FEBS Letters xxx (2013) xxx–xxx

FEBS Letters

journal homepage: www.FEBSLetters.org

Interaction of cationic antimicrobial peptides with *Mycoplasma pulmonis*

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ARTICLE INFO

Article history:
Received 7 June 2013
Revised 7 August 2013
Accepted 9 August 2013
Available online xxxx

Edited by Renee Tsolis

Keywords:
Antimicrobial peptide
Mycoplasma
Phospholipid
Liposome
Action mechanism

ABSTRACT

We investigated the mode of action underlying the anti-mycoplasma activity of cationic antimicrobial peptides (AMPs) using four known AMPs and *Mycoplasma pulmonis* as a model mycoplasma. Scanning electron microscopy revealed that the integrity of the *M. pulmonis* membrane was significantly damaged within 30 min of AMPs exposure, which was confirmed by measuring the uptake of propidium iodine into the mycoplasma cells. The anti-mycoplasma activity of AMPs was found to depend on the binding affinity for phosphatidylcholine, which was incorporated into the mycoplasma membrane from the growth medium and preferentially distributed in the outer leaflet of the lipid bilayer.

1. Introduction

Mycoplasmas, which are the smallest self-replicating organisms in nature, have become one of the major concerns in human and veterinary medicine since they are known to be etiological agents for a variety of infectious diseases [1]. Although they are descendents of Gram-positive bacteria, mycoplasmas have many peculiar features that differ distinctly from cell wall synthesizing bacteria [2]. For example, they lack a rigid cell wall and are bound by a single plasma membrane that interacts directly with their environment. Additionally, most mycoplasmas strictly require host sterols and are generally cultured in serum-containing medium that supplies the choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SPM), which are incorporated into the plasma membrane of the mycoplasma [3,4]. It has been postulated that these two phospholipids are involved in the immune evasion and virulence of mycoplasmas [5,6]. Indeed, microbial cell surface components such as peptidoglycans, lipopolysaccharides, lipoteichoic acid, mannan and β-glucan are major target molecules in the recognition of microbial pathogens by the host defense system [7]. Accordingly, it is important to elucidate how the wall-less mycoplasmas interact with host defense systems.

Over the last three decades, cationic antimicrobial peptides (AMP) have been shown to play pivotal roles in defense systems of animals ranging from insects to humans [8]. AMPs are capable of directly killing microbial pathogens and/or modulating host defense reactions to cope with invading microbes [8]. To exert their antimicrobial effects, AMPs interact with unique molecular patterns on the surface of pathogens. It is well known that most cationic AMPs recognize microbes via their specific binding to anionic components occurring in the cell wall of bacteria and fungi [9]. However, it is not known how they recognize and kill mycoplasmas without cell walls. To date, there have been a few studies conducted to investigate the anti-mycoplasma activity of cationic AMPs although no attempts to demonstrate the mode of action underlying their anti-mycoplasma potency [10–12]. The studies showed that the anti-mycoplasma activity of AMPs was not in accordance with their antimicrobial activity against bacteria equipped with cell walls [11,12]. These findings suggest that AMPs exert an anti-mycoplasma activity in a fashion that differs from

Abbreviations: AMP, antimicrobial peptide; POPC, 1-palmytoyl-2-oleoyl L-α-phosphatidylcholine; POPG, 1-palmytoyl-2-oleoyl L-α-phosphatidylglycerol; CL, cardiolipin; SPM, sphingomyelin; NaP, sodium phosphate; PI, propidium iodine; SEM, scanning electron microscopy; TLC, two-dimensional thin layer chromatography; EPS, exopolysaccharide; Vsa, variable surface antigen

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0014-5793/$36.00 © 2013 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
http://dx.doi.org/10.1016/j.febslet.2013.08.016

antimicrobial mechanisms that have been identified to date, which are generally mediated by binding to components of the microbial cell wall.

In this study, we evaluated the anti-mycoplasma activity of four known cationic AMPs: HG1, LL-37, MSI-78 and polymyxin B [13–16]. In addition, we conducted diverse experiments in an attempt to elucidate the mechanism of AMPs against mycoplasmas using *Mycoplasma pulmonis* as a model mycoplasma. This study is the first to shed light on the mode of action by which cationic AMPs kill mycoplasmas.

2. Materials and methods

2.1. Peptides and reagents

Three AMPs (HG1, LL-37 and MSI-78) used in this study were synthesized using an automated solid-phase peptide synthesizer (Pioneer Applied Biosystems, USA) at Peptron, Inc. (Daejon, Korea), and then purified to over 95% with a C18 reversed-phase HPLC column (Vydac 218TP54: The Separation Group, Hesperia, USA). Polymyxin B (81334) and kanamycin (K1876) were purchased from Sigma. In addition, four phospholipids were also purchased from Sigma: 1-palmytoyl-2-oleoyl L-α-phosphatidylcholine (POPC, P3556), 1-palmytoyl-2-oleoyl L-α-phosphatidylglycerol (POPG, P8 318), cardiolipin (CL, C0563), and sphingomyelin (SPM, S0756).

2.2. Mycoplasma strains and culture medium

The mycoplasmas were grown in PPLO broth (Difco, 255420) supplemented with 10% (v/v) horse serum (GIBCO, 16050-122), yeast extract, glucose, ampicillin and phenol red at 30 °C. The pH of the medium was adjusted to 7.8. To prepare PPLO agar plates, the broth was supplemented with 1% bacto agar (Difco, 214010) without phenol red. Wild-type *M. pulmonis* strain CT and its two mutants (*M. pulmonis* CTG1701 and CTG2028) were used as models to investigate the interaction between mycoplasmas and AMPs. CTG1701 and CTG2028 strains are exopolysaccharide (EPS) mutants that have a long variable surface antigen (Vsa) and an intermediate-length Vsa protein, respectively [17].

2.3. Scanning electron microscopy (SEM)

Washed *M. pulmonis* (10^9 CFU) were incubated with three AMPs (HG1, 16 μg/ml; LL-37, 32 μg/ml; MSI-78, 64 μg/ml) or kanamycin (2 μg/ml) at 37 °C for 30 min and 3 h, respectively. Additionally, one sample was incubated with only 10 mM sodium phosphate (NaP) buffer (pH 7.4) as a control. Each sample was then washed three times with NaP buffer, after which the cells were fixed for 6 h in 50 mM sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde and 2% paraformaldehyde. The fixed cells were subsequently washed extensively with 50 mM sodium cacodylate and then dehydrated with a graded series of ethanol concentrations (50%, 70%, 90%, 95%, 100% × 2). A drop of each sample in 100% ethanol on a slide was then subjected to critical-point drying, after which the cells were coated with gold–palladium. Micrographs were obtained using a Quanta FEG 200 scanning electron microscope (FEI, Netherlands).

2.4. Propidium iodone (PI) uptake assay

M. pulmonis cells were washed and suspended in NaP buffer at 10^9 CFU/ml. Next, 100 μl aliquots of the stock sample were dispensed into each well of a 96-well fluorescence assay plate (SPL, 30196). To pre-stain the cells, 5 μl of PI (SIGMA, P4170) was added into each well to a final concentration of 5 μM and the sample was incubated for 15 min in the dark. Peptide or kanamycin was then added into each well to give a final concentration of 16 μg/ml. At the predetermined time, the fluorescence intensity of each mixture was measured using a microplate reader (BioTek Laboratory Instruments, USA) with excitation and emission values of 530 and 620 nm, respectively. For a positive control (100% PI uptake), the sample was treated with 1% (v/v) Triton X-100 instead of peptides or kanamycin.

2.5. Two-dimensional thin layer chromatography (TLC)

Membrane lipids of *M. pulmonis* CT were extracted according to the procedure described by Bligh and Dyer [18]. Briefly, 10^9 *M. pulmonis* cells were used to obtain membrane lipids and chloroform/methanol (1:2 by vol.) was used as the extractant. Membrane lipids were separated using HPTLC silica gel 60 plates (Merck, 105631), after which they were developed at room temperature by a two-dimensional system using chloroform/methanol/ammonia (65:35:4 by vol.) for the first dimension and chloroform/methanol/acetic acid/water (85:25:5:4 by vol.) for the second dimension. Lipids spots were detected by 10% (v/v) H_2SO_4 and identified by comparison with those of standard lipids.

2.6. Preparation of liposome and calcein leakage assay

The lipid vesicles were prepared and the calcein leakage assay was performed according to the procedure described previously [19]. Calcein-entrapped vesicles were obtained by elution with 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl on a Sephadex G-50 column. In the calcein leakage assay, the fluorescence intensity was measured using a fluorometer (VARIAN, USA) with excitation and emission values of 490 and 520 nm, respectively. The percent dye-release was evaluated by the following equation: leakage (%) = $(F - F_0)/(F_{max} - F_0) \times 100$, where F_0 was the fluorescence intensity of the control vesicle, and F and F_{max} were the fluorescence intensities achieved by peptides and 1% (v/v) Triton X-100, respectively.

2.7. Anti-mycoplasma assay

The MICs of four AMPs and kanamycin were determined via a broth dilution assay conducted in accordance with recommendations of the Clinical and Laboratory Standard Institute [20]. Briefly, mycoplasma cultures were diluted in fresh growth medium to a final concentration of 2×10^5 CFU/ml. Each peptide and kanamycin were then prepared in acidified water (0.01% acetic acid) at 640 μg/ml, after which the samples serially diluted 2-fold in acidified water to 10 μg/ml. Next, 100 μl aliquots of mycoplasma were dispensed into each well of a 96-well microtiter plate (Costar 3790, Corning), after which 11 μl of sample solution were added and adjusted to a total assay volume of 111 μl. A change in the color of the growth media containing phenol red as a pH indicator was monitored to observe mycoplasma growth. The MIC was defined as the lowest concentration that completely inhibited the growth. To verify the effects of POPC or POPG on the antibacterial activity of the four AMPs against *Escherichia coli*, a radial diffusion assay was conducted as previously described [21]. Briefly, each peptide was mixed with phospholipid liposome without calcein (peptide: POPC, 1:40 by wt; peptide: POPG, 1:10 by wt) and incubated for 30 min at 37 °C, after which 5 μl aliquots of the sample mixture were subjected to radial diffusion assay. For the colony count assay, each peptide at a concentration corresponding to the MIC value was mixed with POPC or POPG liposome at the same ratio as in the case of the radial diffusion assay. The total volume of peptide and liposome was 50 μl. After incubation for 30 min at 37 °C,

Please cite this article in press as: Park, H.J., et al. Interaction of cationic antimicrobial peptides with *Mycoplasma pulmonis*. FEBS Lett. (2013), http://dx.doi.org/10.1016/j.febslet.2013.08.016