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Review

Membrane interactions of antimicrobial peptides from Australian tree frogs

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Abstract

The skin secretions of amphibians are rich in host defence peptides. The membrane interactions of the antimicrobial peptides, aurein 1.2, citropin 1.1 and maculatin 1.1, isolated from Australian tree frogs, are reviewed. Although all three peptides are amphipathic α-helices, the mode of action of these membrane-active peptides is not defined. The peptides have a net positive charge and range in length from 13 to 21 residues, with the longest, maculatin 1.1, having a proline at position 15. Interestingly, alanine substitution at Pro-15 leads to loss of activity. The effects of these peptides on phospholipid bilayers indicate different mechanisms for pore formation and lysis of model membranes, with the shorter peptides exhibiting a carpet-like mechanism and the longest peptide forming pores in phospholipid bilayer membranes.
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Keywords: Peptide–lipid interactions; Model membranes; Antibacterial peptides; Solid-state NMR; Pore formation

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1. Introduction

Host defence peptides are known to protect amphibians against a variety of pathogens [1]. The skin secretions of Australian tree frogs are rich in anti-bacterial peptides [2]. In order to exert their bioactive effects, the peptides must penetrate the cell membrane and the means by which they destroy bacteria is possibly by membrane lysis. The membrane interactions of peptides from Australian tree frogs have been studied, in particular, maculatin 1.1, citropin 1.1 and aurein 1.2; and also the peptides caerin 4.1 and caerin 1.1 but to a lesser extent. The amino acid sequences of these peptides [3–6] are given in Table 1.

The focus of this review is the antibacterial effect of these peptides. However, the peptides are known to demonstrate other bioactivity, including e.g. anti-cancer (aurein 1.2, caerin 1.1, citropin 1.1, maculatin 1.1) and both fungicidal and specific neuronal nitric oxide synthase inhibition (caerin 1.1, citropin 1.1, maculatin 1.1) [2]. Four of the peptides demonstrate anti-bacterial activity against both Gram-positive and Gram-negative species. The remaining peptide, caerin 4.1, shows a more specific range of antibacterial effect, preferentially lysing Gram-negative bacteria, including *Pasteurella haemolytica*, which causes swine fever. The antibacterial effects of the peptides are listed in Table 2.

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Table 1
Amino acid sequence of selected antibacterial peptides from Australian tree frogs

Peptide	Amino acid sequence	MW	AA	Net charge
Aurein 1.2 [3]	GLFDIIKKIAESF-NH_2	1478	13	+1
Caerin 1.1 [4]	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	2582	25	+3
Caerin 4.1	GLWQKIKSAAGDLASGIVEGIKS-NH_2	2326	23	+4
Citropin 1.1 [5]	GLFDVIKKVASVIGGL-NH_2	1613	16	+2
Maculatin 1.1 [6]	GLFGVLAKVAAHVVPAIAEHF-NH_2	2145	21	+3

Host defence peptides are produced by Australian tree frogs as inactive three part peptides: a signal peptide, a spacer peptide and the anti-bacterial peptide. After synthesis the peptides are transported to storage glands on the dorsal surface of the animal, where the signal peptide is cleaved by an endopeptidase. The resulting spacer-active peptide combination does not exhibit antibacterial effect. Upon appropriate stimulation, the spacer peptide is cleaved by a second endopeptidase and the active peptide is secreted onto the dorsal surface of the amphibian [7]. The peptides are deactivated by a third endopeptidase, deactivation occurring between 5–30 min after secretion depending upon the species of frog [8]. The enzymes appear to be membrane proteins [8] and deactivate these membrane-active peptides by removal of residues from the N-terminus [5]. The peptides, many of which have major wide-spectrum antibacterial properties, are expressed in the skin secretions when the frog is stressed.

2. Common structural motifs

The peptides discussed in this study consist of between 13 (aurein 1.2) and 25 (caerin 1.1) amino acid residues. Each of the peptides are cationic around neutral pH, with a net positive charge between +1 (aurein 1.2) and +4 (caerin 1.1). Features common to these peptides include a tendency towards random coil arrangement in aqueous solution and an α-helical structure in membrane mimetic environments [3–6]. The helixes are amphipathic with polar side chains aligning along one face of the α-helix.

The primary structures of the peptides have several notable features. The N-terminus of each peptide consists of two common residues, glycine and leucine. Aurein 1.2, citropin 1.1 and maculatin 1.1 share a third common N-terminal amino acid, phenylalanine. Three of the peptides share the motif of adjacent basic amino acids that are essential for anti-bacterial activity [2]. Aurein 1.2 and citropin 1.1 contain lysine residues at positions 7 and 8, while caerin 1.1 contains a lysine–histidine arrangement at positions 11 and 12. Maculatin 1.1 does not contain adjacent basic amino acids, but does have a lysine residue at position 8. Each of the peptides is also C-terminal aminated and, again, this functional group is essential for anti-bacterial action [9].

These peptides may be divided into two groups based upon peptide length and conformation when in membrane mimetic environments. The shorter peptides aurein and citropin both adopt a single continuous α-helix upon membrane binding. The longer peptides comprise a flexible hinge region separating two α-helices. Maculatin 1.1 contains one proline residue while caerin 1.1 has two proline residues, which act to form a hinge region [6,10]. The presence of the proline residue is known to modulate the efficacy of maculatin 1.1 [11] and caerin 1.1. [12]. The region of conformational flexibility of caerin 4.1, on the other hand, contains two glycine residues [13].

3. Models of peptide interaction with lipid membranes

Two principal modes of action for membrane-perturbing peptides have been proposed: pore formation across the lipid bilayer or a 'carpet' mechanism, lysing the membrane in a detergent-like manner [14]. The transmembrane models involve the peptides forming pores through the bacterial outer membrane: the 'barrel-stave' [15] and toroidal pore [16,17] mechanisms. In these models, the peptides oligomerize to form pores through the membrane. The pores act as non-selective channels for ions, toxins and metabolites, thus preventing the bacterium from maintaining homeostasis. Peptides with 20 or more amino acids lend themselves to these mechanisms, as they are able to span the lipid bilayer when in an α-helical conformation.

A key difference between these two mechanisms is the positioning of the head group region of the lipid molecules with respect to the peptide. In the barrel-stave mechanism, the headgroups remain located along the membrane surface, while the pore is formed by the interaction of the peptide within the hydrophobic core of the membrane. The transmembrane pore is lined by the hydrophilic surface of the peptide. By contrast, toroidal pores are formed when the peptides insert in such a way as to cause the inner and outer membrane leaflets to curve and

Table 2
Antibacterial activity of selected peptides from Australian tree frogs [2]

	Aurein 1.2	Caerin 1.1	Caerin 4.1	Citropin 1.1	Maculatin 1.1
Bacillus cereus	50	50	–	50	25
Leuconostoc lactis	6	1.5	–	6	3
Listeria innocua	6	25	–	25	100
Micrococcus luteus	100	12	12	12	12
Staphylococcus aureus	–	3	–	25	6
Staphylococcus epidermidis	50	12	–	12	12
Streptococcus uberis	100	12	–	25	3
Escherichia coli [a]	–	–	25	–	–
Pasteurella multocida [a]	–	25	–	–	50

Minimum inhibitory concentration (μg/mL).
[a] Gram negative bacteria.

the lumen is lined by the hydrophilic surface of the peptide interspersed by the phospholipid headgroups [15]. Preference for barrel-stave vs. toroidal pore may depend on several factors including the peptide length and membrane thinning effect induced by the peptide [15,18]. The α-helical peptide, melittin, the toxin in bee venom, has been shown by solid-state NMR to adopt a transbilayer orientation in aligned phospholipid bilayers [19]. Under similar conditions, melittin appears to form a toroidal pore [15] and, interestingly, the peptide rotates about the helical axis in fluid phase bilayers and this long axis rotation is stopped when the lipid is in the gel phase [19]. Peptides that lyse cells via a pore forming mechanism are predicted to be aligned perpendicular to the membrane surface at high peptide concentrations [18]. Moreover, these transmembrane mechanisms would not solubilize the lipids into smaller (and thus faster tumbling) aggregates.

Alternatively, peptides may lie along the membrane surface before disrupting the bilayer in a detergent like manner. After a threshold concentration of peptides is reached, the peptides permeate the membrane leading to disintegration. In this model, lipids are solubilized by the proteins to form mixed micelles, which could give an isotropic ^{31}P NMR spectral component. This mechanism of membrane perturbation is known as the 'carpet' model [20,21], although it has been suggested this model is simply an extreme form of the toroidal pore model [22]. In the case of the carpet mechanism, the cell membrane is destabilized while for the pore-forming models more gradual leakage would be expected. Upon cell lysis, a random orientation with respect to the bilayer is expected for peptides that cause membrane disruption via the carpet mechanism.

Several experimental techniques have been used in order to determine which of these mechanisms are demonstrated by antibacterial peptides. In the case of the peptides from Australian tree frogs, solid-state NMR, circular dichroism (CD), attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), fluorescence imaging and Langmuir–Blodgett studies of monolayer collapse, have been utilised. The complementary data from these methods allows us to gain a multifaceted insight into the mode of action of these antibacterial peptides.

4. Antibacterial effect—an NMR study

As noted above, several peptides isolated from amphibian skin secretions exhibit broad-spectrum antibacterial activity. A range of experiments has shown that the antibacterial effect of these peptides is caused by lysing the bacterial membrane rather than specific inhibition of a critical enzyme or receptor. Most significantly, Chia et al. have shown that maculatin 1.1 synthesised from all D-amino acids has equivalent activity to the naturally occurring peptide consisting of all L-isomers [6], i.e. chirality has no influence on the activity of the peptide. Moreover, ^{31}P solid-state NMR spectroscopy of live Gram-positive bacteria showed that inoculation with maculatin 1.1 or caerin 1.1 causes a loss of integrity of the bacterial membrane resulting in an isotropic signal (although some residual anisotropic character remained) [23]. The change in the ^{31}P spectra was interpreted as showing that the bacterial membranes were lysed and resulted in the formation of micelle-like structures. By the same token, as expected, the narrow spectrum antibacterial peptide caerin 4.1 (Table 2) did not appear to cause disruption of the Gram positive bacteria *Bacillus cereus* and *Staphylococcus epidermidis* used in the same study. Although these experiments indicate that maculatin 1.1 and caerin 1.1 may operate via the carpet model, due to the increase in isotropic signal of the ^{31}P NMR spectra, studies in model phospholipid membranes suggest a pore-forming mechanism [24]. Electron microscopy results show that maculatin 1.1 severely disintegrates cells of *S. aureus* [6,9], which suggests that in the case of the live bacteria, an extreme case of toroidal pores could be the cause of the isotropic ^{31}P NMR signal. However, since model membranes are used more generally [25], it is difficult to draw definitive conclusions from one NMR study using live bacteria.

5. Surface activity of antibacterial peptides-monolayer studies

Surface interactions of the peptides with lipids have been studied using the Langmuir monolayer method [26]. Application of lateral pressure to a monolayer established at the air/water interface of a water trough reports on the surface properties of the monolayer material. Once the monolayer is compressed to a critical point, the monolayer collapses [27]. The collapse pressure point (Π_c) is a measure of the ability of the system to resist compression and hence is related to the stability of the monolayer. Changes in Π_c upon lipid/peptide mixing are interpreted as a measure of the stabilizing or disrupting effect of the peptide on the lipid monolayer. The effect of the lytic peptide, melittin, on lipid monolayers has been studied using this technique [28].

Monolayer studies of maculatin and citropin have been used to assess the interaction of these peptides with the neutral lipid palmitoyl-oleoyl-phosphatidylcholine (POPC), which is used as a model for eukaryotic membranes, and the anionic lipid palmitoyl-oleoyl-phosphatidylglycerol (POPG), as a model of a bacterial membrane [24]. POPC and POPG monolayers exist in the liquid expanded phase state at room temperature [29]. When maculatin or citropin are mixed with the neutral POPC two separate Π_c events are observed, which was interpreted as the peptide and lipid forming domains in the monolayer. By contrast, in films with the anionic POPG, the peptides showed miscible behaviour and may relate to the specific lytic activity of these peptides in bacterial membranes as opposed to red blood cells. With the application of lateral stress to pure maculatin monolayers with a basic subphase, a discontinuity occurs in the compression isotherm that could be due to a structural reengagement of the peptide. Interestingly, this was not seen for citropin and may be due to the proline hinge in maculatin.

6. Orientation of peptides in lipid bilayers

The orientation of a number of antimicrobial peptides in model membranes has been determined using solid-state NMR, including KIGAKI [30], MSI-78 [31], LL-37 [32],

PGLa [33], pardaxin [34], polyphemusin [35], MSI-843 [36], gramicidin S [37], magainin [38], melittin [19] and gramicidin A [39] (also, see articles within this issue by Bechinger, Hong, Nomura, Ramamoorthy, Ulrich and co-authors). Solid-state NMR studies of the interaction of antimicrobial peptides from Australian tree frogs have been undertaken using model membranes of the zwitterionic phospholipid, dimyristoylphosphatidylcholine (DMPC) [40,41]. Deuterated DMPC was used to ascertain the interaction of the peptides with the lipid acyl chains. The order and dynamics of the lipid headgroups and alkyl chains were measured by ^{31}P and ^{2}H solid-state NMR, respectively. Changes were seen in the NMR spectra of both the headgroup (^{31}P) and alkyl chain regions (^{2}H) of the lipid molecules upon addition of the peptides to bicelles or mechanically aligned bilayers of DMPC. Each of the peptides (aurein 1.2, caerin 1.1, citropin 1.1 and maculatin 1.1) at lipid/peptide molar ratios of 15:1 caused changes to the chemical shift anisotropy of the headgroup ^{31}P atoms [41]. Also in the case of maculatin 1.1 broadening of the NMR signal was also observed, due to changes in lipid mobility. However, the interaction with DMPC is only peripheral and the lipid bilayer phase was preserved even under high peptide concentrations.

Furthermore, ^{15}N NMR of specifically labelled peptides in aligned DMPC bilayers was used in an effort to determine the orientation of the peptides within the lipid bilayer. When the bilayer normal is aligned perpendicular to the magnetic field of the magnet, a single resonance is seen in the ^{15}N spectrum at a chemical shift consistent with the peptide oriented at approximately the magic angle (54.7°) to the membrane surface [41]. However, a similar chemical shift is found for aurein, the shortest peptide, when the bilayer normal is aligned parallel to the direction of the applied magnetic field [40]. Since the ^{15}N resonance of the peptide appears to have the same chemical shift when the bilayers are aligned both parallel and perpendicular to the magnetic field, and the value is close to the isotropic position, then most likely motional averaging of the ^{15}N signal is taking place. Notably for the ^{15}N studies [40,41] cross polarisation was extremely difficult to achieve, which suggests that the peptides are highly mobile. In addition, since the ^{31}P and ^{2}H spectra, were consistent with a fluid phase lipid bilayer with little effect of peptides at relatively high concentrations, the data were taken to show that the peptides do not appear to interact significantly with zwitterionic lipids.

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) was used previously to determine the insertion of maculatin 1.1 into DMPC and DMPG membranes [24]. Moreover, deuterium exchange results showed that the peptide has a preference for the anionic lipids. In zwitterionic lipids, around 5% of the peptide residues were protected, whereas 70% of the residues were protected from exchange by DMPG lipids. ATR-FTIR also gives an estimation of the angle that the peptide inserts into the membrane. The angle of the helix axis of maculatin in DMPG membranes was determined to be 27°–35° relative to the bilayer normal. Such an angle would suggest that maculatin may be pore-forming in bacterial membranes. Further, a similar angle of insertion was observed for the small percentage of the peptide that entered the DMPC membranes. This result is consistent with the angle determined by NMR for the peptide in DMPC bilayers and by oriented CD measurements [41]. However, both the aligned NMR and CD studies were carried out in conditions without excess water, which could favour insertion of the peptide into the bilayer. These results are consistent with the observation that maculatin 1.1 lyses bacterial cells at much lower concentrations than red blood cells [2]. For the ATR-FTIR, the peptide was added after DMPC/DMPG membrane formation whereas for the NMR experiments, maculatin was reconstituted with DMPC, which would promote peptide incorporation. However, despite the sample conditions favouring insertion, the peptide showed little preference for neutral membranes.

7. Membrane lysis-fluorescence studies

Fluorescence spectroscopy has been used to determine the lytic concentrations of membrane perturbing peptides, such as melittin [42]. This technique was applied to aurein 1.2, citropin 1.1, maculatin 1.1 and two single point mutants of maculatin, where the proline residue is replaced by alanine or glycine [43]. Concentrations of peptide required to cause leakage from large (LUV) and giant unilamellar vesicles (GUV) were determined. The vesicles were formed from POPC and mixed POPC/POPG (1:1, molar). Maculatin and the derived peptides showed similar levels of lytic activity in zwitterionic LUV. However, when Pro-15 is replaced by Gly or Ala, the lytic potency is reduced ten fold in anionic vesicles. This suggests that proline in maculatin may play a key role in the antibacterial activity, allowing the peptide to adopt an optimal amphiphilic conformation at the interface leading to membrane damage.

A novel method of determining the mechanism by which antibacterial peptides lyse model membranes was used to study leakage of two differently sized fluorescent molecules (Alexa^{488}-dextran, M_w 10 kDa; Alexa^{546}-maleimide, M_w 1.3 kDa) from GUV [43]. GUV are completely destroyed by addition of the shorter sequence peptides, citropin and aurein, and in contrast to the maculatin peptides, a high peptide to lipid ratio is needed to achieve dye leakage. Addition of maculatin peptides caused differential quenching of the two fluorescence probes indicating that these peptides form pores though which the smaller probe is able to escape from the GUV. The technique appears to distinguish between two models of lytic action of antimicrobial peptides. The difference in peptide length may be the origin of the distinction because the longer peptides are able to adopt a transbilayer configuration, leading to oligomerization and formation of a pore while the bilayer character is preserved. The shorter sequence peptides interact with the membrane but due to a mismatch between the peptide length and the bilayer thickness, the 'carpet' mechanism leads to membrane destabilization.

The fluorescent dye results for native maculatin showing similar behaviour in POPC and POPC/POPG vesicles contrast somewhat to the ATR-FTIR in DMPC and DMPG bilayers [24] and results with POPC and POPG monolayers [26], which indicate a preference for the anionic lipids. Further work is needed to explore these differences obtained using a saturated

phospholipid bilayer (DMPC, DMPG) and an unsaturated (POPC, POPG) monolayer. Evaluation of cell lysis based on peptide fluorospectroscopy [14] is another avenue that has not been explored due to the lack of a fluorescent residue (Table 1), and use of peptides with Trp substitutions may yield useful insights.

8. Concluding remarks

The lytic activity of antibiotic peptides from Australian tree frogs, aurein 1.2, citropin 1.1 and maculatin 1.1, appear to adhere to the so-called carpet model for the shorter peptides and the pore-forming model for the longer peptide, maculatin, However, maculatin may exhibit different lytic mechanisms depending on the model membrane systems and it remains to further explore the effect of cholesterol and non-bilayer forming phospholipids such as phosphatidylethanolamine on the activity of these antimicrobial peptides. Better model systems are required to understand the mode of action. Different mechanisms may be at work resulting in the formation of an isotropic signal in the ^{31}P NMR spectrum of live bacteria [23] and the escape of small molecules from GUV [43].

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