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The role of electrostatic interactions in the membrane binding of melittin

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The binding of melittin and the C-terminally truncated analogue of melittin (21Q) to a range of phospholipid bilayers was studied using surface plasmon resonance (SPR). The phospholipid model membranes included zwitterionic dimyristylphosphatidylcholine (DMPC) and dimyristylphosphatidylethanolamine (DMPE), together with mixtures DMPC/dimyristylphosphatidylglycerol (DMPG), DMPC/DMPG/cholesterol and DMPE/DMPG. Melittin bound rapidly to all membrane mixtures, whereas 21Q, which has a reduced charge, bound much more slowly on the DMPC and DMPC/DMPG mixtures reflecting the role of the initial electrostatic interaction. The loss of the cationic residues also significantly decreased the binding of 21Q with DMPC/DMPG/Cholesterol, DMPE and DMPE/DMPG. The role of electrostatics was also highlighted with NaCl in the buffer, which affected the way melittin bound to the different membranes, causing a more uniform, concentration dependant increase in response. The biosensor results were correlated with the conformation of the peptides determined by circular dichroism analysis, which indicated that high α-helicity was associated with high binding affinity. Overall, the results demonstrate that the positively charged residues at the C-terminus of melittin play an essential role in membrane binding, that modulation of peptide charge influences selectivity of binding to different phospholipids and that manipulation of the cationic regions of antimicrobial peptides can be used to modulate membrane selectivity. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: Mellitin; Biomembrane binding; Surface plasmon resonance; Electrostatic interactions

INTRODUCTION

Melittin is one of the most studied antimicrobial and cytolytic peptides to date. It is the principal active peptide from the sting of the European honey bee *Apis mellifera* making up some 50 per cent of the dry venom. It is a non-selective, cytolytic peptide with high antimicrobial activity and well-documented potent haemolytic activity (Habermann, 1972; DeGrado *et al*., 1981; DeGrado *et al*., 1982; Hider *et al*., 1983; Tosteson *et al*., 1985; Dempsey, 1990; Blondelle and Houghten, 1991a, b; Blondelle *et al*., 1993; Saberwal and Nagaraj, 1994; Rudenko and Patelaros, 1995; Rivett *et al*., 1996; Castano *et al*., 1999; Subbalakshmi *et al*., 1999; Raghuraman and Chattopadhyay, 2005) and has also shown an ability to reduce HIV-1 production (Wachinger *et al*., 1992). Melittin is 26 residues long and although it is mostly hydrophobic, it has a net charge of +6 at physiological pH (Table 1). The amino terminus (residues 1–20) consists of mostly hydrophobic residues and the carboxyl terminus is strongly cationic with four positively charged residues (Lys-Arg-Lys-Arg), the remaining two positive charges are located at Lys-7 and the N-terminus.

Melittin is cleaved from its precursor during its biosynthesis in the bee (Habermann, 1972). The released peptide is unstructured in solution, but in the presence of membranes (or suitable membrane mimics) it adopts an α-helical structure (Brown and Wuthrich, 1981; Brown *et al*., 1982; Chandani and Balasubramanian, 1986; Vogel, 1987; Inagaki *et al*., 1989; Dempsey, 1990; Ikura *et al*., 1991; Dempsey and Butler, 1992; Weaver *et al*., 1992; Bismuto *et al*., 1993; Ghosh *et al*., 1997; Ladokhin and White, 2001; Yang *et al*., 2001; Raghuraman and Chattopadhyay, 2003; Raghuraman and Chattopadhyay, 2004a, b, c; Raghuraman *et al*., 2006). The hydrophobic and hydrophilic residues are distributed on opposite sides of this helix, so forming an amphipathic structure (Dathe and Wieprecht, 1999) as shown in Figure 1. Although melittin contains numerous hydrophobic residues, it is very soluble in water and is reasonably soluble in methanol. The peptide is believed to be monomeric at low peptide concentration (μM) and forms a tetramer (four α-helices) at high concentrations (mM) under certain conditions of high pH and/or high salt concentration (Brown *et al*., 1980; Lauterwein *et al*., 1980; Dempsey, 1990).

The exact mechanism by which melittin disrupts membranes is still elusive. It was originally believed to act via the barrel-stave pore forming model (Vogel and Jahnig, 1986; Sansom, 1991), but recent evidence using techniques such as oriented CD and neutron diffraction (Yang *et al*., 2001) and molecular simulations (Lin and Baumgaertner, 2000) have suggested that melittin may form toroidal pores (Yang *et al*., 2001) in a manner similar to that proposed for the action of magainin (Ludtke *et al*., 1996; Matsuzaki *et al*., 1996). However, studies have also shown that melittin may act via a more general membrane disruption such as the carpet-like mechanism (Ladokhin *et al*., 1997; Ladokhin and White, 2001; Papo and Shai, 2003). It is likely that the mechanism by which melittin exerts its effect is dependent on the specific conditions. Variables such as the concentration of the peptide, the ionic strength and pH of the solution and the composition of the target membrane (including the proportion of positively charged lipids or presence of cholesterol) have all been shown to influence the way melittin interacts with membranes (Toraya

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Journal of Molecular Recognition

Table 1. Sequences of melittin and 21Q, with the molecular mass, number of amino acids (AA), the net charge and hydrophobic moment (μ_H) of each peptide. The 21Q analogue has the five underlined residues removed from the C-terminus of melittin

Peptide	Amino acid sequence	M_r	AA	Charge	μ_H
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2847	26	+6	0.39
21Q	GIGAVLKVLTTGLPALISWIQ-NH_2	2150	21	+2	0.48

et al., 2004; Vogel and Jahnig, 1986; Vogel 1987; Yang *et al*., 2001; Frey and Tamm, 1991; Hristova *et al*., 2001).

As charge has previously been shown to be important for antimicrobial peptide activity, one aspect of melittin that has been investigated is the effect of the cationic tail. Previous work has shown that residues 21–26 of the peptide are crucial for lytic activity (Schroder *et al*., 1971). Sequential deletion of the cationic C-terminus have shown a gradual reduction in lytic activity (Werkmeister *et al*., 1993), although these peptides are still able to bind to erythrocytes (Schroder *et al*., 1971).

In this study, we have explored the role of charge and electrostatic interaction in membrane binding through a comparison of the binding properties of melittin and a truncated analogue (21Q) in which the cationic C-terminal tail has been removed (see Table 1 for sequence). This modification effectively hinders the ability of melittin to rapidly bind with membranes. Although numerous studies have contributed to our current understanding of the molecular mechanism of melittin, the exact nature of the peptide–membrane interaction in terms of affinity and the effect on membrane morphology and lysis is still uncertain. In this study, the interaction of melittin and 21Q with model membranes was investigated using mimics of both eukaryotic and microbial membranes. The membrane binding affinity and kinetics were explored by SPR and the results were correlated with the secondary structure determined by CD, in buffer and in the presence of the model liposome mixtures. Comparison of the membrane interaction of melittin with that of the 21Q analogue, allowed the contribution of the cationic tail to the biological activity of melittin to be investigated and provided new insight into their mechanism of action.

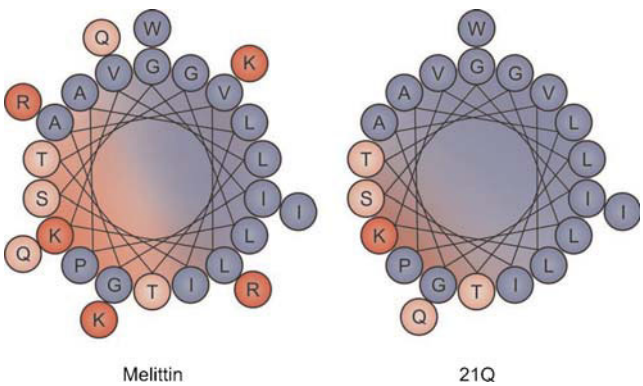

Figure 1. The Edmondson helical wheel diagrams of Melittin and 21Q. Hydrophobic residues and areas are shown in blue, clustering on one side of the helix forming a non-polar face. On the other side are hydrophilic residues and areas are shown in pink and positively charged residues in red, creating a polar face.

EXPERIMENTAL METHODS

Chemicals and reagents

Sodium Phosphate Dibasic/Monobasic, 3-(N-Morpholino)-propanesulphonic acid (MOPS), (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulphonate) (CHAPS), Sodium Hydroxide (NaOH), 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC), 1,2-Dimyristoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (Sodium Salt) (DMPG), 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphoethanolamine (DMPE) and cholesterol were purchased from Sigma (St Louis, MO, USA). Melittin was purchased from Auspep (Parkville, VIC, Australia). 21Q was provided by Dr Jerome Werkmeister (CSRIO, Division Molecular Science, Australia). The sequence of both peptides is listed in Table 1. Chloroform and methanol purchased from Merck (Kilsyth, VIC, Australia). Sodium chloride was purchased from Spectrum Chemical (Gardena, CA, USA). Water was quartz-distilled and deionized in a Milli-Q system (Millipore, Bedford, MA, USA).

Liposome preparation

DMPC and cholesterol were dissolved in chloroform, DMPG was dissolved in a mixture of chloroform/methanol (3:1 v/v) and DMPE was dissolved in a mixture of chloroform/methanol (1:1 v/v) to create individual stock solutions. These stock solutions were then aliquoted out into test tubes in the desired ratios: DMPC, DMPC/PG (4:1 v/v), DMPC/PG/cholesterol (16:4:5 v/v), DMPE, DMPE/PG (4:1 v/v). The solvent was then evaporated under a gentle stream of N_2 and vacuum desiccated overnight. For circular dichroism experiments, lipids were resuspended in 20 mM phosphate buffer with 150 mM NaCl (pH 7.4) with vortexing to a concentration of 1.36 mM. The resultant lipid dispersion was then sonicated with a bath type sonicator until clear. For surface plasmon resonance experiments, lipids were resuspended into buffer with vortexing to a concentration of 0.5–1 mM. The lipid dispersion was then sonicated in a bath type sonicator until nearly clear. Small unilamellar vesicles (SUV; 50 nm) were created via extruding the sonicated mixture through a 50 nm membrane filter (ATA scientific, Lucas Heights, NSW, Australia).

Circular dichroism (CD)

CD measurements were carried out on a Jasco J-810 Circular Dichroism Spectropolarizer (Jasco, Tokyo Japan). Using quartz cells of 1 mm path length, scans between wavelengths of 190 and 260 nm were done with a scan speed of 20 nm/min and a bandwidth of 1.0 nm. The resolution was 0.1 nm with a 1 s response time, 5 scan accumulations. The quartz cell temperature was controlled with a peltier temperature controller at 25°C. The CD instrument was calibrated with (+)-10-camphorsulphonic acid. The different lipid liposomes were prepared as above. The concentration of these lipids was 1.36 mM. Once the lipid solution was ready, melittin was then added to a peptide/lipid ratio of approximately 1:100. This was then sonicated very briefly just

before measurements. The same peptide concentration was used for samples with melittin in buffer solution alone. The CD spectra were measured for melittin in phosphate buffer solution and in the presence of the different lipid liposomes. Solubility problems prevented the acquisition of CD spectra for 21Q. The final spectra obtained for each was the average of the five scans accumulated. Spectra were smoothed using the Jasco Fast Fourier transform algorithm and baseline corrected. Following baseline correction, the percentage of helix was calculated for the peptide in buffer and in the presence of the different lipid solutions. This was calculated from the mean residue ellipticity [θ] at 222 nm (deg cm^2 dmole^{-1}) according to the relationship as follows (Chen *et al.*, 1972):

$$\%\alpha = \frac{100 \times [\theta]_{222}}{\theta_f} \text{ and } \theta_f = -39500 \times \left(\frac{1 - 2.57}{n}\right)$$

where α is the amount of helix.

Surface plasmon resonance (SPR)

SPR experiments were carried out with a Biacore 3000 analytical system with on L1 sensor chip (Biacore, Uppsala, Sweden). The system was cleaned using the 'Desorb and Sanitize' protocol with a maintenance chip and then allowed to run overnight with water. The L1 chip was docked and first washed with an injection of 5 μl of 20 mM CHAPS at a flow rate of 5 μl/min to clean the chip surface. SUVs in immobilization buffer (20 mM phosphate buffer 150 mM NaCl pH 7.4) were then immediately applied to the chip surface with injections of 80 μl at a low flow rate of 2 μl/min. To remove any multi-lamellar structures from the lipid surface and to stabilize the baseline, 30 μl of 10 mM NaOH was injected at 50 μl/min. All solutions were freshly prepared, degassed and filtered through a 0.2 μm filter.

The peptide solutions were prepared by dissolving melittin and 21Q in the running buffer (20 mM phosphate buffer 150 mM NaCl pH 7.4) creating 8 serial dilutions of 0.5–10 μM. 100 μl of these solutions were injected at a flow rate of 30 μl/min having a total injection time of 200 s. On completion of injection, buffer flow continued to allow a dissociation time of at least 600 s. All binding experiments were carried out at 25°C. The affinity of the antimicrobial peptide–lipid binding event was determined from analysis from a series of response curves in each case, where the resultant sensorgrams were collected from peptide injections at different concentrations over each different lipid surface. For experiments conducted in the absence of NaCl, the running buffer was 20 mM phosphate buffer pH 7.4.

RESULTS AND DISCUSSION

Melittin is one of the most extensively studied membrane lytic peptides, and is essentially a model system for monitoring mechanisms of pore formation and membrane disruption. One of the key features of melittin that is believed to be critical to its activity is the stretch of cationic residues at the C-terminus. 21Q has an identical sequence to melittin, but with five of the C-terminal residues removed. The resultant peptide has a lower net positive charge, with a greater hydrophobic moment that forms an amphipathic helix similar to melittin. The specific aims of this study were to explore the role of electrostatic interactions in the membrane binding of melittin through analysis of (1) the effect of NaCl in the buffer to probe the role of electrostatic interactions and (2) comparison of the membrane binding properties of 21Q on five different lipid mixtures (DMPC, DMPC/DMPG, DMPC/DMPG/cholesterol, DMPE and DMPE/DMPG) with the binding of melittin.

Secondary structure determination by CD

Figure 2 shows the CD spectra observed for melittin in buffer and in the presence of the different lipid liposomes and Table 2 lists the % helix in each liposome. Solubility problems prevented the acquisition of CD spectra for 21Q. Melittin exhibited an α-helical structure with the double dip minima at 208 and 222 nm in the presence of buffer (15%), DMPC (27% helix), DMPC/DMPG (4:1) (44%), DMPC/DMPG/Cholesterol (16:4:5) (51%) and DMPE/DMPG (4:1) (27%). This is consistent with previous studies that have shown that melittin requires a membrane environment (or suitable hydrophobic mimic) such as micelles, reverse micelles and liposomes to adopt an α-helical structure (Brown and Wuthrich, 1981; Brown *et al.*, 1982; Vogel, 1987; Inagaki *et al.*, 1989; Ikura *et al.*, 1991; Dempsey, 1992; Weaver *et al.*, 1992; Bismuto *et al.*, 1993; Ghosh *et al.*, 1997; Ladokhin and White, 2001; Mozsolits *et al.*, 2001; Yang *et al.*, 2001; Raghuraman and Chattopadhyay, 2007; Naito *et al.*, 2000). No structure was seen in the presence of DMPE, which was most likely due to the lack of solubility of the lipid. Pure DMPE does not easily form liposomes due to the limited hydration of the headgroup and its tendency to adopt a hexagonal structure, although this is less predominant for the shorter acyl chains such as dimyristoyl (Epand *et al.*, 2003; Leekumjorn and Sum, 2007a, b; Bouchet *et al.*, 2009; Qin *et al.*,

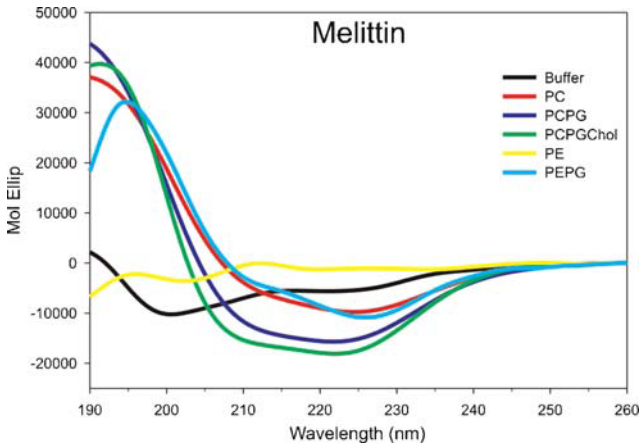

Figure 2. CD spectra of melittin in solution and in the presence of the different lipid liposomes. Molar ellipticity spectra from 190 to 260 nm.

Table 2. Per cent alpha helical content of melittin in buffer and in the presence of the five different liposome mixtures derived from circular dichoism spectra

	% of helical content
Buffer	15
DMPC	27
DMPC/DMPG (4:1)	44
DMPC/DMPG/Chol (16:4:5)	51
DMPE	3
DMPE/DMPG (4:1)	27

Journal of Molecular Recognition

2009). While CD spectra could not be obtained in this study, previous studies have shown that 21Q also adopts an α-helical structure in the presence of liposomes (Mozsolits *et al*., 2001). Studies with CD and Raman spectroscopy suggest that the melittin helix contains around 20 residues in model membranes (Vogel and Jahnig, 1986; Vogel, 1987), suggesting that the positively charged carboxyl tail may not form a helix.

Membrane binding determined by SPR

Melittin (phosphate buffer without NaCl)

Melittin bound in a similar way to each of the lipid layers containing DMPC (DMPC, DMPC/DMPG (4:1) and DMPC/DMPC/Chol (16:4:5)). The lowest concentrations (0.125–1 μM) had a slow association with very little binding. The 2 μM concentration was an intermediate between the lowest and highest concentrations with a very slow association. The higher concentrations (4–12 μM) had a very fast initial binding immediately after injection with the highest concentration (12 μM) binding to just over 5000 RU on each of these three different lipid layers. The higher concentrations showed two distinct phases in the association with a rapid initial binding phase that reached an equilibrium, which was maintained for the rest of the injection. Peptide concentrations below 2 μM showed complete dissociation after completion of the injection. The 2 μM concentration bound nearly irreversibly with the vast majority of mass remaining on the surface at the 600 s time point. Concentrations above 2 μM all showed similar dissociation with an initial fast loss of mass in the first few seconds that slowed to a more gentle linear dissociation. There was still some material on the lipid layer after 600 s with a response of 1000–2000 RU at the higher concentrations.

Overall melittin showed a greater binding response on the lipid layers containing DMPE (DMPE and DMPE/DMPG (4:1)) than DMPC. Again the lower concentrations (0.125–2 μM) showed very little binding. The RU level at 4 μM was intermediate on these lipids with a slow association rate. The higher concentrations (6–12 μM) bound rapidly in the first few seconds after injection, in a similar way to the DMPC containing lipids. However, the overall response was greater with the highest concentration reaching just below 7000 RU on DMPE and ∼9000 RU on DMPE/DMPG (4:1). Again after the sharp increase in initial response the interaction reached equilibrium for the remainder of the injection. After completion of the injection, almost all material dissociated from the surface with the lower concentrations (0.125–4 μM). The 4 μM injection showed a slow dissociation with around half the response remaining at 600 s on DMPE and a substantial amount left on DMPE/DMPG (4:1) at 600 s. The higher concentrations of melittin (6–12 μM) had a gradual dissociation, although this was faster than the peptide on the membrane layers containing PC. There was still a large amount of peptide remaining on the surface at 600 s with some 2000–3000 RU remaining on DMPE and 4000–5000 RU remaining on DMPE/DMPG (4:1).

Melittin has previously been shown to strongly interact with DMPC membranes (Kuchinka and Seelig, 1989; Beschiaschvili and Seelig, 1990; Mozsolits *et al*., 2001; Kriech and Conboy, 2003) and studies by SPR have shown that it binds strongly to both DMPC and DMPG membranes (Mozsolits *et al*., 2001; Blondelle *et al*., 1999). However, our previous studies with melittin and 21Q (Lee *et al*., 2001; Mozsolits *et al*., 2001) used HPA chips on which a hybrid bilayer is created and complete insertion is prevented. Papo and Shai (2003) have also studied the interaction of melittin with PC/cholesterol and PE/PG membranes on the hybrid bilayer of an HPA chip and adsorbed liposomes of the L1 chip. The differences in the binding properties on these two surfaces suggested that melittin inserts into PC/cholesterol but is more surface bound on PE/PG. Melittin has also previously shown bilayer-stabilizing effects when mixed with DMPE-containing membranes under conditions where the pure lipid arranges in a hexagonal phase (Batenburg *et al*., 1988) and DMPE has also been shown to inhibit membrane lysis by melittin (Benachir and Lafleur, 1995; Monette and Lafleur, 1995; Hincha and Crowe, 1996; Ghosh *et al*., 1997; Pott *et al*., 2001). Like DMPC, DMPE is zwitterionic having no net charge, but DMPE has a relatively smaller head group and therefore larger tendency to promote tighter membrane packing compared to DMPC (Lohner and Blondelle, 2005). It is likely that this difference in membrane organization is responsible for the differences in binding response.

The increased binding of melittin to DMPE/DMPG relative to DMPE is also consistent with previous studies that showed that melittin interacts selectively (Beschiaschvili and Seelig, 1990; Gromova *et al*., 1992; Ghosh *et al*., 1997; Kleinschmidt *et al*., 1997; Sheynis *et al*., 2003) and with a much greater affinity with negatively charged lipids (Batenburg *et al*., 1988; Lee *et al*., 2001). Previous SPR studies have also shown that melittin has a higher affinity for DMPG hybrid bilayers than DMPC hybrid bilayers (Mozsolits *et al*., 2001). Greater helical content was also observed in the liposome mixtures containing the DMPG compared to those composed of only zwitterionic lipids. The addition of the anionic charge most likely strengthens the electrostatic interaction leading to an increase in binding response, which may also lead to greater helical content.

It has been suggested that the mechanism of action of melittin is dependent on a critical concentration (DeGrado *et al*., 1982), which may be reflected here in the large gap in response between the low (0.125–2 μM) and the high concentrations (4–12 μM) on each of the membrane mixtures. At low concentrations there may not be sufficient peptide bound to the membrane to proceed to further membrane insertion or destabilizing effects so the peptide dissociates away from the surface easily. With the intermediate concentrations (2–4 μM), it is possible that just enough peptide is bound for the peptide to reorient in the membrane with stronger affinity, as very little material dissociates from the membrane at these concentrations. Concentrations above 4 μM associated quickly then reached equilibrium as the second re-orientation in the membrane would be the rate-limiting step.

Melittin with 150 mM NaCl included in the running buffer

The results above revealed a nonlinear increase in binding with increasing melittin concentration. This result may be caused by a strong electrostatic contribution to the interaction, due to the low salt concentration. The binding of melittin to the different lipids was therefore determined in the presence of 150 mM NaCl on DMPC, DMPC/DMPG (4:1) and DMPE/DMPG (4:1). Figure 4 shows the SPR binding response of 0.5–10 μM melittin with 150 mM NaCl in the buffer on the three different lipid mixtures. The results were very different to those which were obtained without NaCl. With NaCl included in the buffer, melittin bound with a higher response on DMPC. The lower concentrations (0.5–1 μM) showed no binding to the membrane while concentrations from 2–10 μM showed a steady increase in binding response with the top concentration (10 μM) binding to 6500 RU. Melittin also showed

an increasing association rate between the 2 μM peptide with a slow association, up to the 10 μM with a rapid association rate. The highest concentrations showed a fast initial association that reached equilibrium in a similar way to melittin without NaCl. The dissociation was slow with around half the peptide still remaining on the surface at 600 s.

The binding response levels were lower for melittin on DMPC/DMPG (4:1) compared to both the response on DMPC and the response of melittin without NaCl in the buffer on DMPC/DMPG (4:1). The shape of the curves was similar to that on DMPC but the top concentration (10 μM) had a lower maximum response at 4000 RU. The lowest concentration (0.5–1 μM) had very little interaction with this membrane. Association and dissociation rates were similar to that on DMPC. Around half the amount of bound peptide at the end of injection remained at 600 s.

Melittin bound very differently to DMPE/DMPG (4:1) in the presence of NaCl. The association rate was slower compared to the peptide on the other two lipids and to that of melittin without NaCl in the buffer. This result suggests that the addition of NaCl changed the mechanism by which the peptide interacts with DMPE and exhibits binding selectivity for certain phospholipids. The top concentration (10 μM) had a slow association rate that eventually reached equilibrium at the end of injection with a maximum response of 3600 RU. The addition of NaCl also resulted in a binding response which was linearly dependant on concentration. The dissociation was slow, with around half the peptide remaining on the surface at 600 s similar to the other two lipid layer mixtures.

Overall, the segregation in response between low and high peptide concentrations was much less apparent when NaCl was added to the buffer, with a more gradual, concentration dependent increase in response observed. This provides further evidence for the role of electrostatics in the interaction. Previous studies by fluorescence have shown that melittin is monomeric at low salt concentrations (Quay and Condie, 1983; Raghuraman *et al*., 2006; Faucon *et al*., 1979) and aggregates at high peptide concentration or high salt concentration at a neutral pH (Talbot *et al*., 1979; Raghuraman *et al*., 2006). However, under the low NaCl and peptide concentration conditions used in this study, melittin exists as a monomer. It is more likely therefore, that addition of 150 mM NaCl to the buffer changed the electrostatic dynamics of the solution and modulated peptide binding to each lipid layer mixture compared to the experiments in the absence of NaCl (Raghuraman *et al*., 2006).

The binding of 21Q to model membranes

Figure 5 shows the sensorgrams for the binding of 0.125–12 μM 21Q on the five different model membrane layers. Overall, the sensorgrams were very different to those obtained for melittin. 21Q did not show the same response 'gap' between the lower and higher concentrations that was seen with melittin on DMPC. The association was slower and the response was more linearly concentration dependant. The association rate was also partially dependant on concentration showing increase in association rate at higher concentrations. Despite the association being much slower than melittin on this lipid, the top concentration (12 μM) bound to a similar response of ~4800 RU. The majority of material dissociated quickly and completely from the surface with the lower concentrations (0.125–4 μM) and only a small amount of material was left with the 6 μM concentration at 600 s. The higher concentrations (8–12 μM) had a fast initial drop in response that levelled into a gradual dissociation. Around 2000 RU remained on the surface at 600 s. Overall, this result is similar to previous SPR investigations of 21Q on DMPC with a hybrid bilayer, suggesting that 21Q does not penetrate the membrane to any significant extent (Mozsolits *et al*., 2001; Mozsolits and Aguilar, 2002).

The 21Q peptide behaved in a similar way on the DMPC/DMPG (4:1) lipid layer showing a dependence of response units on concentration. 21Q also showed similarly shaped association curves although the response was less on this lipid with the top concentration reaching 3200 RU. The dissociation was also similar to that on DMPC with around 1000 RU remaining on the surface at 600 s for the higher concentrations (8–12 μM).

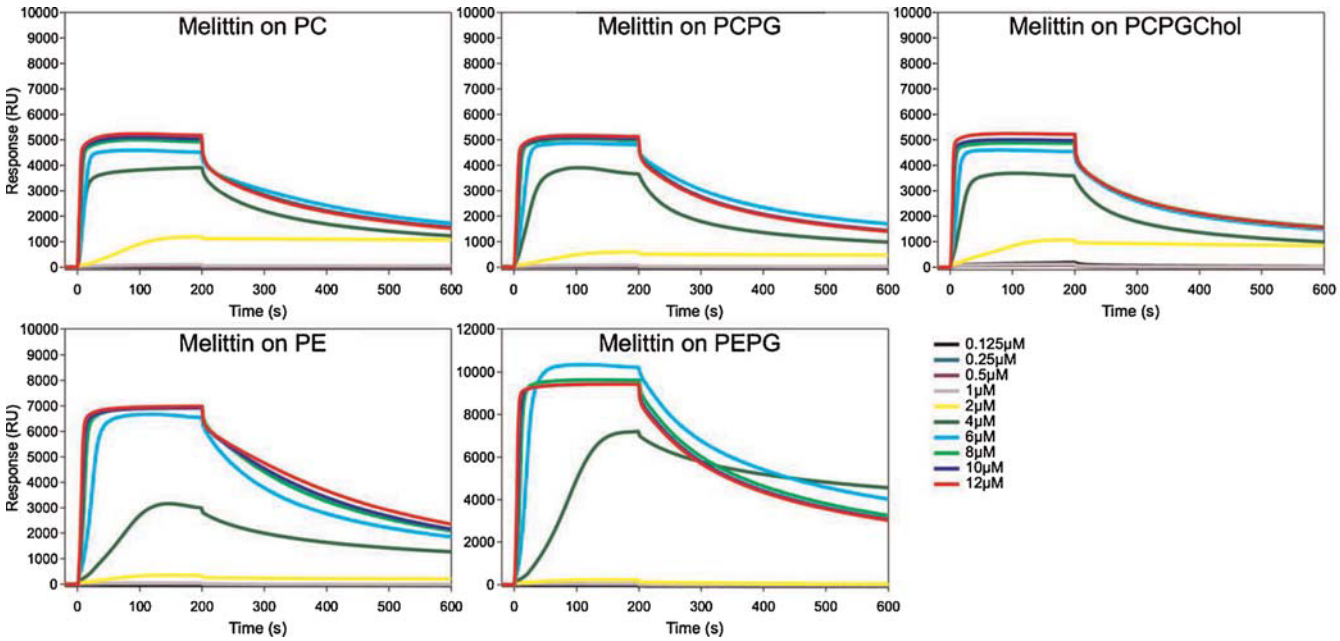

Figure 3. Typical sensorgrams SPR of melittin binding to the different liposome mixtures in the absence of NaCl. Ten different concentrations between 0.125 and 12 μM are shown. Peptide was injected over 200 s at 30 μl/min ($t = 0-200$ s). The peptide was then allowed to dissociate for a further 400 s ($t = 200-600$ s) as buffer continued to flow through the system. The running buffer was 20 mM phosphate buffer pH 7.4.

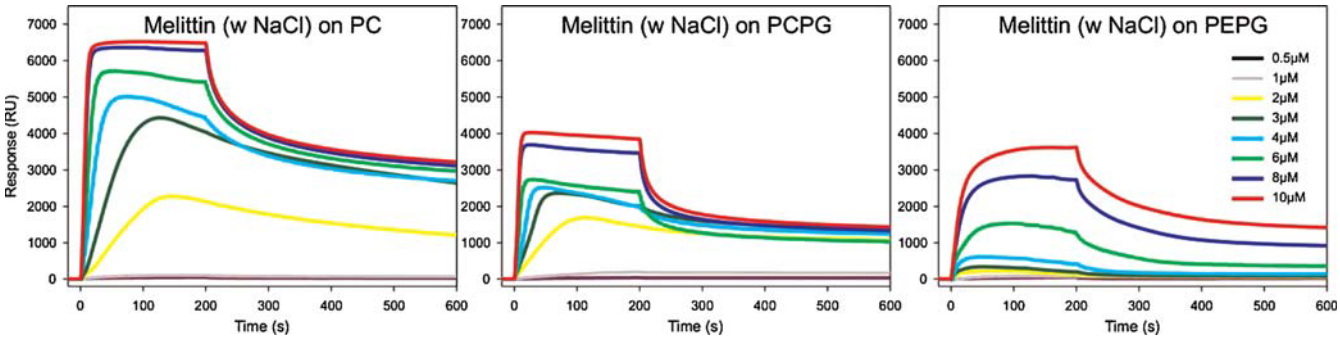

Figure 4. Typical sensorgrams SPR of melittin binding to three different liposome mixtures in the presence of NaCl. Eight different concentrations between 0.5 and 10 μM are shown. Peptide was injected over 200 s at 30 μl/min ($t = 0$–200 s). The peptide was then allowed to dissociate for a further 400 s ($t = 200$–600 s) as buffer continued to flow through the system. The running buffer was 20 mM phosphate buffer 150 mM NaCl pH 7.4.

A very different response was observed with this peptide on the DMPC/DMPG/Cholesterol (16:4:5) layer. While the same concentration dependant response was seen, the response was much lower compared to the other two DMPC-containing lipids. There was a fast initial association, with the response levelling out quickly with the top concentration only reaching 1000 RU. The dissociation was rapid on completion of the injection and complete with no peptide remaining on the surface after 600 s with all concentrations. Cholesterol is known to play a crucial role in eukaryotic membrane organization, dynamics, function and sorting (Yeagle, 1985; Liscum and Underwood, 1995; Simons and Ikonen, 2000; Pucadyil and Chattopadhyay, 2006), resulting in denser lipid packing and increased deformation energy (Needham, 1995). Cholesterol has also been shown to inhibit the lytic activity of melittin (Raghuraman and Chattopadhyay, 2004a; Allende *et al*., 2005) and increase in amounts of cholesterol has been shown to lower melittin binding to DOPC liposomes (Raghuraman and Chattopadhyay, 2004a). While this decrease in binding was not observed in this study, it is possible that the membrane interaction of 21Q (predominantly hydrophobic forces) is more susceptible to this increased stability of the membrane, and therefore accounting for the decrease in lysis of erythrocytes (Raghuraman and Chattopadhyay, 2005).

The response of 21Q on DMPE and DMPE/DMPG (4:1) was similar to that on DMPC/DMPG/Cholesterol (16:4:5) which is opposite to the increase in response seen with melittin on these lipids. While the highest concentration bound to a somewhat lower response of 800RU on both lipids, there was a similar fast association to a maximum level and then rapid complete dissociation on completion of the injection with all concentrations. The reduction in the cationic tail has lead to a complete change in specificity of binding to the DMPC lipids (those without cholesterol). Again the binding selectivity between DMPC and DMPE appears to be heavily influenced by electrostatic forces.

The absence of the cationic C-terminal tail therefore results in a slower rate of association due to the inability to bind via electrostatic interactions. 21Q also shows a concentration dependant increase in association, but does not show the same pronounced concentration response 'gap' between the high

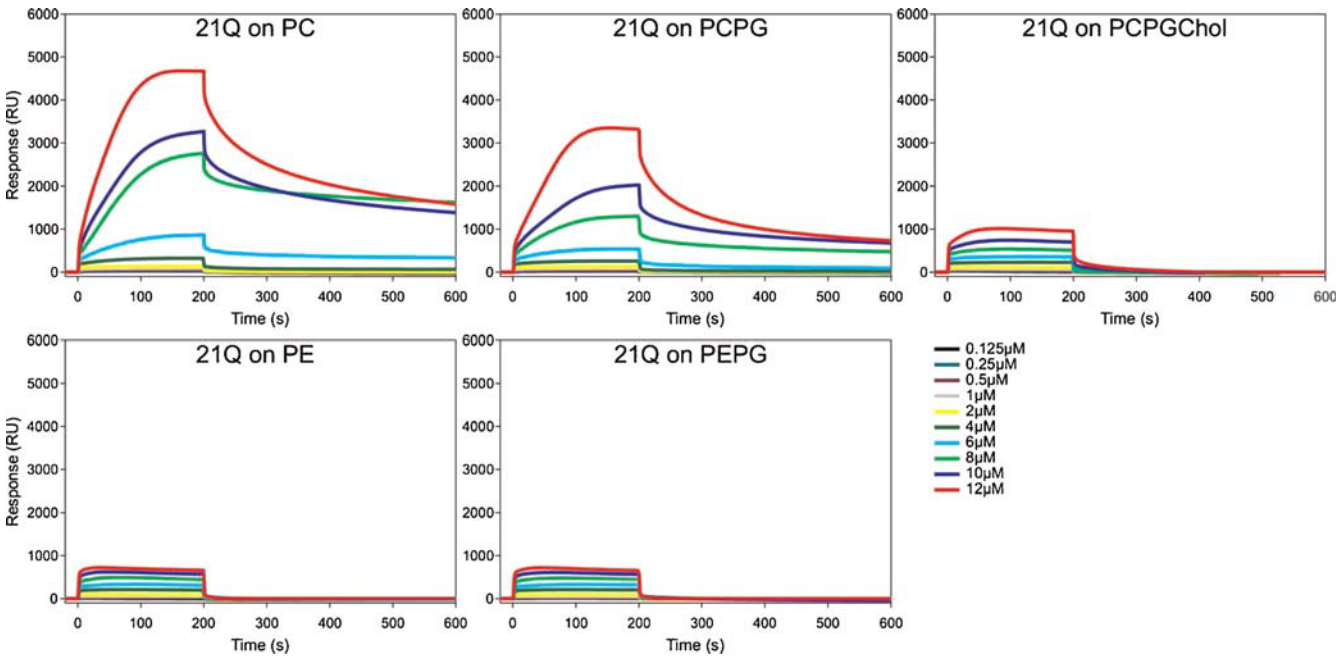

Figure 5. Typical sensorgrams SPR of 21Q binding to the different liposome mixtures in the absence of NaCl. Ten different concentrations between 0.125 and 12 μM are shown. Peptide was injected over 200 s at 30 μl/min ($t = 0$–200 s). The peptide was then allowed to dissociate for a further 400 s ($t = 200$–600 s) as buffer continued to flow through the system. The running buffer was 20 mM phosphate buffer pH 7.4.

and low concentrations as observed for melittin. These results clearly demonstrate the importance of charge for the association of melittin with the membrane, and the importance of electrostatic forces in the initial 'fast step' interaction with the membrane. A second slow step was also seen where the peptide binding reaches equilibrium towards the end of the injection. It is likely that hydrophobic forces predominate in this second phase of association and 21Q still shows a strong interaction, with a slow dissociation and with peptide still remaining on the surface at 600 s on DMPC and DMPC/DMPG. The peptide is still very hydrophobic which is reflected in this relatively strong binding.

Quantitative analysis of SPR sensorgrams

Kinetic analysis of the sensorgrams shown in Figures 3–5 was performed via curve fitting to the different models detailed previously (Hall *et al*., 2003). Curve fitting via the 1:1 Langmuir model was very poor (results not shown). The fit for the parallel reactions model was better than the Langmuir model, but best fits were obtained from the two state reaction model. However, the χ^2 values were still very high (>2000 RU), which is a reflection of the changing shape of the sensorgrams with increase in melittin or 21Q concentration. Overall, it was not possible to make quantitative conclusions about the relative changes in affinity based on this analysis. This clearly demonstrates the complexity of the interaction and the difficulties in determining the overall rate and affinity constants.

In order to allow a semi-quantitative comparison between the two peptides, the RU at the end of the association phase (at 200 s) was plotted against peptide concentration (Hall and Aguilar, 2009) and shown in Figure 6 for melittin (a) in the absence of NaCl, (b) in the presence of NaCl and (c) 21Q (no NaCl), respectively. These plots clearly illustrate the nonlinear dependence of RU on concentration between lower and higher concentrations and confirm that there is a threshold concentration below which minimal binding does not occur. The presence of NaCl partially ameliorates this threshold

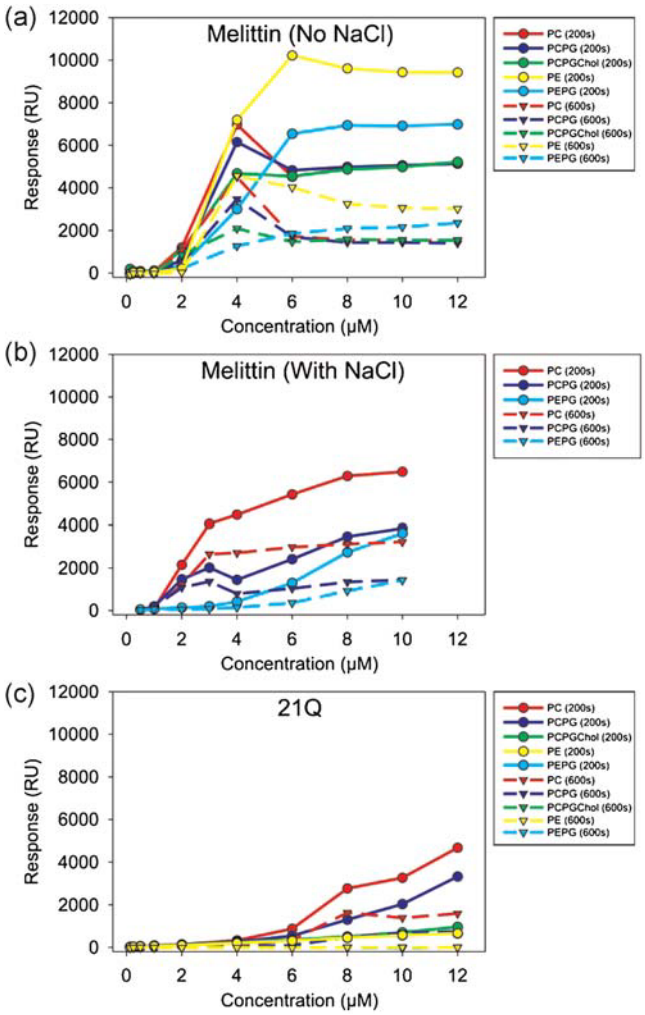

Figure 6. Plot of RU versus peptide concentration for (a) melittin in the absence of NaCl in the running buffer, (b) melittin in the presence of NaCl in the running buffer and (c) 21Q. Solid lines (and filled circles) correspond to RU at the end of the association phase at 200 s, and the dashed lines (and inverted triangles) correspond to RU at the end of the dissociation phase (600 s). Red, DMPC; Blue, DMPC/DMPG (4:1); Green, DMPC/DMPG/Cholesterol (16:4:5); Yellow, DMPE and Cyan, DMPE/DMPG (4:1).

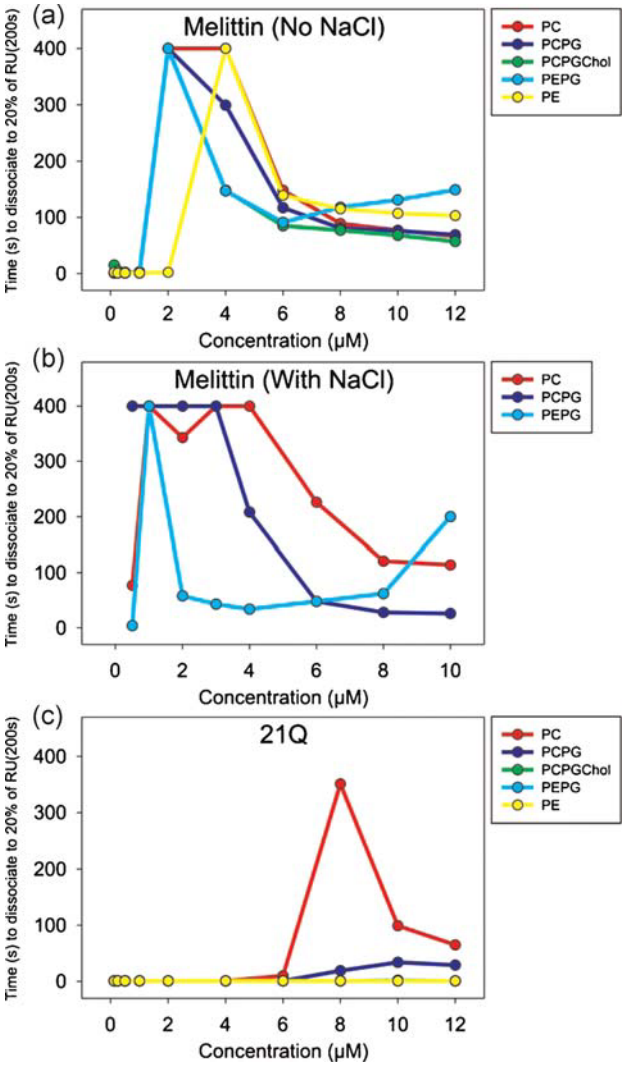

Figure 7. Plot of time (s) to dissociate to 60% of RU (200 s) versus peptide concentration for (a) melittin in the absence of NaCl in the running buffer, (b) melittin in the presence of NaCl in the running buffer and (c) 21Q. Red, DMPC; Blue, DMPC/DMPG (4:1); Green, DMPC/DMPG/Cholesterol (16:4:5); Yellow, DMPE and Cyan, DMPE/DMPG (4:1).

effect with melittin on the DMPC-containing membranes (Figure 6(b)), but significant binding is still not apparent on DMPE/DMPG until 4 μM. The influence of the positively charged tail on peptide binding is further illustrated in Figure 6(c) for 21Q with significant levels of binding only emerging at 6 μM.

Also plotted in Figure 6 is the RU at the end of the dissociation phase at 600 s. This data clearly illustrates the differences in the extent of dissociation between melittin and 21Q whereby 21Q almost completely dissociates (with the exception of DMPC and DMPC/DMPG) while there is approximately 50–70% residually bound melittin depending on the bilayer composition. The extent of dissociation was further analysed through the dependence on peptide concentration of the time taken during dissociation for the RU to reach 60% of the RU at 200 s, which is plotted in Figure 7. The data in Figure 7(a) and (b) show that there is no effect of NaCl on the rate of dissociation of melittin, with similar rates of dissociation occurring on DMPC and DMPC/DMPG in the presence and absence of NaCl. The effect of NaCl on dissociation was more evident on DMPE/DMPG with somewhat faster rates of dissociation at 2–4 μM, but similar at other concentrations. This compares to the results in Figure 6 (a) and (b) which showed that the presence of NaCl strongly influences the extent of association with varying melittin concentration and suggests that hydrophobic interactions are the dominant factor influencing dissociation (independent of NaCl) while electrostatic forces are involved in association (dependent on NaCl). Figure 7(c) shows the corresponding data for 21Q which dissociates more rapidly than melittin on all lipid mixtures. The exception is the results at 8 μM on DMPC in which dissociation was very slow which may represent a transition in the dissociation mechanism.

Overall, the membrane interaction of melittin showed two distinct phases of association, with a fast initial electrostatic phase followed by a second slower phase that is likely dominated by hydrophobic forces. This is similar to previous haemolysis studies of melittin that showed fast and slow kinetics (DeGrado *et al*., 1982; Hider *et al*., 1983; Ghosh *et al*., 1997; El Jastimi and Lafleur, 1999; Gomara *et al*., 2003; Allende *et al*., 2005), and previous membrane binding studies by SPR (Mozsolits *et al*., 2001). In particular, DeGrado *et al*. (1982) interpreted the initial fast phase as the initial binding and accumulation of melittin in the outer leaflet of the erythrocyte and the slow phase as the reorganization of the peptide into favourable packing geometry.

The general differences in membrane binding behaviour of melittin and 21Q are highlighted in Figure 8 which shows a comparative binding schematic for the two peptides. The upper panel depicts the binding of melittin to all lipid mixtures which is mediated initially by electrostatic interactions and is then followed by subsequent insertion/reorientation of melittin into

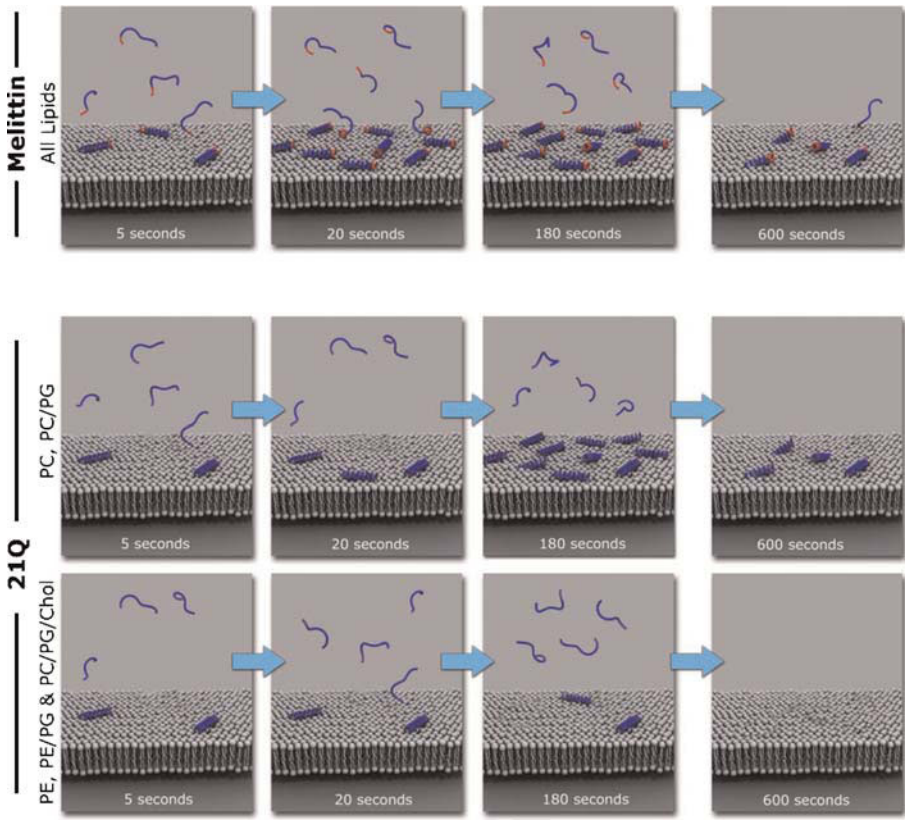

Figure 8. A comparative binding schematic illustrating the binding of melittin and 21Q to the different membrane layers. Soon after the initial injection of peptide, similar amounts of melittin and 21Q are bound to the membrane surface. After approximately 50 s, the cationic C-terminus (red tail) facilitates a stronger electrostatic attraction that results in a higher level of binding of melittin compared to 21Q on DMPC and DMPC/DMPG. Even less 21Q binds to DMPE, DMPE/DMPG and DMPC/DMPG/cholesterol. Towards the end of injection at 180 s, similar amounts of melittin bound on all lipids, and 21Q on DMPC and DMPC/DMPG. There was very little 21Q bound at this stage on DMPE, DMPE/DMPG and DMPC/DMPG/cholesterol. On completion of the injection, buffer continues to flow, and around 30–40% of melittin on all lipids, and 21Q on DMPC and DMPC/DMPG remained bound to the surface at the 600 s time point. No 21Q remained on the surface DMPE, DMPE/DMPG and DMPC/DMPG/cholesterol at 600 s.

the lipid bilayer. In comparison, the binding of 21Q is depicted in the middle panels for DMPC and DMPC/DMPG mixtures which show much slower initial binding but a similar level of binding to melittin is eventually reached. In contrast, the lower panels show the binding of 21Q to DMPC/DMPG/Cholesterol and DMPE/DMPG mixtures which show a slow initial binding which does not change throughout the injection.

CONCLUSIONS

Comparison of the membrane interaction of melittin with that of the 21Q analogue has allowed the contribution of the cationic tail to the biological activity of melittin to be investigated. We have shown that the interaction of melittin with membranes is highly dependent on electrostatic interactions as shown by the effect of NaCl and the removal of the C-terminal cationic amino acid residues. In particular, the positively charged C-terminus is responsible for the initial fast binding to all lipid mixtures. Moreover, the removal of the initial electrostatic interactions also resulted in significant selectivity changes and suggests that modulation of peptide charge can be used to manipulate phospholipid selectivity. Although melittin and 21Q are too haemolytic to be safely considered for future therapeutics, the knowledge derived from these biophysical studies provides important insight into the role of cationic residues in membrane binding and assists in not only understanding the mechanism of action of these peptides, but the potential for greater insight into the mechanism of action all membrane-lytic peptides.

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