; (C) DOPC/chol, 1 : 1. Other details are the same as Fig. 1. The peak indicated by an asterisk is an artefact.

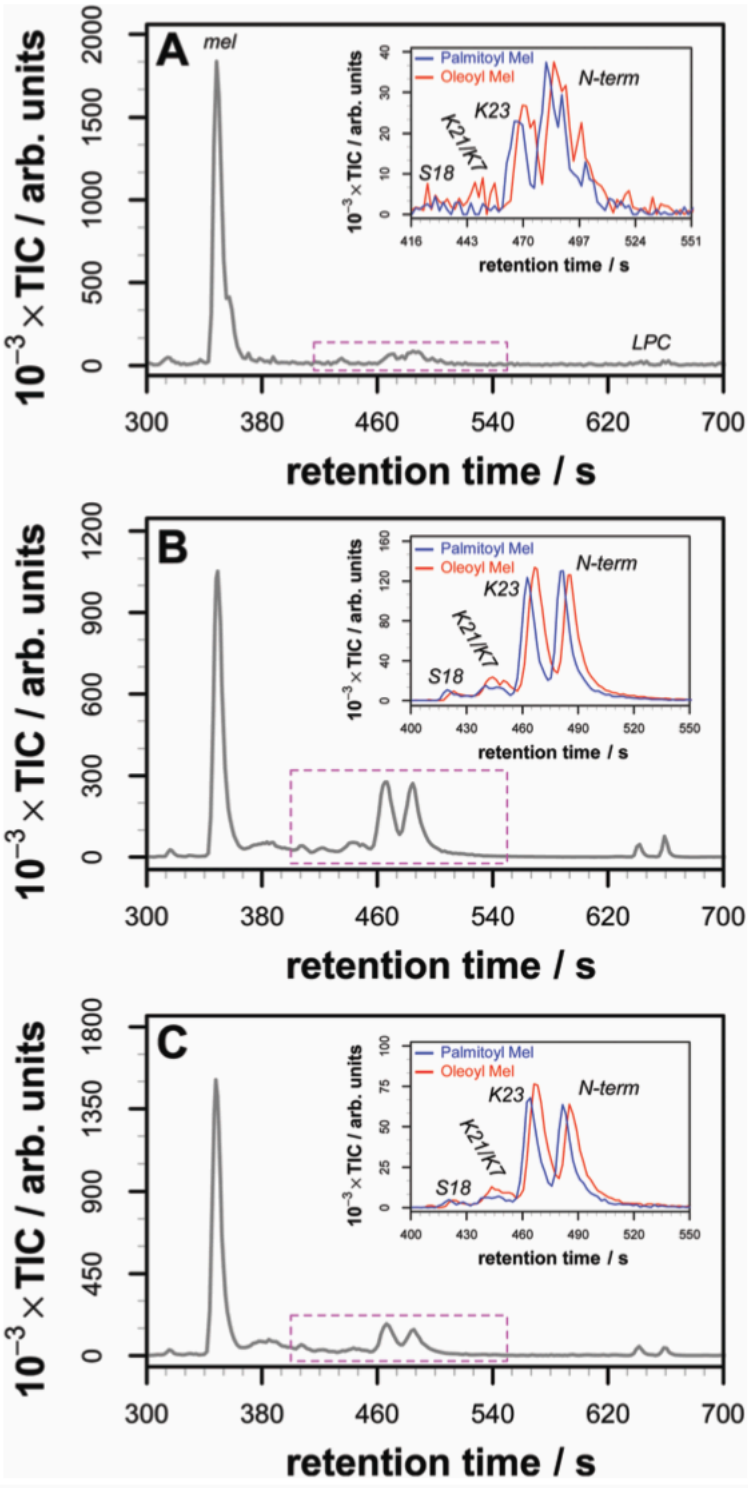


Figure 3: LC-MS analysis (total ion chromatograms, TICs) of melittin/POPC liposome mixtures after 24 h incubation. Conditions: [melittin], 26 mM; [POPC + chol], 0.26 mM (P : L, 1 : 10); [NaCl], 90 mM; [NaHCO3], 10 mM; pH 7.4, 37 1C. (A) POPC; (B) POPC/chol, 4 : 1; (C) POPC/chol, 1 : 1. Other details are the same as Fig. 1.

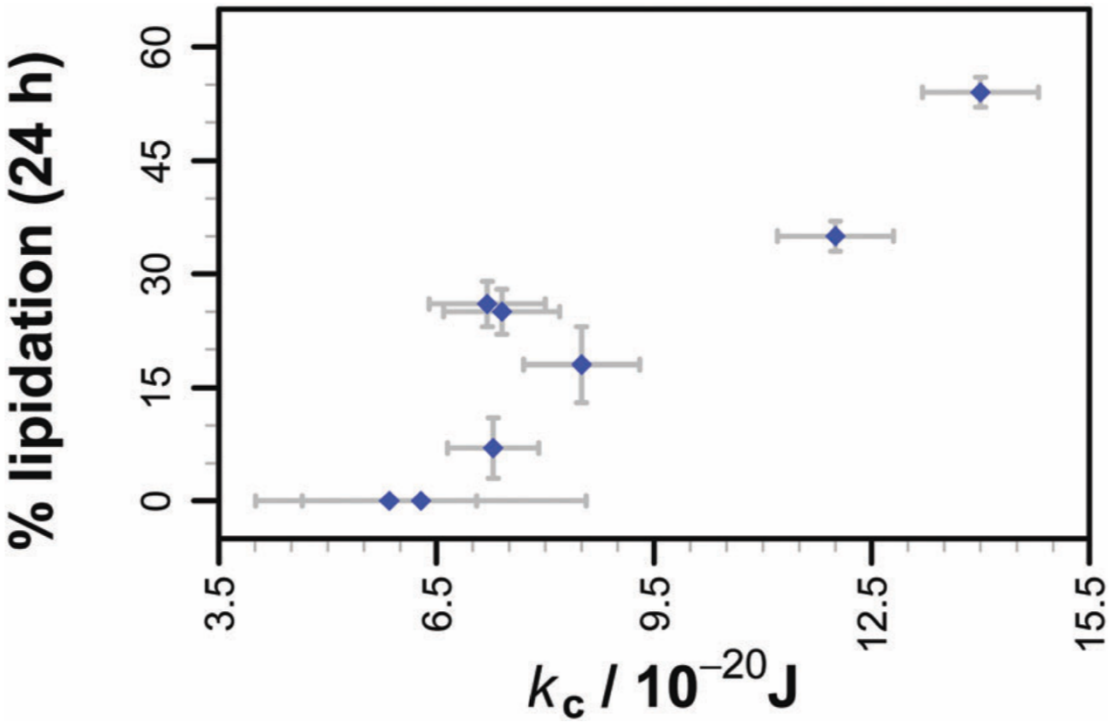


Figure 4: Correlation between the extent of lipidation (expressed as peak area for lipidated melittin as a % of the total peptidic peaks) and the curvature modulus k_c.

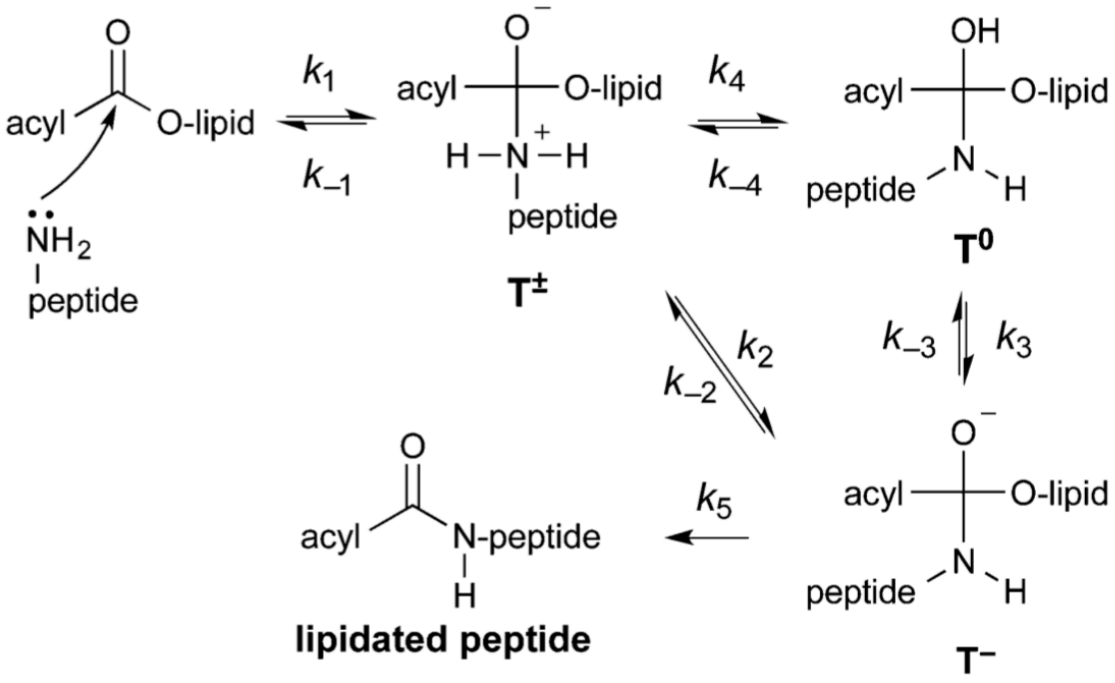


Figure 5: Intermediates in aminolysis.

		Peak area[a] (% after 24 h)			
Composition	Mel	*N*-acyl	K23-acyl	(K21 + K7)-acyl[b]	S18-acyl
DMPC	100 ± 0	0	0	0	0
DPPC	100 ± 0	0	0	0	0
DPPC/chol, 4:1	74 ± 3	12 ± 2	13 ± 3	2 ± 1	0
DPPC/chol, 1:1	90 ± 4	5 ± 3	5 ± 3	0	0
DOPC	93 ± 4	5 ± 2	2 ± 1	0	0
DOPC/chol, 4:1	74 ± 3	16 ± 1	6 ± 2	2 ± 1	1
DOPC/chol, 1:1	75 ± 3	13 ± 2	9 ± 2	3 ± 1	0
DOPC/DPPC/chol, 1:1:1	100 ± 0	0	0	0	0
POPC	82 ± 5	9 ± 3	6 ± 2	1 ± 1	1 ± 1
POPC/chol, 4:1	46 ± 2	21 ± 2	23 ± 3	6 ± 3	3 ± 2
POPC/chol, 1:1	65 ± 2	13 ± 3	14 ± 3	5 ± 2	3 ± 2

Table 1: Relative peak areas for peptidic peaks in LC-MS traces obtained following melittin (26 mM) incubation with LUVs (0.26 mM) of varying composition for 24 h

[a] Peak assignments are based on literature precedents.17,18 Areas were determined by fitting an exponentially modified Gaussian function to the EIC of each species. [b] These could not be resolved due to peak overlap.

Lipid	% lipidation[a]	K_c[b]/10^{-20} J	Temp.[c]/°C
DMPC	0	6.3 ± 2.8	29–40
DPPC	0	5.85 ± 1.2	50
DOPC/chol, 8 : 2	26 ± 3	7.2 ± 0.8	30
DOPC	7 ± 4	7.3 ± 0.6	30
POPC	18 ± 5	8.5 ± 0.8	30
POPC/chol, 4 : 1[d]	54 ± 2	14 ± 0.8	30
POPC/chol, 1 : 1[d]	35 ± 2	12 ± 0.8	30
DOPC/chol, 1 : 1[e]	25 ± 3	7.4 ± 0.8	30

Table 2: Lipidation and bending modulus data used for Fig. 4

[a] After 24 h. [b] Values are from X-ray scattering methods from ref. 42, 43, and 22 and are mean ± s for n > 3, the mean ± combined error for n = 2 and the reported error for n = 1. [c] The reported temperature for the determination of Kc. [d] Data are for SOPC/chol, as the nearest match. [e] Extrapolated from literature data.44,45