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Lipid-specific binding of the calcium-dependent antibiotic daptomycin leads to changes in lipid polymorphism of model membranes

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ABSTRACT

Daptomycin is a cyclic anionic lipopeptide with an antibiotic activity that is completely dependent on the presence of calcium (as Ca^{2+}). In a previous study [Jung et al., 2004. Chem. Biol. 11, 949–957], it was concluded that daptomycin underwent two Ca^{2+}-dependent structural transitions, whereby the first transition was solely dependent on Ca^{2+}, while the second transition was dependent on both Ca^{2+} and the presence of negatively charged lipids that allowed daptomycin to insert into and perturb bilayer membranes with acidic character. Differences in the interaction of daptomycin with acidic and neutral membranes were further investigated by spectroscopic means. The lack of quenching of intrinsic fluorescence by the water-soluble quencher, KI, confirmed the insertion of the daptomycin Trp residue into the membrane bilayer, while the kynurenine residue was inaccessible even in an aqueous environment. Differential scanning calorimetry (DSC) indicated that the binding of daptomycin to neutral bilayers occurred through a combination of electrostatic and hydrophobic interactions, while the binding of daptomycin to bilayers containing acidic lipids primarily involved electrostatic interactions. The binding of daptomycin to acidic membranes led to the induction of non-lamellar lipid phases and membrane fusion.

1. Introduction

Daptomycin is a cyclic anionic lipopeptide antibiotic that is produced as a fermentation end product from a strain of *Streptomyces roseosporous* (Tally et al., 1999). It is composed of 13 amino acid residues, which include 3 D-amino acid residues (D-asparagine, D-alanine, and D-serine) and 3 uncommon amino acids (ornithine, (2*S*,3*R*)-3-methyl-glutamic acid and kynurenine), as well as a *n*-decanoyl fatty acid chain at the N-terminus (Debono et al., 1988). Daptomycin has been shown to be bactericidal against a wide variety of Gram-positive bacteria, including vancomycin-resistant Enterococci, coagulase-negative Staphylococci, glycopeptide intermediate-susceptible *Staphylococcus aureus*, and penicillin-resistant *Streptococcus pneumoniae* (Eliopoulos et al., 1986; Fass and Helsel, 1986; Jones and Barry, 1987; Tally and DeBruin, 2000; Tally et al., 1999). Its antimicrobial activity is entirely dependent on calcium as Ca^{2+}. In September 2003, daptomycin became the first lipopeptide from the class of Ca^{2+}-dependent antibiotics (Boeck et al., 1990; Hojati et al., 2002) to be approved by the US FDA for the treatment of complicated skin-infections caused by Gram-positive organisms (Kirkpatrick et al., 2003). The mechanism of action of daptomycin is still under investigation; however, the prevailing hypothesis involves the disruption of the cytoplasmic membrane leading to depolarization of the cytoplasmic membrane and cell death (Silverman et al., 2003). Recently we solved the structure of daptomycin and were able to demonstrate that daptomycin in the presence of Ca^{2+} interacted differently with acidic and neutral membranes (Jung et al., 2004). Based on these studies, we proposed that daptomycin underwent two Ca^{2+}-dependent structural transitions, these being a transition upon interaction of daptomycin with Ca^{2+}, and a further transition that was dependent on interaction with the bacterial acidic phospholipid, phosphatidyl glycerol, and provided data suggesting that the latter Ca^{2+}-dependent transition led to an increased perturbation of the cytoplasmic membrane.

A large proportion of the membrane lipids of *S. aureus* are the negatively charged phospholipids, phosphatidyl glycerol, cardiolipin, and lysyl phosphatidyl glycerol (Hayami et al., 1979), which can modulate the interaction and insertion of peptides into membranes (Liu and Deber, 1997). The structural organization and dynamics of such membranes is influenced by thermotrophic phase transitions that affect whether lipids adopt rigid, gel-like or fluid lamellar (bilayer) or non-lamellar (non-bilayer hexagonal or micellar) organizations. The phase that a given bilayer adopts and the

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transition temperature between phases are linked to the packing of the phospholipids within the bilayer which in turn is directly related to the lipid composition and environmental conditions. For example, the zwitterionic lipid phosphatidyl ethanolamine most favourably adopts a non-lamellar phase, while the negatively charged lipid phosphatidyl glycerol favours the lamellar phase (Foht et al., 1995). Non-lamellar lipid phases influence membrane bilayer structure by inducing either negative (hexagonal phase) or positive (micellar phase) curvature, that has been linked to the transbilayer movement of both phospholipids and water-soluble molecules, as well as fusion between membrane bilayers (Cullis and de Kruijff, 1979; Ellens et al., 1986; Gerritsen et al., 1980a,b). Changes in the organization of membrane lipids have also been linked to the membrane interaction of several cationic antimicrobial peptides (Lohner et al., 1997, 1999; Lohner and Prenner, 1999; Prenner et al., 1997).

In this study, we further examined the interaction of daptomycin with both neutral and acidic membranes in the presence of Ca^{2+} to gain additional insight into the mechanism of interaction of daptomycin with membranes. Our results collectively favour the conclusion that daptomycin binds near the surface of both acidic and neutral membranes in a Ca^{2+}-dependent manner in somewhat different ways, driven respectively by electrostatic and hydrophobic/electrostatic interactions. In particular, the binding of Ca^{2+}-conjugated daptomycin to acidic membranes resulted in a reduction in the transition temperature to the non-lamellar hexagonal phase, consistent with the enhanced disruption of acidic membranes. Furthermore daptomycin increased the lamellar to inverted hexagonal phase transition temperature of phosphatidyl ethanolamine indicating the induction of positive curvature strain.

2. Materials and methods

2.1. Liposome preparation

1,2-Dimyristoyl-*sn*-glycero-3-phospho-*rac*-1-glycero (DMPG) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Sigma–Aldrich (Oakville, ON). Liposomes were prepared as described in our previous report (Jung et al., 2004). In summary, lipids were weighed and dissolved with $CHCl_3$. The $CHCl_3$ was evaporated off with N_2, and the lipids were further dried under vacuum. The dried lipid films were resuspended with 20 mM HEPES buffer, pH 7.4, unless otherwise stated, and freeze–thawed five times. The resulting lipid suspensions were extruded 10 times through two 0.1 μm filters (AMD Manufacturing Inc., Mississauga, ON). Daptomycin was provided by Cubist Pharmaceuticals (Lexington, MA).

2.2. Fluorescence quenching studies

For potassium iodide (KI) quenching studies, aliquots of a 5 M KI stock solution (prepared with the addition of sodium thiosulfate (800 μM) to prevent the formation of I_3^-, which absorbs at the same wavelength as Trp) were added to the sample to create 20 mM increments in KI concentration. A final daptomycin concentration of 2 μM was used and added to 10 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES) buffer, pH 7.2. For samples containing lipids, unilamellar liposomes of either 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or an equimolar mixture of POPC and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (POPG) (Avanti Polar lipids Inc., Alabaster, AL) (final lipid concentration of 0.5 mM) and 5 mM $CaCl_2$ were made by vortexing and then sonicating to clarity.

Liposomes containing 10 mol% spin-labelled lipids were prepared as previously described (Epand and Surewicz, 1984). Briefly, unlabelled large unilamellar liposomes composed of POPC or 1:1 POPC/POPG mixtures containing 10 mol% of either 1,2-diacyl-*sn*-glycero-phosphotempocholine, 1-palmitoyl-2-stearoyl (5-DOXYL)-*sn*-glycero-3-phosphocholine, or 1-palmitoyl-2-stearoyl (12-DOXYL)-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids Inc., Alabaster, AL) were prepared by the resuspension of a dried lipid film into 20 mM HEPES (pH 7.4), 150 mM NaCl and extrusion.

For both the nitroxide spin-labelled lipid quenching and KI quenching studies, the emission spectra at room temperature of Kyn-13 from 400 to 520 nm were individually collected using an excitation wavelength of 365 nm, while that of Trp-1 utilized an excitation wavelength of 280 nm and emission wavelengths from 300 to 400 nm, using a PerkinElmer 650-10S fluorescence spectrometer (PerkinElmer Inc., Norwalk, CT). The slit widths were set at 8 nm. Each spectrum was baseline corrected by subtracting a sample containing all of the added components except for daptomycin.

2.3. Differential scanning calorimetry (DSC)

Multilamellar vesicles of DMPG, DMPC, or 1:1 DMPC/DMPG with a final lipid concentration of 25 mg/ml were prepared by first dissolving the lipids in chloroform, drying under a stream of nitrogen and subsequently under vacuum overnight to remove any residual solvent, adding daptomycin to the dried lipid sample at the desired ratio of daptomycin:lipid, re-suspending in 20 mM HEPES, pH 7.0, 100 mM NaCl and vortexing vigorously three times, while heating the sample above the phase transition peak temperature for 5 min between each vortexing step. Samples were degassed for 5 min prior to loading the sample into a multicell differential scanning calorimeter (Calorimetry Sciences, Corp., UT) located at the UBC Centre for Biological Calorimetry. The samples were heated and cooled over a temperature range of 5–90 °C at a rate of 0.333 °C/min. The resulting data were converted to units of molar heat capacity after baseline correction by subtracting a blank buffer scan. The corrected data was analyzed using Prism Graphpad version 4.0 (Graphpad Software Inc., San Diego, CA).

For experiments involving DiPoPE (1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine), lipid, peptide and calcium (5 mM final concentration) were co-dissolved in chloroform:methanol (2:1) at a peptide to lipid ratio of 1:500, dried under a stream of N_2 and held under vacuum for a minimum of 4 h. The peptide–lipid film was suspended in 10 mM Tris, 100 mM NaCl, pH 7.4 to yield multilamellar vesicles at a working lipid concentration of 7.5 mg/ml. The fluid lamellar (L_α) to inverted hexagonal (H_{II}) phase transition temperature of the lipids was measured with a MicroCal VP-DSC differential scanning calorimeter (Northampton, MA). A minimum of three scans was performed from 10 to 60 °C with a temperature increase of 1 °C/min and a 15 min equilibration period before each scan. In all cases, the thermogram of buffer alone was subtracted prior to plotting and analysis using MicroCal Origin 7.0.

2.4. ^{31}Phosphorous nuclear magnetic resonance (NMR) spectroscopy

^{31}P NMR spectra were collected with a Varian Unity 500 spectrometer at the UBC Laboratory for Molecular Biophysics. Multilamellar vesicle samples were prepared as for DSC. A lipid:peptide ratio of 100:1 with a final lipid concentration of 25 mg/ml was used. The spectra were collected at specified temperatures that were above and below the transition temperatures determined by DSC.

The resulting spectra were processed and analyzed using the program MestRe-C. All spectra were referenced to external 85% H_3PO_4 (Sigma–Aldrich, Oakville, ON).

2.5. Vesicle size determination

The size of a population of unilamellar liposomes consisting of either 1:1 POPC/POPG or POPC was determined using a Beckman Coulter N4 Plus laser particle sizer (Mississauga, ON). Liposome samples containing either 5 mM $CaCl_2$ alone or with added daptomycin were incubated at 23 °C for 15 min prior to measuring. The measurements were taken at 23 °C at a 90° angle. The resulting data were analyzed using a unimodal distribution curve.

2.6. Fluorescence resonance energy transfer (FRET)

Fluorescence spectra were collected at room temperature between the emission wavelengths of 300 and 522 nm with an excitation wavelength of 280 nm at a slit width of 5 nm, at a final daptomycin concentration of 8 μg/ml in solution, or in the presence of either 180 μM PC:PG (1:1) or PC liposomes and 5 mM $CaCl_2$ in a buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl. All spectra were baseline corrected by subtracting the spectra of a sample containing all of the added components except for daptomycin. FRET efficiency was calculated using the equation $E = 1 - (F_{ad}/F_a)$, where F_{ad} was equal to the fluorescence intensity of Trp in the presence of Kyn and F_a was equal to the fluorescence of Trp in absence of Kyn. F_a was determined by measuring the emission spectrum of free Trp at a concentration of 0.1 μg/ml.

To monitor membrane fusion as outlined by Struck et al. (1981) unilamellar (1:1) POPC/POPG liposomes were labelled with 0.6 mol% 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphoethanolamine and 0.6 mol% 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Avanti Polar Lipids Inc., Alabaster, AL) using a final lipid concentration of 180 μM in 5 mM HEPES, pH 7.4, 100 mM NaCl. Varying concentrations of daptomycin with 5 mM $CaCl_2$ were added to the lipid and membrane fusion was measured at an emission wavelength of 530 nm and an excitation wavelength of 465 nm at a slit width of 8 nm. The percent membrane fusion was calculated with the equation % Fusion = $100 \times (F_t - F_p)/(F_t - F_o)$, where F_o, F_p, and F_t were respectively equal to initial fluorescence, fluorescence with daptomycin and Ca^{2+}, and maximum fluorescence (which was determined by the addition of 0.01% Triton X-100).

3. Results

3.1. Membrane interaction of daptomycin: fluorescence studies

Our previously published data indicated that when Ca^{2+}-conjugated daptomycin interacted with liposomes, it caused a blue shift and dramatic increase in fluorescence, consistent with the daptomycin entering the hydrophobic core of the membrane (Jung et al., 2004). To probe this further, we examined the ability of the aqueous quencher KI to quench fluorescence, a property that reflects its accessibility to the fluorophor (i.e. the extent of exposure of the fluorophor to the solution bathing the membranes). Both Trp-1 and Kyn-13 were investigated, as these show similar fluorescence profiles when mixed together as free amino acids in solution (Muangsiri and Kirsch, 2001). Fig. 1 illustrates the Stern–Volmer plots for KI quenching of either daptomycin Trp-1 or Kyn-13 in solution, in negatively charged POPC/POPG liposomes and in neutral POPC liposomes. Quenching efficiency was expressed using the dynamic quenching constant K_{sv} derived from the slope of the

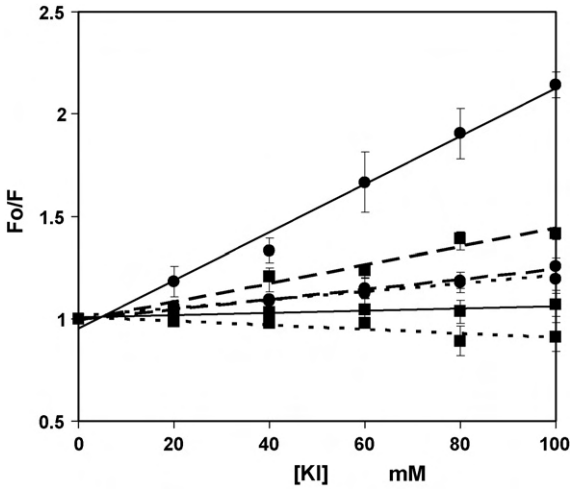

Fig. 1. Fluorescence quenching of the fluorophors Trp-1 and Kyn-13 in daptomycin by potassium iodide. Stern–Volmer plots of Trp-1 (●) and Kyn-13 (■) in solution (solid lines) (1:1) POPC/POPG large unilamellar liposomes (dotted lines), and POPC large unilamellar liposomes (dashed lines) with increasing concentrations of KI.

Stern–Volmer plots of the fluorescence intensity in the absence and presence of quencher as a function of KI concentration. A measured K_{sv} value of $12 \pm 1\ M^{-1}$ indicated that Trp was relatively solute-accessible when in solution (Kropacheva et al., 2005). In the presence of POPC and POPC:POPG liposomes, the K_{sv} value declined to $2.4 \pm 0.5\ M^{-1}$ and $2.0 \pm 0.6\ M^{-1}$, respectively, indicating that the Trp residue became relatively inaccessible to KI, due to insertion into the membrane. In contrast, Kyn was poorly quenched by KI in solution ($K_{sv} = 1.3 \pm 0.6\ M^{-1}$) indicating that it was relatively inaccessible. Similarly KI retained relatively poor accessibility to Kyn in POPC/POPG liposomes ($K_{sv} \sim 0\ M^{-1}$), with more modest quenching being observed in POPC liposomes ($K_{sv} = 4.3 \pm 0.2\ M^{-1}$). The larger quencher accessibility for Kyn in POPC than in solution alone may indicate that daptomycin undergoes a structural change in the presence of neutral PC lipids (Scott et al., 2008).

A previous study of the fluorescence characteristics of daptomycin by Lakey and Ptak (1988) concluded that the fluorescence peak of Trp was weakened due to the presence of Kyn and interpreted this by suggesting that Kyn was able to absorb the energy emitted by Trp and re-emit this energy at the Kyn emission maximum. According to Förster (Weinryb and Steiner, 1971), the efficiency of fluorescence resonance energy transfer (FRET) between fluorophors is related to the distance between the fluorophors as a reciprocal of the sixth power. The relative fluorescence of daptomycin excited at the excitation wavelength of Trp, 280 nm, is shown in Fig. 2. In our hands, daptomycin exhibited emission peaks at 362 nm (typical of Trp) and at 467 nm (typical of Kyn) in solution. In the presence of neutral POPC liposomes with 5 mM $CaCl_2$ there was (relative to daptomycin in solution) a minor increase in fluorescence intensity and an 8-nm shift of the emission peak at 362 nm, and an eightfold increase in fluorescence with an 8-nm shift of the emission peak at 467 nm. In the presence of acidic (1:1) POPC/POPG liposomes and 5 mM Ca^{2+}, there was a fivefold reduction in fluorescence intensity of the Trp emission peak and a 40-fold increase in intensity coupled with a 20-nm shift of the Kyn emission peak compared to daptomycin in solution. Based on these changes in fluorescence intensity, FRET efficiencies of 0.99, 0.94 and 0.92 were calculated for daptomycin in 5 mM Ca^{2+}/POPC/POPG liposomes, free solution and 5 mM Ca^{2+}/POPC liposomes, respectively. Note that changes in fluorescence intensity which arise from the interaction of Trp or Kyn (assumed to interact in the same manner as Trp) with the membrane were taken into account. The higher

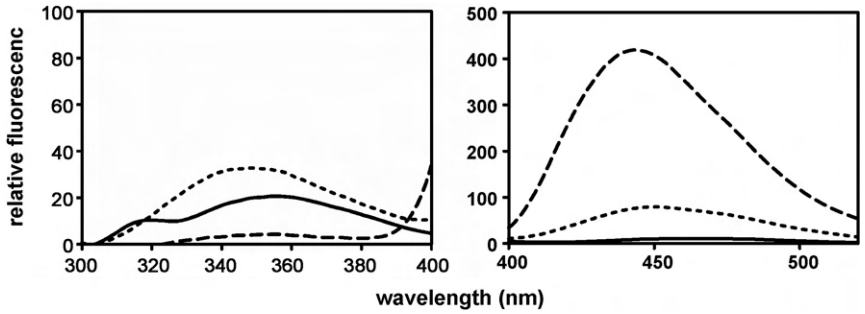

Fig. 2. Fluorescence emission spectra of daptomycin in aqueous solution without 5 mM Ca^{2+} (solid line), in (1:1) POPC/POPG liposomes with 5 mM Ca^{2+} (large dashed lines), and in POPC liposomes with 5 mM Ca^{2+} (small dashed lines). Emission spectra were recorded with an emission wavelength between 300 and 522 nm and an excitation wavelength of 280 nm, previously used to study daptomycin (Grunewald et al., 2005). A final lipid to peptide molar ratio of 30:1 was used with the final concentration of daptomycin being 5 μM.

FRET efficiency in POPC/POPG liposomes indicates that Kyn and Trp were closer together when interacting with acidic lipid bilayers, consistent with a conformational change in daptomycin in acidic liposomes relative to that in neutral liposomes.

3.2. Membrane interaction of daptomycin: differential scanning calorimetry (DSC)

At defined temperatures, phospholipids undergo phase transitions that reflect changes in the structural organization and dynamics of the lipids. DSC was used to examine the effects of daptomycin on lipid phase transitions of DMPG, DMPC, and a 1:1 mixture of DMPC/DMPG. DSC endothermic scans of DMPG bilayers in the presence and absence of Ca^{2+} and/or daptomycin at a lipid:peptide ratio of 100:1 are depicted in Fig. 3. In the absence of both Ca^{2+} and daptomycin, bilayers composed of DMPG produced a single endothermic transition peak at 23 °C (Fig. 3a and b, solid line), in agreement with the literature (Riske et al., 2003). Adding varying amounts of Ca^{2+} to DMPG had a number of effects on the appearance of the DSC endotherms. Addition of 2 mM Ca^{2+} to DMPG caused the transition peak temperature to increase to 25 °C (Fig. 3c, solid line), which can be attributed to changes in the surrounding electrostatics in the lipid bilayer resulting from the binding of Ca^{2+} to the negatively charged phospholipid headgroup.

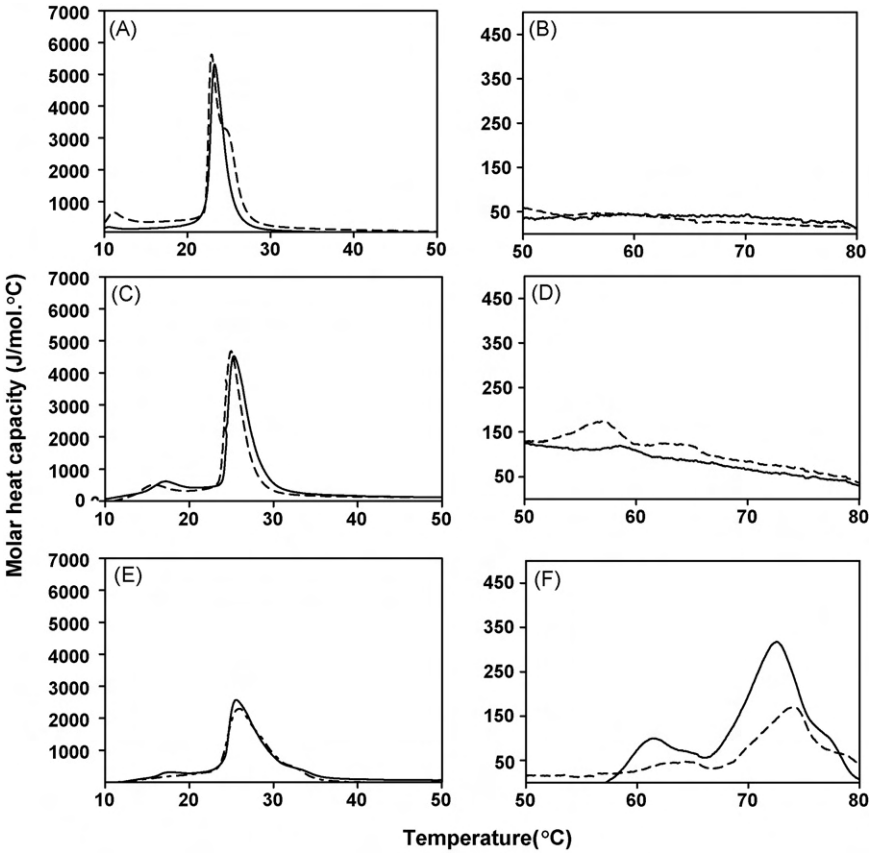

Fig. 3. DSC endothermic scans of DMPG as a function of the presence/absence of daptomycin and/or calcium. (A) and (B) Lower and upper transition temperature scans of DMPG alone (solid line) and with daptomycin (dashed line), (C) and (D) lower and upper transition scans of DMPG with 2 mM Ca^{2+} (solid line) and with 2 mM Ca^{2+} and daptomycin (dashed line), and (E) and (F) lower and upper transition scans of DMPG with 5 mM Ca^{2+} (solid line) and with 5 mM Ca^{2+} and daptomycin (dashed line). All scans were taken between 5 and 90 °C at a rate of 0.333 °C/min. A final concentration of 25 mg/ml of lipid was used with a lipid to peptide molar ratio of 100:1.

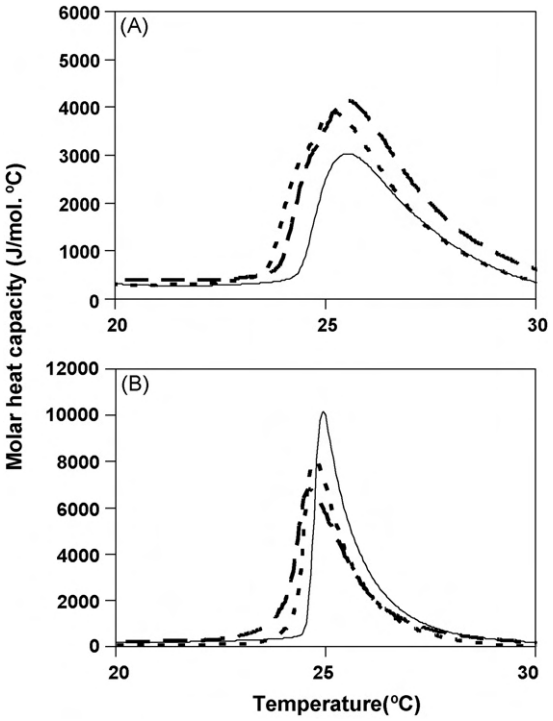

Fig. 4. DSC endothermic scans of increasing amounts of daptomycin with 5 mM Ca^{2+} in DMPG (A), and DMPC (B). Scans were taken between 5 and 90 °C at a rate of 0.333 °C/min with a lipid to peptide molar ratio of 500:1 (solid line), 75:1 (small dash line), and 50:1 (large dash line).

Increasing the added Ca^{2+} concentration from 2 to 5 mM caused a further increase in the low transition temperature to 26 °C and a broadening of the transition peak (Fig. 3e, solid line), indicative of a reduction in cooperativity of the phase transition. At both Ca^{2+} concentrations, a minor transition peak at 16 °C was observed. In addition, a single weak peak with a higher transition temperature, 59 °C, was detected with the addition of 2 mM Ca^{2+} (Fig. 3d, solid line), while the addition of 5 mM Ca^{2+} to DMPG led to a prominent higher temperature transition peak at 74 °C (Fig. 3f, solid line).

The addition of daptomycin to DMPG alone, DMPG + 2 mM Ca^{2+} or DMPG + 5 mM Ca^{2+} had no substantial effect on the main transition temperature (Fig. 3a, c and e, dashed lines). However daptomycin addition to DMPG + 2 mM Ca^{2+} resulted in the appearance of a transition peak at 56 °C and a broad peak around 64 °C (Fig. 3d), whereas its addition to DMPG + 5 mM Ca^{2+} caused a shift to higher temperature of both transitions at 61 and 72 °C observed for the calcium + DMPG case (Fig. 3f, dashed line). Most importantly, the presence of 5 mM calcium and daptomycin decreased the transition enthalpy for these high temperature transitions relative to calcium alone. Finally, increasing the concentration of daptomycin by up to 10-fold did not significantly change the main transition temperature, but it caused a modest increase in the transition enthalpy for this transition temperature (Fig. 4a).

For the mixture of DMPC/DMPG (1:1), the DSC thermogram exhibited two endothermic peaks, one at 16 °C and another at 25 °C (Fig. 5). In the presence of 5 mM Ca^{2+}, an increase of 9 °C in the transition peak temperature was observed with a broadening and reduction in the overall enthalpy of both transition peaks. Adding daptomycin to (1:1) DMPC/DMPG in the presence of 5 mM Ca^{2+} caused a 2 °C reduction in the phase transition temperature of the higher temperature phase transition (Fig. 5). Finally, the DSC endotherm for the neutral DMPC bilayers displayed a major transition peak at 23 °C, and a minor pre-transition peak at 16 °C. Addition

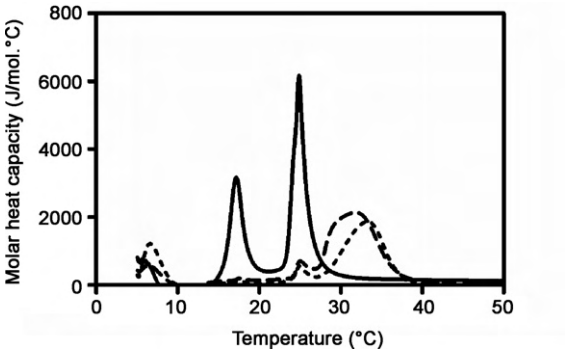

Fig. 5. DSC endothermic scans of DMPC/DMPG (1:1) as a function of the presence/absence of daptomycin and/or calcium: DMPC/DMPG alone (solid line); DMPC/DMPG with 5 mM Ca^{2+} (small dashed line); and DMPC/DMPG with 5 mM Ca^{2+} and daptomycin (large dashed line). All scans were taken between 5 and 90 °C at a rate of 0.333 °C/min. A final concentration of 25 mg/ml of lipid was used with a lipid to peptide molar ratio of 100:1.

of Ca^{2+} in this case had no effect (data not shown), as would be expected since electrostatics do not play a role in this system. Furthermore addition of daptomycin in the presence of 5 mM Ca^{2+} did not result in any new phase transition peaks, causing only a minor decrease in the transition temperature and a modest decrease in the transition enthalpy (Fig. 4b). Overall, daptomycin most perturbed the DMPG containing bilayers in the presence of high calcium concentrations. Note that daptomycin added to either DMPC, DMPG, or DMPC/DMPG without calcium did not have any effect on the transition temperatures nor the enthalpies of the lipids. This indicated that daptomycin binding to the membrane is mediated by Ca^{2+}.

To further characterize the effect of daptomycin on model membrane bilayers, DSC was also used to determine the effects of daptomycin with or without Ca^{2+} on membrane curvature by monitoring the L_α to H_{II} phase transition temperature (T_H) of DiPoPE. The DSC thermogram of pure DiPoPE demonstrated a T_H of 43.4 °C as indicated by the vertical line (Fig. 6). Addition of daptomycin alone, at daptomycin to lipid ratios of 1:500, increased the transition temperature by nearly 2 °C, while 5 mM Ca^{2+} caused a 3 °C decrease in the transition. When daptomycin was added in the presence of 5 mM Ca^{2+}, a modest increase in the phase transition temperature was observed (relative to the case of 5 mM Ca^{2+} + DiPoPE), signifying that an overall positive curvature strain was induced.

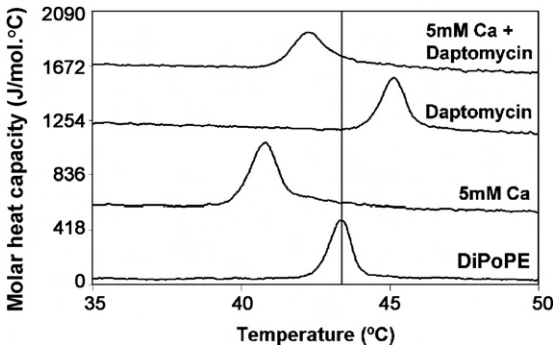

Fig. 6. Differential scanning calorimetry of the lamellar (L_α) to invert hexagonal (H_{II}) phase transition of DiPoPE under the influence of daptomycin and calcium with, from bottom, DiPoPE alone, DiPoPE and 5 mM calcium, DiPoPE and daptomycin and DiPoPE, 5 mM calcium and daptomycin. Peptide, lipid and calcium were co-dissolved in a chloroform/methanol solution, dried and suspended in 10 mM HEPES, 150 mM NaCl, pH 7.4 to yield multilamellar vesicles at a working concentration of 7.5 mg/ml lipid. Where applicable, peptide to lipid molar ratio was 1:500 and calcium concentration was 5 mM. The vertical line indicates the transition temperature of pure DiPoPE (T_H = 43.4 °C) with no added peptide.

3.3. ^{31}Phosphorous NMR spectroscopy

^{31}P NMR was used to further characterize the lipid phase transitions, since changes from lamellar (bilayer) to non-lamellar (hexagonal and micellar) phase alter the orientation of the phosphorous headgroup of lipids, leading to changes in the shape of the ^{31}P NMR powder spectrum. Therefore ^{31}P NMR spectra (Fig. 7) were recorded at selected temperatures, under the conditions corresponding to those employed for the DSC studies.

The ^{31}P NMR spectrum of DMPG multilamellar vesicles (MLVs) at temperatures below the gel to liquid crystalline transition was a typical bilayer powder spectrum with a chemical shift anisotropy (CSA) of ca. 78 ppm, in agreement with previous literature reports (Heimburg and Biltonen, 1994; Richard et al., 2002). As the temperature was increased past the transition temperature of 23 °C, the CSA was reduced to approximately 45 ppm at 31 °C, indicative of a change from gel to fluid phase (Fig. 7, left column). A further increase in temperature up to 80 °C resulted in a ^{31}P spectrum which consisted of a powder pattern with a CSA of around 40 ppm superimposed on an isotropic peak. The addition of 5 mM Ca^{2+} to DMPG did not significantly affect the peak shape and CSA observed at the lower temperatures (Fig. 7, middle column) (Cullis and De Kruyff, 1976), consistent with the results observed for DSC. At higher temperatures (≥62 °C) (Fig. 7), however, the width of the spectrum was narrower, with reversed symmetry compared to the bilayer spectra observed at lower temperature. This is typical of the non-lamellar hexagonal phase and has previously been observed upon addition of Ca^{2+} to phosphatidylethanolamine (Cullis and Verkleij, 1979). Addition of daptomycin to DMPG in the presence of 5 mM Ca^{2+} further affected the appearance of the ^{31}P NMR spectra (Fig. 7, right column). Below the main transition temperature, the spectrum at 17 °C consisted of a powder pattern with a CSA of approximately 78 ppm, overlapping with a sharp peak at 0 ppm. This isotropic peak at 0 ppm was present at higher temperatures as well and was dominant at a temperature of 62 °C and higher. The presence of an isotropic peak could be attributed to a number of different effects of daptomycin on DMPG including (i) distortion of the membrane at the lipopeptide binding site; (ii) the formation of small vessicles or micelles; and/or (iii) development of lipid structures with high degrees of curvature (Heimburg and Biltonen, 1994).

Additional spectra in DMPC and DMPC/DMPG (1:1) mixtures were also acquired at the lower temperatures in the presence/absence of 5 mM Ca^{2+} and/or daptomycin (data not shown). In the zwitterionic lipid DMPC, no significant changes in the CSA or shape were observed upon addition of calcium and daptomycin, indicating that the interactions between DMPC and these components are weak. This further supported the DSC data discussed above. In the 1:1 mixture of DMPG and DMPC, bilayer phase was observed at both 17 and 32 °C, with the only unusual observation being a decrease in peak width from 80 ppm for both lipid types to 70 and 40 ppm, respectively, indicating that in this lipid mixture daptomycin/5 mM Ca^{2+} caused increased motion of the lipid headgroup.

3.4. Membrane fusion

It is believed that membrane fusion proceeds through the formation of fusion-intermediates that involve the transition from lamellar bilayer to non-lamellar hexagonal phases. Both

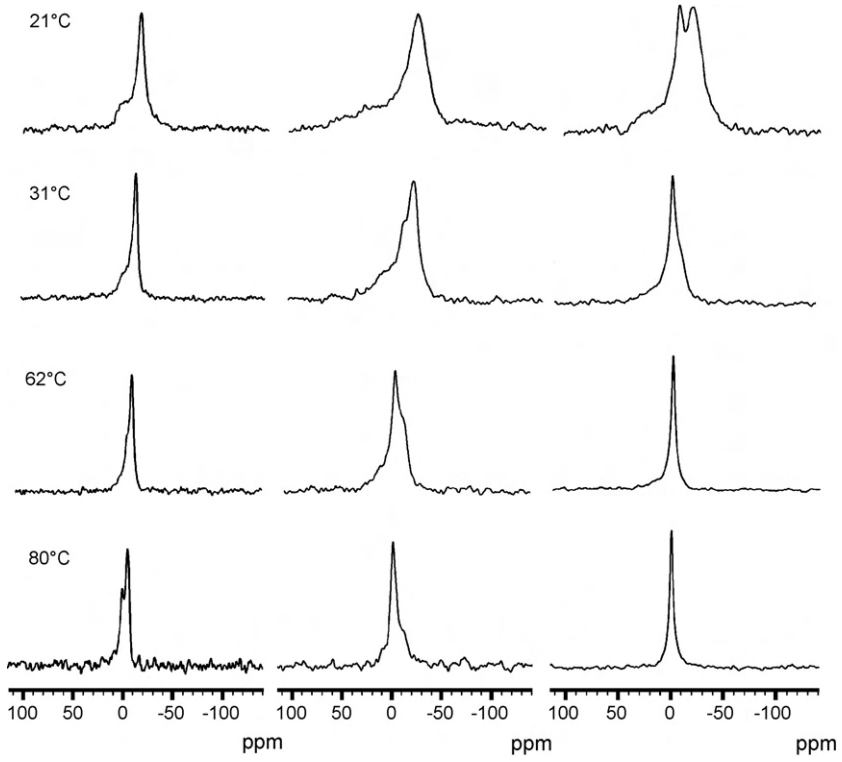

Fig. 7. ^{31}P NMR spectra of DMPG alone (left column), DMPG with 5 mM Ca^{2+} (middle column), and DMPG with 5 mM Ca^{2+} and daptomycin (right column). Spectra were collected using a lipid to peptide molar ratio of 100:1, with the final lipid concentration being 25 mg/ml. The peaks were fitted to determine the percentage for each phase under each condition. These are for DMPG alone (going from 21 to 80 °C): gel (100%); fluid (100%); fluid (100%); and fluid (61.5%) and hexagonal phase (38.5%). For DMPG with 5 mM Ca^{2+} (middle column), going from 21 to 80 °C: gel (100%); fluid (100%); hexagonal (100%); and hexagonal phase (100%). And finally, for DMPG with 5 mM Ca^{2+} and daptomycin (right column), going from 21 to 80 °C: fluid (45%) and hexagonal (55%); hexagonal (100%); isotropic (100%); and isotropic phase (100%). The spectra were referenced to external 85% H_3PO_4 (0 ppm).

the above DSC and ^{31}P NMR data indicated that the addition of daptomycin/Ca^{2+} to acidic membranes greatly reduced the transition enthalpies at which these phase changes occurred. Vesicle sizes of large unilamellar liposomes in the presence and absence of 5 mM Ca^{2+} and daptomycin are shown in Fig. 8a. The addition of 5 mM Ca^{2+} or daptomycin alone to large unilamellar liposomes consisting of either POPC or (1:1) POPC/POPG caused no significant increase in vesicle size. Similarly, there was also no significant size increase when daptomycin/5 mM Ca^{2+} was added to POPC liposomes. However, when daptomycin/5 mM Ca^{2+} was added to POPC/POPG (1:1) liposomes, a sixfold increase in vesicle size occurred. To confirm that the vesicle size increase of large unilamellar (1:1) POPC/POPG liposomes was the result of membrane fusion, we monitored the fluorescence resonance energy transfer due to fusion of (1:1) POPC/POPG liposomes labelled with either NBD-PE or Rh-PE and mixed together in the presence of daptomycin/5 mM Ca^{2+} (Fig. 8b). The results indicated that daptomycin was able to induce a significant amount of membrane fusion at all concentrations tested with maximum fusion occurring at 8 μg/ml daptomycin.

4. Discussion

Earlier studies on daptomycin (Silverman et al., 2003) showed that daptomycin inserts into membranes in a Ca^{2+}-dependent manner. In our previous work (Jung et al., 2004), we concluded through a series of detailed structural and functional studies that daptomycin underwent one structural transition upon the binding of Ca^{2+} in solution and another upon the binding of Ca^{2+} in the presence of acidic membranes. From these observations, we proposed that the mechanism of action of daptomycin involved two calcium-dependent structural transitions with the first transition resulting in an increase in the amphipathicity and a reduction in the overall net negative charge, which enables it to interact with both acidic and neutral liposomes and cause lipid flip-flop. In Straus and Hancock (2006), we proposed that these effects probably reflect instead oligomerization of daptomycin. The second transition was proposed to result in deeper insertion of daptomycin into acidic liposomes leading to greater perturbation of acidic membranes and membrane leakage. Here we have provided further evidence in support of this latter concept and demonstrate that the insertion of daptomycin/Ca^{2+} into membranes containing acidic phospholipids leads to a perturbation of lipid structure.

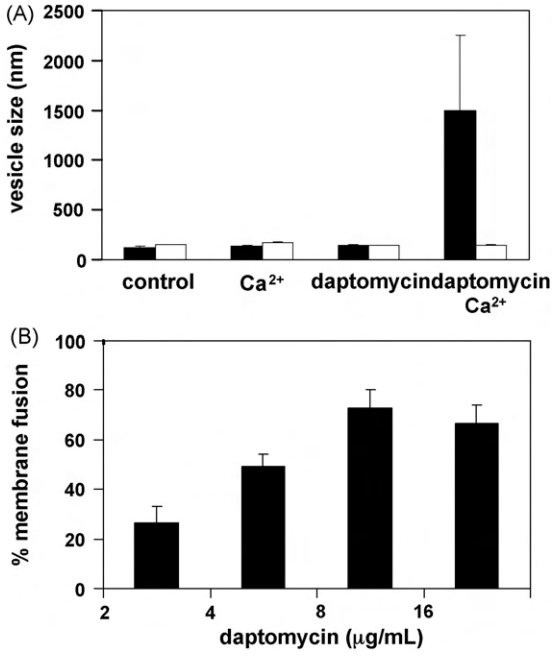

Fig. 8. Membrane fusion caused by daptomycin. (A) Size determination of large unilamellar (1:1) POPC/POPG liposomes (black bars) and POPC liposomes (white bars) alone, with 5 mM Ca^{2+}, daptomycin, and daptomycin with 5 mM Ca^{2+}. (B) Dose-dependent fusion of POPC/POPG liposomes caused by the addition of daptomycin with 5 mM Ca^{2+}.

Fluorescence analysis of the two fluorophores in daptomycin (Trp-1 and Kyn-13) revealed that the Trp residue was relatively accessible to KI in solution but not in the presence of either neutral or acidic membranes, while Kyn was not accessible to KI in solution or acidic membranes. Consistent with the concept of a structural transition in daptomycin upon interaction with acidic membranes, FRET studies indicated that the Trp residue moved closer to the Kyn residue in acidic (PC/PG) membranes, relative to its positioning in solution or in neutral (PC) liposomes. The inaccessibility of Kyn to KI in solution suggests that Kyn was somehow hidden from the aqueous environment, possibly by aggregation. Indeed both we (Straus and Hancock, 2006) and others (Ball et al., 2004; Rotondi and Gierasch, 2005) were recently able to demonstrate using NMR that daptomycin is able to self-aggregate in solution in the presence of calcium. We previously noted a large change in the CD spectrum of daptomycin/Ca^{2+} induced by the presence of acidic (PC/PG) liposomes (Jung et al., 2004). This change involved a flip from positive to negative molar ellipticity between the wavelengths 210 and 220 nm, and from negative to positive molar ellipticity at 233 nm. Although it was previously concluded that these changes in molar ellipticity reflected a conformational change in daptomycin, the data presented here are consistent with the alternative or additional explanation that they reflect aggregation, and particularly changes in the local environments of Trp and Kyn.

Changes in the transition temperature and in the transition enthalpy of the gel to fluid phase have been used to predict and categorize the binding and interaction of peptides and proteins with model membranes (Epand and Surewicz, 1984). Generally speaking, peptides that bind to membranes at the lipid–water interface through mainly electrostatic interactions are characterized by a progressive increase in transition enthalpy as a function of increasing peptide concentration. Consistent with this, the addition of increasing amounts of daptomycin/Ca^{2+} to acidic membranes caused progressive but modest increases in the transition enthalpy and minor changes in the transition temperature of the gel to fluid phase. Conversely, addition of increasing amounts of daptomycin/Ca^{2+} to neutral membranes lead to a modest (relative, e.g. to LL-37 Henzler-Wildman et al., 2004) reduction in the transition enthalpy and the temperature of the gel to fluid phase, a behaviour that is similar to that of peptides that interact with membranes through a combination of hydrophobic and electrostatic interactions and are normally found partially inserted into the bilayer. Thus we can conclude that daptomycin in the presence of Ca^{2+} binds in a different fashion to acidic and neutral membranes. This finding is supported by previously reported data that demonstrated more substantial changes in the functional properties of acidic membranes (Jung et al., 2004). Presumably the electrostatic bridging by Ca^{2+} of acidic phospholipid head groups with the acidic amino acids in daptomycin is at the root of these differences in DSC profiles.

The DSC and ^{31}P NMR data presented here clearly indicate that the addition of daptomycin had a strong effect on the structural organization of acidic membranes in a manner dependent on Ca^{2+}. The addition of 5 mM Ca^{2+} to pure DMPG bilayers resulted in a bilayer to hexagonal phase transition temperature of $\geq$62 °C, and caused only a minimal increase in vesicle size of large unilamellar POPC/POPG liposomes. The addition of daptomycin in the presence

of 5 mM Ca^{2+} resulted in the presence of an isotropic peak in the ^{31}P NMR spectra at all temperatures measured, and a sixfold, non-uniform, increase in vesicle size of POPC/POPG liposomes. It seems likely that the binding of daptomycin at the lipid–water interface of acidic membranes would neutralize the electrostatic charge of the PG headgroup, thus reducing its effective headgroup area which might lead to the induction of non-lamellar phases that would favour fusion. Interestingly a marked effect on the ^{31}P NMR spectra was also noted in daptomycin–2 mM Ca^{2+} but no major changes were observed even with daptomycin–5 mM Ca^{2+} in neutral DMPC bilayers, consistent with the concept that daptomycin–5 mM Ca^{2+} interacted superficially with neutral membranes. Conversely, by monitoring the L_α to H_{II} phase transition temperature of a lipid that favours structural transitions to non-lamellar phases, DiPoPE, we were able to determine that, in the presence of 5 mM Ca^{2+}, at daptomycin to lipid ratios of only 1:500, a modest increase in the phase transition temperature was observed signifying an overall induction of positive curvature strain analogous to that caused by cationic peptides like LL-37.

The changes in bilayer properties are quite reminiscent of those caused by cationic antimicrobial peptides (Henzler-Wildman et al., 2004; Mani et al., 2004; Thennarasu et al., 2005), leading us to speculate that nature has provided two alternative routes to compounds that perturb membranes as part of their mechanism of action, namely strongly cationic amphipathic peptides and the strongly anionic lipopeptide daptomycin in which the negatively charged residues are partially neutralized by Ca^{2+}.

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