Biochimica et Biophysica Acta 1858 (2016) 1091–1098

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Atomic force microscopy of bacteria reveals the mechanobiology of pore forming peptide action

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ARTICLE INFO

Article history:
Received 1 November 2015
Received in revised form 23 February 2016
Accepted 1 March 2016
Available online 4 March 2016

Keywords:
Atomic force microscopy
Klebsiella pneumoniae
Antimicrobial peptide
Capsular polysaccharide
Membrane interaction
Caerin
Melittin

ABSTRACT

Time-resolved AFM images revealed that the antimicrobial peptide (AMP) caerin 1.1 caused localised defects in the cell walls of lysed *Klebsiella pneumoniae* cells, corroborating a pore-forming mechanism of action. The defects continued to grow during the AFM experiment, in corroboration with large holes that were visualised by scanning electron microscopy. Defects in cytoplasmic membranes were visualised by cryo-EM using the same peptide concentration as in the AFM experiments. At three times the minimum inhibitory concentration of caerin, 'pores' were apparent in the outer membrane. The capsule of *K. pneumoniae* AJ218 was unchanged by exposure to caerin, indicating that the ionic interaction of the positively charged peptide with the negatively charged capsular polysaccharide is not a critical component of AMP interaction with *K. pneumoniae* AJ218 cells. Further, the presence of a capsule confers no advantage to wild-type over capsule-deficient cells when exposed to the AMP caerin.

1. Introduction

Throughout the plant and animal kingdoms, antimicrobial peptides (AMPs) are part of the host organism's innate defence response [1]. AMPs show promise as an alternative to conventional antibiotics [2,3]: their minimum inhibitory concentrations (MICs) are in the low micromolar range, and comparable to the active concentrations of commercially available antibiotics against sensitive organisms [4]. The activity of most AMPs has been attributed to membrane disruption, either via a detergent-like or pore-forming mechanism [5], although studies have proposed mechanisms in which, following membrane permeabilisation, AMPs target intracellular cell components as the killing mechanism [3,6]. In either case, the mechanism of AMP-induced membrane permeabilisation remains unclear, and warrants further investigation. Phospholipid membranes have been used extensively to study the mode of action of AMPs. These model systems lack the complexity of the bacterial surface and cell wall, but have demonstrated that phospholipid charge [7,8] and membrane curvature [9–11] influence the mode of interaction. The size, structure and hydrophobicity of the peptide also affect its selectivity and activity [12]. However, studies of the interactions of AMPs with model membrane systems and live bacteria often yield contradictory results [13–17], which emphasise the importance of in situ studies.

Atomic force microscopy (AFM) is a proven technique for the study of biological samples in situ [18,19]. The physical properties of living biological cells can be studied under physiologically relevant conditions that require little sample preparation and without destruction of the sample [20–23]. Properties such as cytoplasmic turgor pressure, cell wall elasticity and long-range electrostatic interaction can be determined using AFM [24,25]. Of particular relevance to this work are studies that have investigated how AMPs affect cell stiffness and adhesion in live bacteria [15,26–29]. Here, we present AFM data that gives insight into the physiological responses of *Klebsiella pneumoniae* when exposed to the AMP, caerin 1.1.

Caerin 1.1 (GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2) is a major component of the skin gland secretions of the Australian tree frogs of the genus *Litoria* [30]. Like many AMPs, caerin 1.1 is an unstructured random coil in solution, rearranging into an amphipathic α-helix on partitioning into membrane or membrane mimetic environments [11]. It has wide spectrum antibiotic activity [31] although the peptide is more potent against Gram-positive bacteria. This has been attributed to more complex protective structures separating the inner workings of Gram-negative bacterial cells from their environments [11]. Caerin has also shown anticancer [32] and antiviral [33] activity and has been characterised by quartz crystal microbalance [34], surface plasmon resonance [35], circular dichroism and nuclear magnetic resonance [16] studies.

Caerin is one of a group of related AMPs that, depending on their length, act by fundamentally different mechanisms in the same model

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http://dx.doi.org/10.1016/j.bbamem.2016.03.002
0005-2736/

membrane systems. The shorter peptides, aurein and citropin, have been shown to act via a detergent-like mechanism whereas the longer peptides, caerin and maculatin, use a pore-forming mechanism [36]. The propensity of caerin for pore formation in phospholipid membranes makes it ideal to compare with the melittin P14K peptide (GIGA VLKVLTTGL**K**ALISWIKRKRQQ-NH_2) [29]. Previous work has demonstrated that pore formation is less significant for interaction between this melittin-derived peptide in live bacterial cells than in model membrane systems [14]. Like melittin, caerin is also cationic and α-helical in model membranes and differs in length by only one amino acid.

K. pneumoniae is a Gram-negative bacillus and a source of shared antimicrobial resistance such as carbapenem-resistance and the extended-spectrum beta lactamases (ESBLs) [37,38] and is also recognised as a serious cause of hospital-acquired infections. A loosely organised polysaccharide layer (known as the capsule) is assembled around the *K. pneumoniae* cell envelope. It takes the form of a hydrated polyelectrolyte network and can grow up to several hundred nanometers thick [39]. The capsule provides protection from environmental stresses, such as antibiotics, detergents, desiccation and the host's immune response [40]. The exopolysaccharide capsule also plays a role in the formation of biofilms on surfaces [41]. Further, a direct association has been discovered between capsular size and pathogenicity of isolates in animal models [42]. Since AMPs must first pass through this network, the role of the capsule in the mode of action of caerin warrants investigation.

The resistance of a capsule-deficient *K. pneumoniae* mutant to the AMPs, α-defensin-1 and polymyxin B, increased following the addition of purified capsular polysaccharides [43]. The negatively charged bacterial capsule was proposed to act as an effective decoy, enabling encapsulated cells to resist cationic AMPs. The AMPs were postulated to trigger the release of capsular material in vivo, which in turn protect bacteria against AMPs. Previously [29], we used AFM capsule thickness measurements to demonstrate that the entropic drive of the peptide to associate with the bacterial membrane dominates over long-range electrostatic attraction between a cationic peptide and the negatively-charged *K. pneumoniae* capsule. The long-range electrostatic attraction, however, may first attract the peptides to bacterial cells. These results do not negate the idea that extracellular capsular polysaccharides play a role in protecting bacterial cells from AMPs.

In this work we present time-resolved AFM images that revealed caerin caused local defects in the cell wall of lysed *K. pneumoniae*, which supports a pore-forming mechanism of action. These defects continued to grow, becoming large holes that also were visualised using scanning electron microscopy. Further, we present data which demonstrate that the *K. pneumoniae* AJ218 capsule did not confer an advantage over capsule-deficient *K. pneumoniae* AJ218. The *K. pneumoniae* AJ218 capsule appeared to be unaffected by peptide exposure which indicates that ionic interaction of peptide and bacteria-bound capsular polysaccharide is not a critical component of *K. pneumoniae* AJ218 interaction with caerin.

2. Experimental

2.1. Peptides

Caerin 1.1 was purchased from Bio21 Peptide Technology, University of Melbourne. Melittin was purchased from Mimotopes (Clayton, Australia). Peptides were synthesised by solid-phase techniques and were >95% pure.

2.2. Bacterial strains, culture conditions, and harvesting

K. pneumoniae AJ218 (capsule serotype K54) is a human urinary tract infection isolate, identified at the microbiological laboratory of the Alfred Hospital, Melbourne, Australia [44,45]. All strains were maintained on Luria–Bertani (LB) agar at 37 °C. LB broths inoculated with these cultures were grown for 16 h at 37 °C while shaking (180 rpm). Stationary phase cells were then harvested by centrifugation (10 min at 3500 ×*g*) and washed twice with Milli-Q™ water (18.2 MΩ cm^{-1}). The final concentration of bacterial cells in Milli-Q™ water was approximately 2×10^8 cfu mL^{-1}.

A *wzc* mutant of *K. pneumoniae* AJ218, defective in the transporter that enables capsule polysaccharide export, was isolated following random mini-Tn*5Km2* transposon insertion mutagenesis [46] of *K. pneumoniae* AJ218. To confirm transposon insertion within the *wzc* gene, Y-linker ligation PCR and subsequent DNA sequencing analysis of the transposon-flanking region was performed to ensure correct location of the mutation [47].

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of caerin was determined for both wild-type and capsule-deficient *K. pneumoniae* cells using the microdilution broth method outlined by the National Committee for Clinical Laboratory standards [48].

3. Viable count assay

Cell suspensions of *K. pneumoniae* AJ218 wild-type and capsule-deficient strains were incubated in 10 mM HEPES buffer (pH 7.4) at 37 °C in the presence or absence of 6 μM peptide solution. At hourly time-points, aliquots were removed from suspensions and then diluted on agar media to determine the viable number of bacteria (expressed as colony forming units per mL, cfu/mL).

4. Bacterial sample conditions for AFM measurements

Gelatine-coated glass slides were used to immobilise bacteria for AFM measurements. In previous work [24,29] slides coated with polyethylenimine (PEI) were used for wild-type cells. The PEI and gelatine coating methods are described [24]. When treated with caerin 1.1, *K. pneumoniae* AJ218 did not adhere firmly enough to PEI-coated slides for force measurements, so the more adhesive gelatine coating was used. Substrate rigidity is a requirement when measuring cell indentation to ensure that only cell compression contributes to the measurement. Wang et al. [24] have shown that, even though gelatine may be expected to be a softer substrate than PEI, there is no measureable effect of gelatine deformability on the force profiles of the bacteria, which suggests that this method results in a gelatine layer sufficiently thin to present as a rigid surface in AFM force measurements.

All mechanical measurements were performed within 2–3 h of removal of the bacteria from growth media. Bacteria-coated slides were immersed in 10 mM HEPES buffer (pH 7.4) or 6 μM peptide solution (made in 10 mM HEPES buffer, pH 7.4) and kept at rest within the calibrated AFM for at least 40 min before measurements commenced. Previous work has demonstrated that cells remain viable for the duration of the experiment as determined by live/dead cell fluorescence assays using a Molecular Probes Bacterial Viability Kit [24]..

An imaging volume of 5 mL was used. Measurements were conducted first in HEPES buffer and then exchanged for 1.2 μM peptide in HEPES buffer. To establish the lipid to peptide ratio at which experiments were conducted, bacteria attached to the slides were stained with crystal violet following AFM measurements, and viewed at 60× magnification. Counting cells in several areas of known size allowed for an approximation of the number of cells per slide. Ingraham et al. [49] give an approximation of the number of lipids per microbial cell, which allowed us to calculate the number of lipid molecules per slide. The lipid to peptide ratio for all experiments reported here, assuming all peptide is bound, was estimated to be 1:1. This is considerably higher than generally used with model membrane systems to ensure measurable peptide–cell interaction within the timeframe of the experiment.

5. Atomic force microscopy and force measurements

AFM measurements were performed using an MFP-3D instrument (Asylum Research, Santa Barbara, CA). Silicon nitride cantilevers were