Biochimica et Biophysica Acta 1834 (2013) 996–1002

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Novicidin's membrane permeabilizing activity is driven by membrane partitioning but not by helicity: A biophysical study of the impact of lipid charge and cholesterol

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ARTICLE INFO

Article history:
Received 14 December 2012
Received in revised form 5 March 2013
Accepted 25 March 2013
Available online 2 April 2013

Keywords:
Antimicrobial peptide
Secondary structure
Thermodynamics of membrane binding
Partitioning coefficient
Equilibrium dialysis
Membrane permeabilization

ABSTRACT

We have investigated the interactions between the antimicrobial peptide Novicidin (Nc) and vesicles containing the phospholipid DOPC, with various amounts of DOPG and cholesterol using circular dichroism spectroscopy, calcein release, equilibrium dialysis and isothermal titration calorimetry. Nc adopts a random coil structure in the absence of lipids and in the presence of vesicles containing 100% DOPC. Lipids with 25–40% DOPG induce the highest level of helicity in Nc; higher DOPG levels lead to lower helicity levels and an altered tertiary arrangement of the peptide. However, the ability of Nc to permeabilize vesicles correlates not with helicity but rather with its overall membrane affinity, which is enthalpically favorable but opposed by entropy. Permeabilization declines with increasing mole percentage PG. Changes in helicity correlate with changes in enthalpy, reflecting the enthalpy of helix formation, but not with affinity. There is also a large favorable enthalpic interaction between Nc and lipids in the absence of negative charge and structural changes. Cholesterol slightly reduces membrane permeabilization but has little effect on Nc affinity and secondary structure, and probably protects the membrane by inducing the liquid ordered state. We conclude that helicity is not a prerequisite for activity, and charge–charge interactions are not the only major driving force for AMP interactions with membranes. Our data are compatible with a model in which a superficial binding mode with a large membrane surface binding area per peptide is more efficient than a more intimate embedding within the membrane environment.

1. Introduction

The rapid evolution of microbial resistance to antibiotics makes the development of new antibiotics a permanent arms race [1]. As part of this effort, there has been increasing focus on antimicrobial peptides (AMPs), which are part of the innate immunity of all higher organisms [2]. Currently, only a few AMPs are either in clinical trials (e.g. Protegrin IB-367) or on the market (e.g. Neuprex™ and Mycoprex™) [3–5]. AMPs such as cecropins have a broad-spectrum activity [6], while bacteriocins are specific to a sub-class of microbes [2]. Most AMPs are cationic, which is believed to allow them to target the bacterial cell membranes – both the inner and outer membranes – due to their content of anionic phospholipids. In addition, bacterial membranes contain no cholesterol. In contrast, the mammalian cell membranes have predominantly zwitterionic phospholipids interspersed with anionic lipids largely in the inner lamella of the membrane, and cholesterol in both the lamellae [7].

Cholesterol occurs in mammalian cell membranes, existing at mole percentages up to 50% in red blood cells [8]. It is important for membrane integrity, membrane organization, interaction of membrane proteins with receptors, membrane dynamics etc. [7]. The molecule provides rigidity to the membrane [8], due to its lipid acyl chain ordering property that reduces the *trans* → *gauche* isomerization of the acyl chains of the lipid molecules in its vicinity, thereby reducing the membrane surface area. Biophysical studies using bilayers with homogeneous lipids have shown that, this membrane cohesive and stiffening property of cholesterol thus prevents excessive binding of AMPs [8,9] to destructive levels as seen for MSI-78 [10], Pardaxin [11], melittin [5] and LL-37 [12,13]. Whereas, some membrane active peptides like δ-lysin [14] bind actively to the mammalian cell membranes by altering their fluidity, despite the presence of cholesterol, and cause cell lysis. However, with heterogeneous lipid bilayer systems that mimic the natural lipid rafts, cholesterol does not reduce

Abbreviations: CD, circular dichroism; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; Nc, Novicidin; ITC, isothermal titration calorimetry; CRA, calcein release assay; AMP, antimicrobial peptide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); MIC, minimum inhibitory concentration; MWCO, molecular weight cut-off; PC, phosphatidyl choline; PG, phosphatidyl glycerol; PS, phosphatidyl serine; MRE, mean residue ellipticity

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http://dx.doi.org/10.1016/j.bbapap.2013.03.025

membrane permeabilization of peptides such as MSI derivatives [10] and Pardaxin [11] to the same extent as in homogeneous lipid systems.

AMPs generally target Gram-negative bacteria better than Gram-positives [15]. The membranes of Gram-positive bacteria are made mainly of anionic phospholipids such as phosphatidylglycerol (57% of membrane composition in *Staphylococcus aureus*) and cardiolipin (5% during growth phase of *S. aureus*), whereas the membranes of Gram-negatives have a mixture of zwitterionic lipids such as PC and PE (90% in outer membrane (OM) and 82% in cytoplasmic membrane (CM) in *Escherichia coli*), along with anionic lipids such as PG (3% in OM and 6% in CM) (see Table 1 in [8]). It has been suggested that a mixture of zwitterionic and anionic phospholipids allows bound cationic AMPs to have increased conformational flexibility, and facilitates the leakage of cell contents at low concentrations [15]. This phenomenon has also been referred to as a '*charge cluster mechanism*' [16,17] in which the existence of an intermediate amount of anionic lipids allows the cationic AMPs to segregate these lipids and form distinct domains with membrane-disruptive consequences. Segregation is promoted by AMPs' tendency to self-associate in membrane environments [18]. The higher content of anionic lipids in Gram-positive membranes should conversely tie down AMPs to a greater extent, leading to higher minimum inhibitory concentrations (MIC). AMPs thought to adopt this mechanism include MSI-103, MSI-469, MSI-843, PR-9 and PI-9 [15]. Charge clustering is also associated with conformational flexibility of the AMP in the membrane, as in the case of the peptide GF-17 D3 [15].

The subject of the present study is the AMP Novicidin, a cationic AMP derived from the sheep peptide SMAP-29. This peptide shares several features with the magainin-2 analog MSI-78 [19] and the neurotoxic peptide Pardaxin [20]. They are all unstructured (or randomly coiled) in solution, but structured in the presence of lipid bilayers, though the bilayer structure varies from one peptide to the next. While MSI-78 forms an antiparallel dimer of amphipathic helices both in zwitterionic, and mixed anionic-zwitterionic membranes [19], Pardaxin forms a helix-turn-helix in zwitterionic membranes [20], although the helicity is greatly reduced in the presence of anionic lipids [21]. Nc is randomly coiled in solution and only shows a low degree of helicity in zwitterionic membranes, whereas in the presence of anionic lipids it adopts a helical conformation [22,23]. Similar to MSI-78 and Pardaxin, structure formation is not a pre-requisite for Nc activity against bacteria, since Nc permeates the zwitterionic phospholipid vesicles more effectively than those with a mixture of zwitterionic and anionic lipids [22,23].

MSI-78 has been reported to form toroidal-type membrane-disruptive pores [19], while Pardaxin, adopts different structures depending on the membrane composition [11,21,24]. These range from barrel-staves to surface-bound helices (carpet-mechanism), depending on lipid phase, acyl chain lengths and head group charge. Our work on Nc has led us to propose that Nc lyses lipid membranes in a carpet-like or detergent-like fashion [22,23].

Like MSI-78 and Pardaxin, from in vitro bactericidal assays, Nc is also found to be an effective broad-spectrum antibacterial peptide, which is active against various Gram-positive bacteria like *Bacillus cereus* [25], *S. aureus* and *Listeria monocytogenes* [26,27] and Gram-negative bacteria like *E. coli*, and *Salmonella enterica* [25]. Nc causes ATP leakage from *S. aureus*, which is an indication of membrane disruption [27]. However the MIC values for Nc are lower against Gram-negative bacteria.

Here we address in more detail the charge dependent activity of Nc with the aim of rationalizing Nc's preference for Gram-negative bacteria through its binding to mixtures of the phospholipids DOPC and DOPG. In addition, we tested the extent of potential mammalian cytotoxicity of Nc by mixing cholesterol with the DOPC/DOPG vesicles.

The secondary structure of Nc in DOPC with varying PG and cholesterol was followed using CD spectroscopy. A calcein release assay was used to quantitate the extent of bilayer perturbation by Nc and the amount of peptide required for maximum release in various amounts of PG. We used ITC to monitor the energetics of binding and structure formation and obtained the apparent partition coefficients by equilibrium dialysis. Our results conclusively demonstrate that membrane composition is far more important than the conformational change of Nc on the lipid bilayers in determining Nc efficiency towards bacterial membranes.

2. Materials and methods

2.1. Materials

DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and DOPG (1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt)) were from Avanti Polar Lipids (Alabaster, AL). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. The buffer used in all experiments was 20 mM sodium phosphate buffer, pH 7.5, filtered through a 0.2 μm filter. Novicidin (Nc) was kindly provided by Drs. Per Holse Mygind and Hans Henrik Kristensen, Novozymes A/S. Dansyl-Nc was prepared as described [23]. Lyophilized Nc was dissolved in buffer and dialyzed against the same buffer with at least three buffer changes overnight using a membrane of molecular weight cut off 1 kDa.

2.2. Preparation of large unilamellar vesicles

≤1 ml samples of LUVs (~100 nm) were prepared as described [28] using eight freeze–thaw cycles. Mixed vesicles were prepared as follows: From chloroform stock solutions (w/w%) of DOPC, DOPG and cholesterol, appropriate volumes were mixed, evaporated overnight in a vacuum desiccator and resuspended in buffer to obtain 5 mg/ml solutions of mixture of the two lipids containing 95–90–82.5–75–62.5–50–37.5–25–17.5–10–5–0% DOPC. The dried films were then re-suspended in buffer and freeze-thawed in 8 cycles, followed by an extrusion through 100 nm polycarbonate membranes in a mini-extruder (Avanti Polar Lipids, Alabaster, AL). The vesicles formed were either used immediately or kept at 4 °C and used within 3 days. To obtain >1 ml of liposomes, a barrel extruder (Northern Lipids, Burnaby, Canada) was used [29].

2.3. Preparation and analysis of calcein-filled LUVs

The dried lipid films prepared as above were suspended in 100 mM calcein (sodium salt) to a solution of 5 mg/ml lipid and LUVs were prepared as above. The extruded vesicles with entrapped calcein were separated from free calcein using a PD-10 column (Sepharose G-25, GE Healthcare, Waukesha, WI). The first three colored fractions of each of the lipid mixture were pooled and diluted to obtain ca. 8 μM lipid (assuming 100% recovery of applied lipid), stored in the refrigerator, and used within 2 days.

2.4. Calcein dye leakage

The release of free calcein from the vesicles and the subsequent increase in fluorescence were monitored by exciting at 490 nm and recording the emission at 515 nm every second using a slit width of 5 nm for both the monochromators, in a spectrofluorometer (Varian Cary, Agilent Technologies, USA). The vesicles entrapped with calcein were diluted to the appropriate final concentrations (~33–34 μM) for each lipid mixture. A 10 mm quartz cuvette with stirring was used, and the sample was allowed to equilibrate at 30 °C for at least 2 min prior to starting the measurement. Emission intensity was normalized with regard to total calcein released by 10 μl of 2% of Triton X-100 as described [30].