Comparison of reversible membrane destabilisation induced by antimicrobial peptides derived from Australian frogs ☆

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ABSTRACT

The membrane destabilising properties of the antimicrobial peptides (AMP) aurein 1.2, citropin 1.1, maculatin 1.1 and caerin 1.1, have been studied by dual polarisation interferometry (DPI). The overall process of peptide induced membrane destabilisation was examined by the changes in bilayer order as a function of membrane-bound peptide mass per unit area and revealed three different modes of action. Aurein 1.2 was the only peptide that significantly destabilised the neutral membrane (DMPC), while all four peptides induced destabilisation of the negatively charged membrane (DMPC/DMPG). On DMPC, citropin 1.1, maculatin 1.1 and caerin 1.1 bound irreversibly at low concentrations but caused a reversible drop in the bilayer order. In contrast to DMPC/DMPG, these three peptides caused a mass drop at the higher concentrations, which may correspond to insertion and bilayer expansion. The critical level of bound peptide necessary to induce membrane destabilisation (peptide:lipid ratio) was determined and correlated with peptide structure. As the most lytic peptide, aurein 1.2 adsorbed strongly prior to dissolution of the bilayer. In contrast, the binding of citropin 1.1, maculatin 1.1 and caerin 1.1 needed to reach a critical level prior to insertion into the membrane and incremental expansion and disruption. Our results demonstrate that sequential events can be monitored in real-time under fluidic conditions to elucidate the complex molecular mechanism of AMP action. In particular, the analysis of birefringence in real time allows the description of a detailed mechanistic model of the impact of peptides on the membrane bilayer order. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

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1. Introduction

Currently, one of the major challenges facing the medical field is the increase in frequency of resistance to multiple antibiotics [1–3]. The antimicrobial peptides (AMPs) are widely distributed and are excellent candidates for the development of effective therapeutics due to their ability to kill a broad spectrum of bacteria, fungi and cancer cells [4], commonly exerting their effects within minutes as compared to hours for common antibiotics via destabilisation of the cell membrane. However, better understanding of the interplay between AMPs and lipid membranes and their selectivity and mechanism of action is necessary for the design of selective and potent antibacterial peptides.

The skin secretions of many amphibians are rich sources of novel compounds that include highly potent AMPs [5]. In this study, we have investigated the membrane interaction of four peptides isolated from several species of Australian tree frogs, namely aurein 1.2, citropin

1.1, maculatin 1.1 and caerin 1.1. Previous studies have shown that these four peptides exhibit selectivity against G(+) bacteria and are less effective or inactive against G(−) bacteria [6]. The sequence and structural features of these peptides are central to their antimicrobial activity and selectivity. Each of these peptides is cationic with a calculated pI between 9.9 and 10.6. Despite differences in length, aurein 1.2 (13 residues), citropin 1.1 (16 residues), maculatin 1.1 (21 residues) and caerin 1.1 (25 residues), all have high sequence homology in the N- and C-termini. The increase in length for each peptide roughly coincides with the insertion of one helical turn [7]. Based on structural analysis using FTIR, CD and NMR, these peptides are predominantly unstructured in aqueous solution. However, they readily adopt an amphipathic α-helical structure in membrane mimetic environments [7]. This ability to fold into an amphipathic α-helical conformation in the vicinity of a membrane has been recognised as a critical factor in facilitating the initial interaction of the peptide with the lipid bilayer [7–9]. NMR studies of these peptides in the membrane mimetic environments show that the shorter peptides, aurein 1.2 and citropin 1.1, adopt a single continuous α-helix while the longer peptides, maculatin 1.1 and caerin 1.1, comprise two α-helices separated by a flexible hinge region induced by the presence of one and two proline residues, respectively [7]. The kink structure caused by the proline residues is crucial for

their activity and may be important for membrane interaction. Based on solid-state NMR and FTIR results together with dye leakage from GUVs, the modes of action of these peptides are closely linked to their helical length [10]. Aurein 1.2 and citropin 1.1 are short peptides, which hinder their ability to fully penetrate the lipid bilayer, and destabilise the membrane via binding to the interfacial region of membrane [7,11]. The longer peptides, maculatin 1.1 and caerin 1.1, may insert and disrupt the membrane through the formation of pores [7,12,13] as a result of their α-helical length allowing the peptide to penetrate and span the lipid bilayer. Characteristics such as sequence, length, secondary structure, overall net charge, hydrophobicity and amphipathicity, are linked to AMP activity which, therefore, make these four peptides a suitable set for comparison of AMP mechanisms of action and their roles in membrane destabilisation.

To date, the exact mode of action of these peptides is debated, with peptide hydrophobicity, hydrophobic moment, and lipid composition and charge of the target cell membrane also proposed to contribute to the peptide ability to perturb the cellular membrane [14]. However, without the information on the role of the lipid bilayer and the structural and dynamic changes of the bilayer during this interaction, our understanding of the mechanism of AMP action may be over-simplified [15]. We have previously studied the membrane binding characteristics of aurein 1.2 and maculatin 1.1 using SPR and DPI [9,12,16,17]. In particular, we demonstrated that both peptides exert a significant effect on the membrane bilayer structure but, in the case of aurein 1.2, the bilayer was irreversibly disrupted [17] while, in the presence of maculatin, the bilayer was able to recover [9,12]. These results demonstrate that understanding changes in the bilayer structure during peptide binding is as important as analysing the peptide structure and membrane affinity. In the present study we have expanded our analysis to investigate the membrane-disrupting properties of citropin 1.1 and caerin 1.1 and allow a comparative analysis of the membrane disruptive properties across the four peptides. We have used model membrane systems to represent both eukaryotic and bacterial membranes. Specifically, zwitterionic phospholipids such as dimyristoylphosphatidylcholine (DMPC) are used to mimic eukaryotic membranes while the anionic dimyristoylphosphatidylglycerol (DMPG) is used to mimic the properties of negatively charged bacterial cell membranes. We deposited stable membranes on a planar silicon oxynitride biosensor chip which were then used to study the changes in membrane structure throughout the process of binding, insertion and membrane lysis by these four frog peptides in real time using DPI technology [18–20]. This technique provides simultaneous quantification of real time changes in the thickness, mass density and birefringence of the membrane. We hypothesise that changes in birefringence as a function of the amount of each peptide bound to the membrane provide unique insight into the mechanism of binding of these four peptides, and that the disruption of the membrane by the four peptides is preceded by substantial membrane structure changes that can be measured by DPI. The aim of this study, therefore, was to examine and compare the changes in the lipid bilayer structure throughout the sequence of events from initial electrostatic binding of the peptide to the final membrane destabilisation induced by aurein 1.2, citropin 1.1, maculatin 1.1 and caerin 1.1.

2. Materials and methods

2.1. Chemicals and reagents

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] sodium salt (DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL). 4-morpholinepropanesulfonic acid (MOPS), sodium chloride, calcium chloride and sodium dodecylsulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MI). Chloroform, methanol, and ethanol were all HPLC-grade solvents purchased from Merck (Darmstadt, Germany). Hellmanex II was obtained from Hellma (Müllheim, Germany). Bovine serum albumin (BSA) was purchased from ThermoFisher Scientific, Scoresby, Australia. Water was quartz-distilled and deionised in a Milli-Q system equipped with UV oxidation to remove organic residue (Millipore, Bedford, MA). Aurein 1.2, citropin 1.1, maculatin 1.1 and caerin 1.1 were purchased from Mimotopes (Melbourne, Australia) and the sequences and molecular properties are listed in Table 1. The purity of peptides (~95%) was analysed using a capillary reversed phase C18 column (Zorbax SB 0.5 × 150 mm, 5 μm, 300 Å, Agilent, St. Clara, CA) using an appropriate 0–60% 0.1% trifluoroacetic acid-acetonitrile gradient. These peptides were further confirmed by ESI-ion trap (1100 series, LC/MSD Trap, Agilent). Peptide concentration was determined by amino acid analysis.

2.2. Liposome preparation

2 mM DMPC stock in chloroform and 2 mM DMPG stock in chloroform/methanol (3:1 v/v) were used for the preparation of dried DMPC and DMPC/DMPG (molar ratio 4:1) films. Appropriate volume of lipid stock solutions was used to make the total lipid amount of 0.8 μmol. The solvent was then evaporated under a gentle stream of N$_2$ gas and further vacuum dried overnight. The dried lipid thin films were hydrated with the running buffer (10 mM MOPS, pH 7.0, 150 mM NaCl) to make a lipid concentration of 1 mM. The liposome solution was then extruded through a 50 nm polycarbonate membrane 21 times using Liposofast extruder (Avestin, Ottawa, Canada). The size distribution of resulting small unilamellar vesicles (SUVs) was characterised by dynamic light scattering with a Malvern Zetasizer 3000 (Malvern Laboratories Ltd., Malvern, UK).

2.3. Dual polarisation interferometry

Dual polarisation interferometry (DPI) is an analytical method for analysing thin films using a dual optical waveguide interferometric technique [18–20]. Alternate dual orthogonal polarisation allows unique combinations of several opto-geometrical properties, including refractive index (RI), density, thickness, mass and birefringence, to be measured in real time for the layer formation of biomolecules. DPI (Analight BIO200, Farfield Group Ltd., Manchester, UK) comprises a dual slab waveguide sensor chip with an upper sensing waveguide and a lower optical reference waveguide illuminated with an alternating polarised laser beam (HeNe, wavelength 632.8 nm). Two orthogonal

Table 1
Characteristics of AMPs from Australian tree frogs used in this study.

Peptide	Sequence																									Mass	No AA	Charge	[H]	[μH]
	1				5					10					15					20					25						
Aurein 1.2	G	L	F	D	I	I	K	K	–	–	–	–	–	–	–	–	–	–	–	–	I	A	E	S	F	1478	13	+1	0.582	0.765
Citropin 1.1	G	L	F	D	V	I	K	K	V	A	S	–	–	–	–	–	–	–	–	V	I	G	G	L	–	1613	16	+2	0.623	0.614
Maculatin 1.1	G	L	F	G	V	L	A	K	V	A	A	–	–	–	–	H	V	V	P	A	I	A	E	H	F	2145	21	+1	0.435	0.435
Caerin 1.1	G	L	L	S	V	L	G	S	V	A	K	H	V	L	P	H	V	V	P	V	I	A	E	H	L	2583	25	+1	0.734	0.321

[H]: Overall hydrophobicity; [μH]: Mean amphipathic moment. The peptides are aligned according to their sequence homology.
Each peptide has a free N-terminus and a C-terminal amide.