Biochimica et Biophysica Acta 1858 (2016) 1317–1327

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Selective membrane disruption by the cyclotide kalata B7: complex ions and essential functional groups in the phosphatidylethanolamine binding pocket

Adam A. Strömstedt [a,*], Per Eugen Kristiansen [b], Sunithi Gunasekera [a], Nathalie Grob [a], Lars Skjeldal [c], Ulf Göransson [a]

[a] Department of Medicinal Chemistry, Division of Pharmacognosy, Uppsala University, Box 574, SE 75123 Uppsala, Sweden
[b] Department of Molecular Biosciences, University of Oslo, Box 1041, 0316 Oslo, Norway
[c] Department of Chemistry, Biochemistry and Food Science, Norwegian University of Life Sciences, N-1432 Ås, Norway

ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 10 February 2016
Accepted 11 February 2016
Available online 12 February 2016

Keywords:
Cyclotide
Antimicrobial peptide
Membrane permeabilization
Peptide-membrane interaction
Liposome
Phosphatidylethanolamine binding

ABSTRACT

The cyclic cystine knot plant peptides called cyclotides are active against a wide variety of organisms. This is primarily achieved through membrane binding and disruption, in part deriving from a high affinity for phosphatidylethanolamine (PE) lipids. Some cyclotides, such as kalata B7 (kB7), form complexes with divalent cations in a pocket associated with the tyrosine residue at position 15 (Tyr15). In the current work we explore the effect of cations on membrane leakage caused by cyclotides kB1, kB2 and kB7, and we identify a functional group that is essential for PE selectivity. The presence of PE-lipids in liposomes increased the membrane permeabilizing potency of the cyclotides, with the potency of kB7 increasing by as much as 740-fold. The divalent cations Mn^{2+}, Mg^{2+} and Ca^{2+} had no apparent effect on PE selectivity. However, amino acid substitutions in kB7 proved that Tyr15 is crucial for PE-selective membrane permeabilization on various liposome systems. Although the tertiary structure of kB7 was maintained, as reflected by the NMR solution structure, mutating Tyr into Ser at position 15 resulted in substantially reduced PE selectivity. Ala substitution at the same position produced a similar reduction in PE selectivity, while substitution with Phe maintained high selectivity. We conclude that the phenyl ring in Tyr15 is critical for the high PE selectivity of kB7. Our results suggest that PE-binding and divalent cation coordination occur in the same pocket without adverse effects of competitive binding for the phospholipid.

1. Introduction

Cyclotides are a family of plant derived macrocyclic peptides [1,2]. Their peptide backbones of about 30 residues are linked head-to-tail and contain six strictly conserved cysteines forming the characteristic cyclic cystine knot motif [3]. This specific motif, in conjunction with the cyclic backbone, gives cyclotides extraordinary stability against denaturation and enzymatic degradation [4]. Cyclotides are divided into the Möbius and bracelet subfamilies according to a *cis*- or *trans*-conformation, respectively, of the proline in loop 5. At present, more than 260 naturally-occurring cyclotides have been characterized, isolated from species within the *Violaceae*, *Rubiaceae*, *Fabaceae*, *Solanaceae* and *Cucurbitaceae* families of plants [5,6].

The first discovered cyclotide, kalata B1 (kB1), was identified as responsible for the uterotonic effect of a decoction of the plant *Oldenlandia affinis*, traditionally used to facilitate childbirth [7]. High homology between loop 3 of kalata cyclotides and the hormone oxytocin seems to explain this uterotonic activity [8]. Since this discovery, many studies have shown that cyclotides have a wide range of antimicrobial activities including: anthelmintic [9], antifouling [10], antiviral [11], antibacterial [12], anticancer and hemolytic [13] bioactivities. Moreover, cyclotides are toxic to snails [14], and the gastrointestinal tracts of insect larvae upon ingestion [15]. These bioactivities imply that the main biological function of the cyclotides is to protect the plants against herbivores and pathogens [16]. The various anti-biological properties appear to result from a mutual mechanism, i.e. permeabilization of the phospholipid membrane, which is likely the primary cause for most of the documented activities [2].

Cyclotides from both the Möbius and bracelet subfamilies bind specifically to phosphatidylethanolamine (PE) membrane lipids [17]. Although this PE interaction was observed some time ago in Möbius cyclotides [18], its importance for membrane disruption and its strict

Abbreviations: AMP, antimicrobial peptide; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; POPE/PC/PG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospholipids; DPC, dodecylphosphocholine; kB1–7, kalata B1-7; cyO2, cycloviolacin O2; TFA, trifluoroacetic acid; DMF, dimethylformamide; ACN, acetonitrile.

* Corresponding author.

http://dx.doi.org/10.1016/j.bbamem.2016.02.013
0005-2736/

dependence on structural elements were discovered recently [19]. The bracelet cyclotide cycloviolacin O2 (cyO2) preferentially disrupt PE-containing membranes rather than those with phosphatidylcholine (PC). The underlying mechanism for this selectivity involves high cyO2 adsorption on PE-containing membranes, augmented by structural affinity, and a subsequent specific extraction of PE-lipids from the bilayer. This results in a thinning of the membrane which, in turn, makes it permeable and sensitive to peptide-induced leakage.

So far, cyclotides are the only molecules that demonstrate a PE-selective lytic activity and that mediate selective extraction of phospholipids from a membrane [19]. The only other family of antimicrobial peptides (AMPs) with known PE affinity, the cinnamycin-like peptides, have a reversed relationship comprising a loss of membrane lytic properties in the presence of PE [20]. Other compounds that exhibit PE-binding qualities are the PepB [21], and possibly the amyloid-β, family [22] of peptides, neither of which is associated with membrane perforation or antimicrobial activity.

The PE selectivity of cyO2 has been linked to a conserved, buried Glu7 residue in loop 1 that stabilizes the helical structure of loop 3 [19]. The Glu7 residue is also conserved within Möbius cyclotides such as kalata B1 (kB1), which shows a NMR chemical shift at that position with increasing levels of ethanolamine phosphate (i.e. PE detached from its glyceryl and fatty acid moieties) [23]. This indicates an interaction with ethanolamine phosphate that may be associated with the specific affinity of cyclotides for PE-lipids in membrane environments.

Besides its link to PE selectivity, Glu7, together with the residue at position 15 and neighboring Thr residues forms a complex with Mn^{2+} in kB1 and kB7 [24,25]. Structural characterization of kB7 in the presence of micelles and Mn^{2+} showed that the divalent cations coordinated within a pocket formed between Tyr15, the conserved Glu7, and the free carbonyls of two proximal Thr residues. [25]. The large, flat tyrosine sidechain protruded, forming a major part of the pocket lining. Tyr15, though hydrophobic, did not penetrate into the hydrophobic micelle interior (unlike loops 2 and 5). Instead, it positions itself, along with the cation coordinated pocket, at the headgroup region of the dodecylphosphocholine (DPC). Thus we hypothesized that divalent cations occupy the same site on kB7 as the PE-headgroups which, in turn, led us to investigate the relevance of this site, and of the presence of complex ions, for membrane activity.

In this study, we investigate how the presence of Mn^{2+}, Mg^{2+}, Ca^{2+} and Na^{+} effects cyclotide membrane permeabilization, and in particular how these ions affect PE selectivity. Furthermore, we examine the role of the Mn^{2+} coordination pocket in PE selectivity by substituting the Tyr15 residue for either: phenylalanine (Y15F), preserving the aromatic group; serine (Y15S), which retains the hydroxyl group; or alanine (Y15A), which has neither functional group (Fig. 1).

Any influences from possible structural rearrangement deriving from the point mutation are excluded by elucidating the kB7 mutant

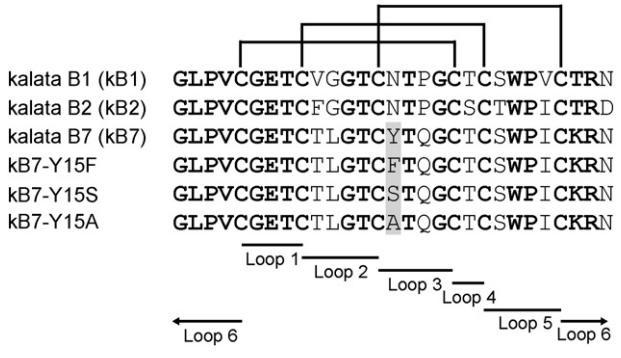

Fig. 1. Amino acid sequences of the Möbius cyclotides used in this study. The disulfide connectivity is indicated by brackets, and the inter-cysteine loops by lines and loop numbers. The mutation site at position 15 is highlighted in gray. All sequences are head-to-tail cyclic, explaining the terminal continuation of loop 6.

3D-structure with NMR and by retention time comparisons from reversed-phase (RP-)HPLC. Two related Möbius cyclotides, kB1 and kB2, serve as control peptides to factor out electrostatic and hydrophobic interactions as driving forces for membrane adsorption.

This experimental layout makes it possible to pinpoint the PE-binding pocket and its critical functional groups, as well as clarify whether PE affinity is affected by the presence of complex ions that might otherwise occupy that same pocket.

2. Materials and methods

2.1. Isolation of cyclotides

Kalata B1, B2 and B7 were isolated from *O. affinis* DC (*Rubiaceae*), as described previously [26]. In short, the polypeptide fraction of the dried plant material was purified through ethanol precipitation and subjected to a silica gel column. A stepwise gradient with decreasing ethanol concentrations was used to separate the different kalata peptides. Each cyclotide-containing fraction was freeze-dried and re-suspended for preparative C18 RP-HPLC.

Isolation of individual cyclotides was performed on an ÄKTA basic HPLC system (Amersham Biosciences, Uppsala, SE) equipped with a UV-detector operating at 215, 254, and 280 nm, and a ReproSil-Pur C18-AQ column (250 × 20 mm i.d., 10 μm, 300 Å). The chromatography was performed using a linear gradient from 10% to 60% acetonitrile (ACN) in 0.05% trifluoroacetic acid (TFA) and water over 45 min, at a flow rate of 5.0 ml/min. Individual peaks were collected and analyzed on a Thermo Finnigan LCQ Deca ion trap mass spectrometer in positive electrospray ionization mode (Thermo Electron, San Jose, CA). The peptides were identified by molecular weight and retention time. The final peptide yields were >95% pure. Both natural and synthetic peptides were subjected to repeated freeze-drying prior to use, and no substantial TFA contamination was observed in the subsequent IR spectrum measurements on a Direct Detect spectrometer (Merck KGaA, Darmstadt, DE).

2.2. Synthesis of kalata B7 mutants

The mutated kalata B7 peptides (Y15A, Y15S and Y15F) were synthesized on a 0.1 mmol scale as linear precursors by microwave-assisted, automated Fmoc solid phase peptide synthesis. A solution of 20% piperidine in dimethylformamide (DMF) served as Fmoc de-protecting agent. Peptides were synthesized to contain a C-terminal diaminobenzoic acid (Dbz) linker and a N-terminal cysteine to facilitate head-to-tail cyclization by native chemical ligation [27]. A TentaGel rink amide resin (0.18 mmol/g; Peptide International, Louisville, KY) was used for the synthesis.

Dbz linker coupling (1 eq relative to the resin) was carried out manually (30 min × 2) using 0.5 M HBTU (2 eq) and DIPEA (6 eq). The first residue, the Gly in loop 3 of kB7, was double coupled manually (2 eq; 30 min) in a mixture of 0.5 M HBTU (2 eq) and DIPEA (6 eq). The rest of the peptide chain was elongated in a Liberty automated peptide synthesizer (CEM Corp., Matthews, NC) with microwave irradiation assistance for coupling and de-protection using previously specified conditions [27]. As the last residue, a Boc-Cys was incorporated to facilitate subsequent peptide cyclization by native chemical ligation.

The peptides were converted into N-acyl-benzimidazolinone (Nbz) peptides on resin by acylation with 4-nitrophenylchloroformate in dichloromethane (16 eq; 0.05 M) for 1 h and activation with DIPEA in DMF (129 eq, 0.5 M) for 20 min. The peptides were then dried in dichloromethane and cleaved using a mixture of TFA:triisopropylsilane:water (*v*/v 95%:2.5%:2.5%). Crude Nbz peptides were precipitated with ether and freeze dried. Cyclization of Nbz peptides was performed with a 24 h incubation in 159 mM sodium phosphate buffer (pH 7.1–7.2) containing 6 M GnHCl, 50 mM 4-mercaptophenyl-acetic acid, and 20 mM TCEP-HCl. The cyclized peptides were then purified by PD10 column