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Membrane-modifying properties of crotamine, a small peptide-toxin from *Crotalus durissus terifficus* venom

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ABSTRACT

Background: Crotamine is a small, highly basic myotoxin from the venom of the South American rattlesnake *Crotalus durissus terifficus*. It is structurally well defined and exhibits some similarities with the β-defensins of vertebrates. An amazing variety of functions and targets that range from analgesia and tumor-related activity to cell penetration have been associated with crotamine. Similar to defensins, it had been argued that crotamine has antimicrobial activity, and this supposition was recently proven. Moreover, it has been argued that the antimicrobial activity of crotamine is due to the membrane permeabilizing properties of the peptide. However, until now, the detailed mechanism of this postulated membrane permeabilization was still unclear.
Methods: In this paper, we used gradient SDS-gels, mass spectroscopy (MALDI-TOF), and monolayer and planar lipid bilayer experiments to investigate the membrane modifying properties of crotamine.
Results: We showed that crotamine itself forms stable monolayers because of its amphipathic structure, is easily incorporated into lipid monolayers and forms well-defined pores with low cationic selectivity in planar lipid bilayers; these properties might account for the antimicrobial activity of crotamine. The pores are probably oligomeric aggregates of crotamine molecules, as suggested by the tendency of crotamine to form oligomers in aqueous solution and the fact that the structure of crotamine does not allow pore formation by monomers.
Conclusions: The membrane modifying and antimicrobial properties of crotamine are probably due to homo-oligomeric pore formation in membranes.
General significance: The results should be highly interesting to researchers in the fields of biophysics, pharmacology, toxicology and antibiotics.

1. Introduction

Crotamine is a small, highly basic polypeptide from the venom of the South American rattlesnake *Crotalus durrisus terifficus* [1]. Its primary [2] and three-dimensional [3,4] structures are well defined: 42 amino acid residues and three disulfide bridges [1] form α-helix, β-sheet and random-coil structures [5,6]. Furthermore, the 3D-structure bears a resemblance to the β-defensins from vertebrates [7]. A wide variety of functions and targets have been assigned to crotamine, including analgesic action [8], anti-tumor activities [9], spontaneous repetitive firings of mammalian skeletal muscle [10], membrane penetration [11,12], irreversible membrane depolarization [13] and others [14]. Additionally it has been shown that crotamine exhibits antimicrobial activity [15], and it has been postulated that this antimicrobial activity is most likely due to the membrane permeabilizing properties of the polypeptide [16].

Based on its antimicrobial activity, crotamine belongs to the family of anti-microbial peptides (AMPs) that share the following features: between 10 and 100 amino acid residues, strongly positive charges, and amphipathic structures [17]. AMPs have an alpha-helical part of variable length, beta-sheets, some disulfide bridges and are usually rich in cysteines [17].

The antimicrobial activity of crotamine against a variety of bacteria has been studied and it has been stated that this activity is likely due to crotamine's membrane permeabilizing properties [16]. Some non-human defensins have previously been shown to induce membrane permeabilization and to sometimes even induce ionic pores in membranes [18]. However, the detailed membrane permeabilizing mechanism of crotamine is currently unclear.

In this paper, we show that crotamine forms stable monolayers by itself due to its amphipathic structure, is easily incorporated in lipid monolayers and induces defined pores in planar lipid bilayers. Because crotamine has a significant tendency to form oligomers in water [19], we hypothesized that crotamine forms pores in bacterial and other cell membranes through a homo-oligomeric process because its structure does not allow for the formation of monomeric pores.

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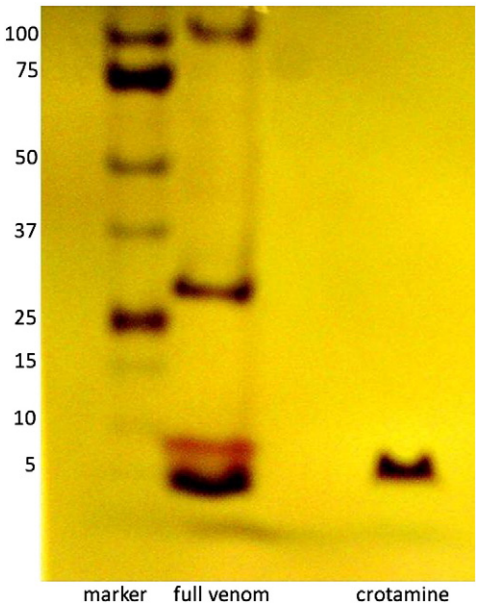

Fig. 1. An SDS Tris–Tricine gradient gel (10–20%, NUSep) of purified crotamine from *Crotalus durissus terrificus* venom is shown. Standard silver staining was used. Left trace: markers (precision plus protein standard, dual Xtra, BioRad) in the molecular weight range of 5–100 kDa. Middle trace: full venom. Right trace: purified crotamine.

2. Materials and methods

Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids. All other lipids and chemicals were obtained from Sigma-Aldrich and were, at minimum, p.a. grade. Purified water was obtained from a laboratory distillery. *Crotalus* venom was purchased from a snake farm in Candeias (IBAMA 001/03/ER Processo 02015.005789/01-14) and delivered in crystalline form.

Crotamine was isolated from the *Crotalus* venom to purity by RP-HPLC (ÄKTA purifier 10, Vydac 218TP-C18 column) with a hydrophilic eluent A (1‰ TFA in ddH_2O) and a hydrophobic eluent B (1‰ TFA in acetonitrile) over 20 column volumes (eluent B gradient 2%–50%). After RP-HPLC purification, the crotamine was concentrated in a vacuum centrifuge. The purity of the crotamine is shown in Fig. 1 using a low molecular weight range SDS Tris–Tricine gradient gel (10–20%). The purified material was also checked by mass spectroscopy (MALDI-TOF). Fig. 2A illustrates that, in the range of 0 to 6000 Da, two double-peaks, the first at approximately 4885 Da (single charged) and the second at approximately 2442 Da (double charged), represent pure crotamine. The double peak structure of crotamine is the consequence of the two isoforms of the crotamine molecule [20,21]. In the mass range above 6000 Da, as shown in Fig. 2B, homo-oligomers of crotamine can be identified in equidistant masses that are up to six times that of the plain crotamine mass.

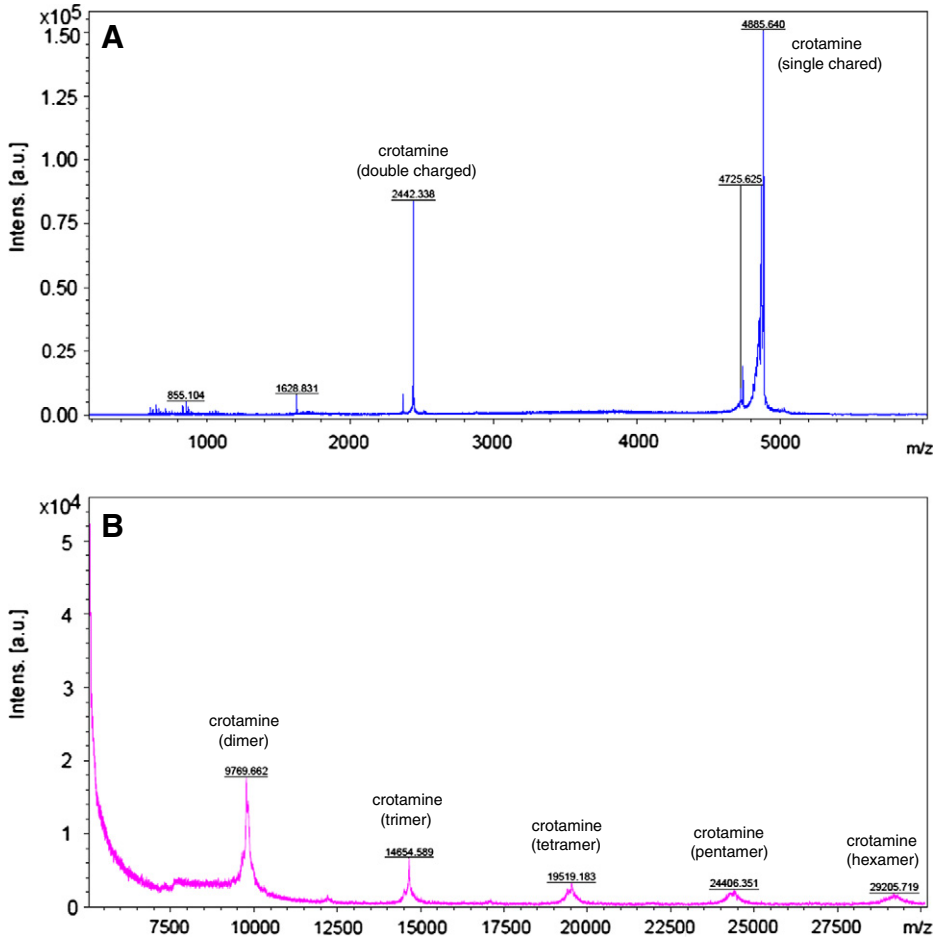

Fig. 2. Mass spectroscopic data of purified crotamine. **A**: Mass range up to 6 kDa. Two double-peaks can clearly be recognized: the first is single-charged with a main mass of 4885.640 Da, and the second is double-charged with a main mass of 2442.338 Da. The double peak results from the two isoforms of crotamine. The other masses were insignificant. **B**: Mass range of 5–30 kDa for the same preparation. The multiples of the crotamine mass indicate the strong oligomerization of this molecule.

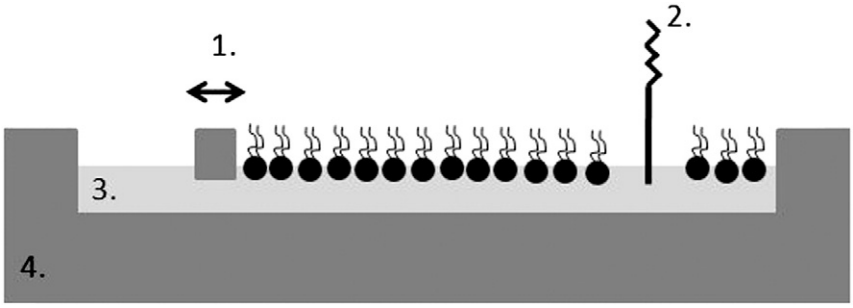

Fig. 3. Illustration of the film balance: 1. movable barrier, 2. force meter with a Wilhelmy-plate, 3. subphase, 4. through. After addition of peptides and incorporation into the lipid monolayer, a decrease in the area was observed.

The monolayer experiments were performed on a Langmuir film balance from NIMA Technologies. These experiments can be used to demonstrate whether peptides or proteins can incorporate into lipid monolayers and lipid bilayers. The principal function of a film balance is shown in Fig. 3. To investigate the monolayer formation of crotamine itself, which, based on its structure, is amphipathic in nature, the isothermic mode (i.e., changes in pressure in relation to area) of the film balance was used. 20 μl of crotamine (equivalent to 250 μg crotamine) was spread on the subphase (100 mM KCl, 10 mM Tris, pH 7.4), and the area was continuously decreased. The incorporation of crotamine into lipid monolayers was studied in the isobaric mode (i.e., changes of area at constant pressure). The aqueous solution used in the film balance was equal to the isothermic measurements with pure crotamine. Lipids (asolectin or PC:PE:PS at a ratio of 8:2:0.5 by weight) were dissolved in chloroform (10 mg/ml), and 2 μl was added to the water-surface by a Hamilton syringe. Crotamine (5 μl equivalent to 62.5 μg) was added to the bath after monolayer formation, again by a Hamilton syringe. The bath was gently stirred.

The bilayer experiments were performed on a homemade classical bilayer set-up (see Fig. 4) that has been previously described [22]. The method of Mueller and Rudin [23] was used for bilayer formation. The lipids were dissolved in decane (30 mg/ml), and the compositions of the aqueous solution are given in the Results section. After bilayer formation, crotamine (2 μl equivalent to 25 μg) was added to the bath solution of the grounded side of the bilayer.

All experiments were performed at room temperature (20 °C to 22 °C).

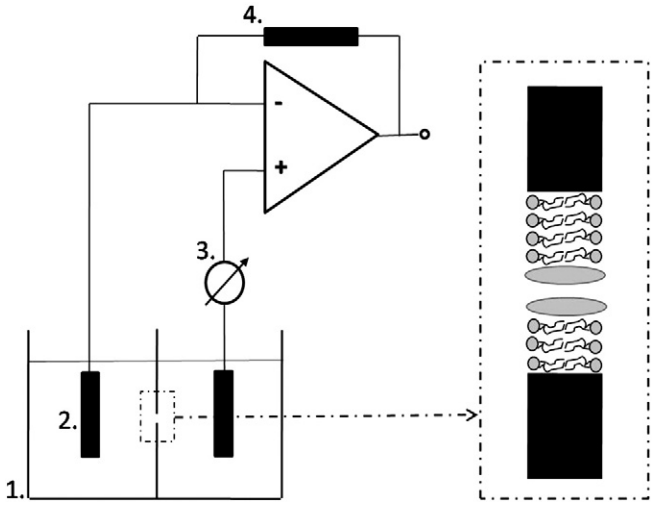

Fig. 4. Illustration of a classical bilayer setup. 1. Chamber system filled with a bath solution, 2. Ag/AgCl-electrodes, 3. voltage encoder, and 4. amplifier. On the right, a close up of the lipid bilayer is shown.

3. Results

As described in the Methods and methods section, crotamine was purified by RP-HPLC, and its tendency to form homo-oligomeric aggregates was demonstrated using mass spectroscopy. The structure of crotamine is amphipathic [24]. Thus, crotamine formed ordered monolayers at water–air interfaces as illustrated in Fig. 3. Crotamine was spread on a bath solution using a film balance. The isothermic mode was used to obtain area-pressure diagrams. As seen in Fig. 5, the area-pressure curve (compression/decompression) has a defined shoulder at a pressure close to 7.5 mN/m. This finding indicates that condensation of at least parts of the molecules occurred at this pressure range.

Next, the incorporation of crotamine into plain lipid monolayers was studied. Lipids (asolectin or PC:PE:PS at a ratio of 8:2:0.5 by weight) dissolved in chloroform (10 mg/ml) were spread on the air–water interface of a film balance. The lipids were chosen to contain negatively charged lipids because bacterial membranes contain a significant amount of negatively charged lipids, and it can be argued that these negatively charged lipids might interact specifically with the positively charged crotamine [12]. After evaporation of the solvent, the film balance was operated in the isobaric mode at a pressure of 7.5 mN/m. At this relatively low pressure, in the constant pressure mode of the film balance, crotamine was expected to be incorporated well; crotamine also exhibited a pseudo phase transition at this pressure that can be seen in the

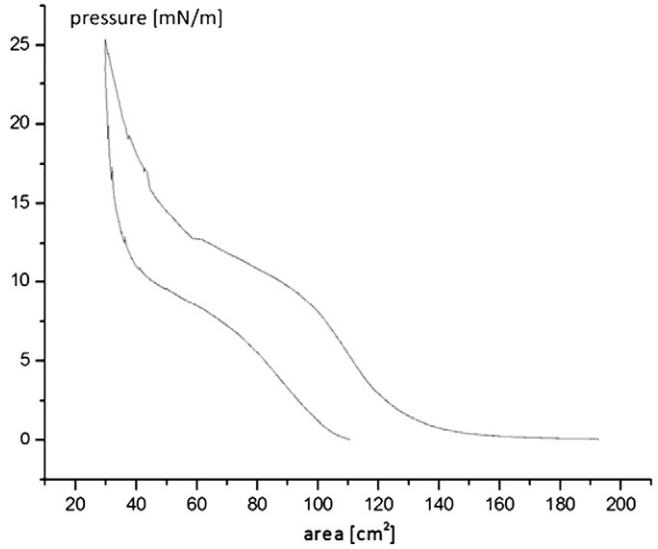

Fig. 5. Isotherm of purified crotamine. The graph shows that crotamine can form stable monolayers on water–air interfaces by itself (subphase: 100 mM KCl, 10 mM Tris, pH 7.4). Therefore, crotamine is amphipathic. Crotamine exhibits a pseudo phase-transition at a pressure of approximately 7.5 mN/m, indicating that a portion of these molecules condensed.

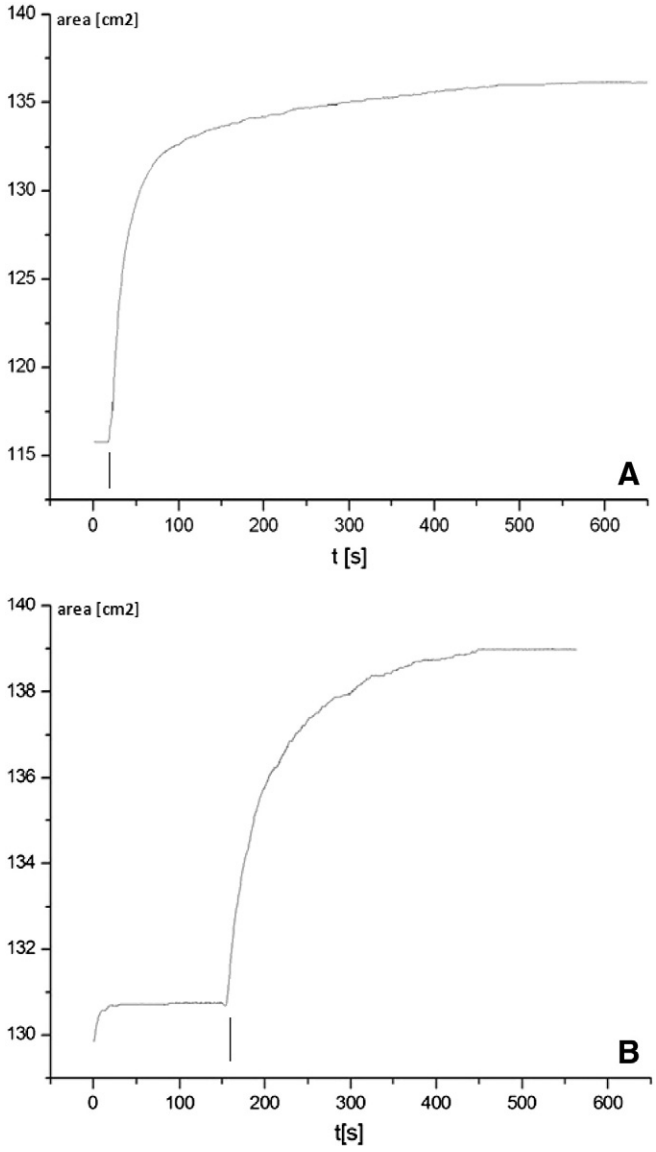

Fig. 6. A: Isobare of an asolectin monolayer after crotamine addition. After formation of the monolayer, crotamine was added to the subphase (point of time marked by a bar below the trace), composed of 100 mM KCl, 10 mM Tris, pH 7.4. Crotamine is incorporated into the bilayer on a timescale of approximately 100 s, as shown by the increasing area. **B**: Isobare of a PC:PE:PS (8:2:0.5 parts weight) monolayer and crotamine (equal subphase to asolectin monolayer). Crotamine is incorporated into the bilayer on a timescale of some 100 s, again shown by the increasing area.

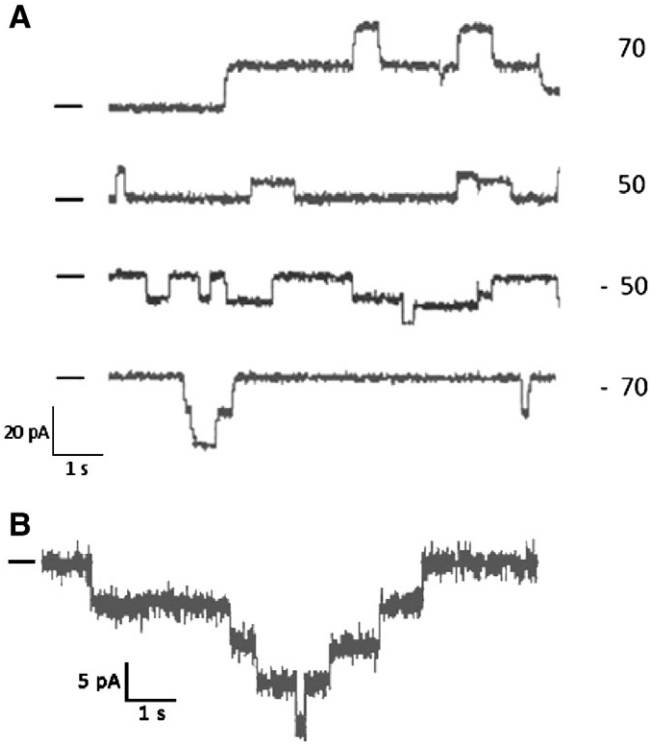

Fig. 7. Pore formation of crotamine in planar lipid bilayers. The bilayers were made from asolectin (30 mg/ml). **A**: Example traces at different holding potentials are shown (bath solution: 1 M KCl, 10 mM Tris, pH 7.4). The baseline is marked at the left of the traces. Pore fluctuations can clearly be seen even within the different open state levels. **B**: Recording of a multi-pore event with four identical levels and shown at −25 mV (bath solution: 1 M KCl, 10 mM Tris, pH 7.4). Channel opening is reflected by downward deflections in current.

area-pressure curve. Crotamine was added to the bath solution, which was gently stirred. As seen in Fig. 6, the increasing area of the film at a constant pressure over a period of around 100 s indicates that crotamine was definitely incorporated into the monolayers.

After showing that crotamine is incorporated well into lipid monolayers, we investigated the membrane-modifying properties of crotamine using the planar lipid bilayer technique. The bilayers were made from asolectin (a lipid mixture from soy beans with PC, PE and Pi; thus, this mixture also has negatively charged lipids) dissolved in decane (30 mg/ml) according to the Mueller–Rudin technique [23]. In these experiments, we found that crotamine forms well-defined pores in lipid membranes as illustrated in Fig. 7. Fig. 7A shows current recordings at different voltages that indicate defined pores with conductances of approximately 200 pS. In addition to this main conductance level, sub-levels of lower conductances are recognizable. Fig. 7B shows a multi-pore recording of 4 equidistant pores with the same conductance. The current to voltage relations of the crotamine pores (Fig. 8) were linear in a range of −100 mV to +100 mV. Additionally, we found that the pores were only weakly cation-selective based on the comparison of the conductivities of K^+ and Na^+ (K^+:Na^+ 1.25:1). In measurements with $CaCl_2$ (100 mM $CaCl_2$, 10 mM Tris, pH 7.4), no pore-forming activity was observed. The calculated open state probability showed that pore formation had no relevant voltage dependency as shown in Fig. 9.

4. Discussion and outlook

Based on previous literature, it has been argued that crotamine, a small basic peptide from *C. durissus terifficus*, exhibits antimicrobial activity [16] and thus belongs to the well-studied group of AMPs. This argument derives particular support from the similarity of crotamine to the β-defensins of vertebrates, which, among other activities, are known to act as antibiotics [25]. In a more recent study [16], the antimicrobial behavior of crotamine was investigated in further detail and verified. Based on the current results, we suggest that this verified antimicrobial activity of crotamine is likely due to a membrane permeabilizing mechanism. However, the detailed nature of this mechanism remains unclear, which, in a more general sense, is also true for the majority of AMPs [26]. Thus, these mechanisms are an important field of pharmacological research.

It has previously been shown that some defensins are able to form stable pores in lipid membranes [18], and it can be argued that

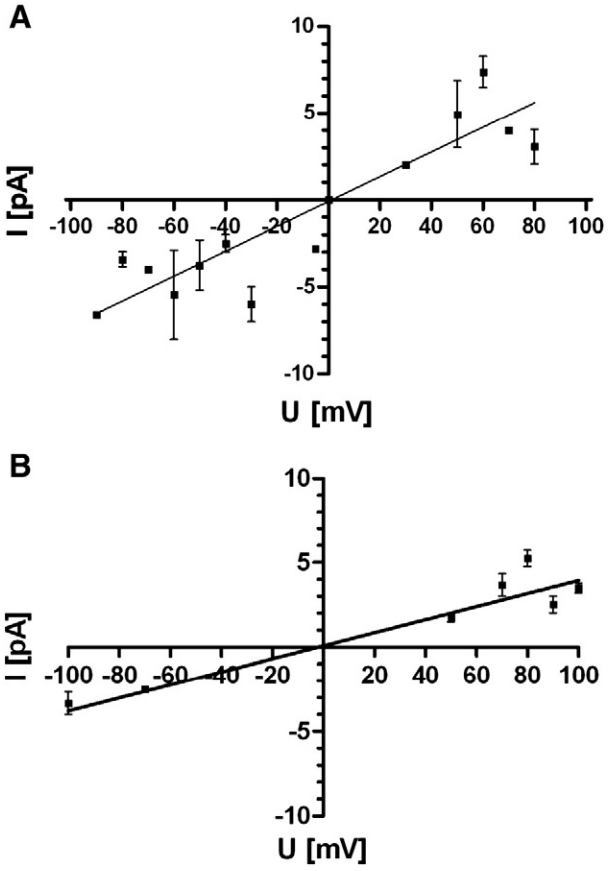

Fig. 8. Current-voltage characteristics of crotamine pores in asolectin bilayers with different bath solutions (the means ± the SDs are shown). **A**: The graph shows a linear conductance of approximately 72 pS from −90 mV to 80 mV (bath solution: 150 mM KCl, 10 mM Tris, pH 7.4). **B**: The graph shows a linear conductance of approximately 38 pS from −100 mV to 100 mV (bath solution: 100 mM NaCl, 10 mM Tris, pH 7.4). The comparison of **A** and **B** indicates that the crotamine pores lacked significant selectivity between K^+ and Na^+ (1.25:1).

crotamine also has this ability. Monolayer studies are widely accepted in the study of the incorporation of AMPs into membranes and, more specifically, for the study of peptide–lipid interactions [27,28]. Thus, we used monolayers to verify the incorporation of crotamine into membranes and to show that crotamine itself is able to form a stable monolayer due to its amphipathic nature. Our experiments allowed us to present data that clearly demonstrate that crotamine is incorporated into lipid monolayers well and forms stable pores with low cationic selectivity in black lipid bilayers. Based on the structure of crotamine, it can be argued that the formation of monomeric pores by this molecule is impossible. Thus, based on the demonstrated oligomerization of crotamine in aqueous solution (shown in Fig. 2B) and its partially amphipathic structure, we propose a homo-oligomeric pore formation model for crotamine. The occurrence of levels of conductance that were higher and lower than the main level, even at low probability, support this argument because the additional levels might be due to aggregates of different numbers of monomers compared to the predominate state.

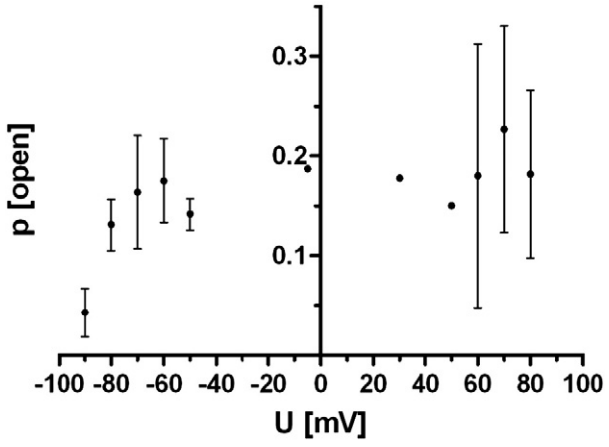

Fig. 9. Open state probability of crotamine pores in asolectin bilayers (bath solution: 150 mM KCl, 10 mM Tris, pH 7.4, the means ± the SDs are shown). No significant voltage dependency of the open state was detected.

A large variety of pore-forming mechanisms for small peptides have been published [e.g., 29], and one of these might be useful for explaining the pore-forming mechanism of crotamine. Although a mixed lipid-peptide pore-forming mechanism is also possible [30], a more specific model cannot be justified at present. The pore-forming properties of crotamine are most likely sufficient for explaining crotamine's membrane modifying properties and thus its antimicrobial activity.

The question of why a snake would have venom components with antimicrobial activity is of additional zoological interest.

Acknowledgements

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