

Ion channel-like activity of the antimicrobial peptide tritrpticin in planar lipid bilayers

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Abstract The cationic peptide tritrpticin (VRRFPWWP-FLRR, Trp3) has a broad action spectrum, acting against Gram-positive and Gram-negative bacteria, as well as some fungi, while also displaying hemolytic activity. We have studied the behavior of Trp3 in planar lipid bilayers (or black lipid membrane – BLM) and were able to demonstrate its ion channel-like activity. Channel-like activity was observed in negatively charged azolectin BLM as a sudden appearance of discrete current fluctuations upon application of a constant voltage across the membrane. Trp3 formed large conductance channels (500–2000 pS) both at positive and negative potentials. In azolectin bilayers, the predominant ion-channel activity was characterized by very regular and discrete current steps (corresponding to openings) of uniform amplitude, which exhibited relatively long residence times (of the order of seconds). Occasionally, multiple conductance steps were observed, indicating the simultaneous presence of more than one open pore. In bilayers of zwitterionic diphytanoylphosphatidyl choline (DPhPC) Trp3 also showed ion-channel activity, but in a much less frequent and less prominent way. Studies of ion selectivity indicated that Trp3 forms a cation-selective channel. These results should contribute to the understanding of the molecular interactions and mechanism of action of Trp3 in lipid bilayers and biological membranes.

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1. Introduction

A vast variety of organisms from both vegetal and animal kingdoms produces substances whose main function is defense against invading pathogenic micro-organisms. An im-

portant sub-class of these substances – antimicrobial peptides – constitutes a substantial component of the innate defense system of plants and animals. A large number of antimicrobial peptides were discovered and characterized over the last decades [1–3]. The importance of antimicrobial peptides stems from the growing problem of pathogenic organisms resistant to conventional antibiotics. Moreover, while many conventional antibiotics disable or kill bacteria over a period of days, antimicrobial peptides act within minutes [4]. In addition, some have a broad action spectrum, acting against bacteria, fungi, viruses, and protozoa. All these facts have raised increasing interest in the pharmacological application of antimicrobial peptides to explore their potential as novel therapeutic agents. Detailed investigations have indicated that the majority of host-defense peptides exert their action by permeabilizing cell membranes. Thus, a major task is to understand the molecular mechanisms underlying peptide-mediated cell lysis [4–6].

The 13-residue cationic peptide tritrpticin (VRRFPWW-WPFLRR, Trp3) is a cathelicidin with a high Arg (30%), Trp (23%), and Pro (15%) content. This high Arg and Trp content, together with three consecutive Trp residues, makes this peptide unique. Trp3 has a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, and some fungi [7], as well as hemolytic activity [8].

Nuclear magnetic resonance (NMR) studies showed that, while Trp3 interconverts between different conformations in solution, upon binding to sodium dodecyl sulfate (SDS) micelles, it acquires a conformation with two adjacent turns around Pro5 and Pro9. Such a positioning of the amino acids causes Trp3 to adopt an amphipathic turn–turn structure, with the Trp clustered together and buried in the hydrophobic core of the micelles and the Arg residues on the opposite side of the structure [9].

Several studies aimed at understanding the mechanism of action of Trp3 peptide in lipid membranes have been performed [8,10–13]. However, no clear description has been obtained thus far. In this work, we studied the behavior of Trp3 in planar lipid bilayers and we demonstrate that the peptide has ion channel-like activity. To our knowledge, this finding has not been reported before. The present results should contribute to the understanding of the molecular interactions and mechanism of action of this unique antimicrobial peptide in biological and lipid membranes.

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Abbreviations: Boc, *tert*-butoxycarbonyl; For, formyl; HPLC, high performance liquid chromatography; DOPC, dioleoylphosphatidyl choline; DOPE, dioleoylphosphatidyl ethanolamine; DOPG, dioleoylphosphatidyl glycerol; DPhPC, diphytanoylphosphatidyl choline; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PBC, phosphate–borate–citrate; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; SDS, sodium dodecyl sulfate; Tos, *p*-toluenesulfonyl; Trp3, tritrpticin

2. Materials and methods

2.1. Materials

Monobasic potassium phosphate, boric acid, and potassium chloride were from Merck S.A. Indústrias Químicas (Rio de Janeiro, RJ, Brazil); sodium citrate was from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Azolectin and *n*-decane came from Sigma Chemical Co., St. Louis, MO; diphytanoylphosphatidyl choline (DPhPC) was from Avanti Polar Lipids, Alabaster, AL. Five millimolar phosphate–borate–citrate (PBC) buffer, pH 7.2, prepared in Milli-Q water was used throughout.

2.2. Peptide synthesis and sample preparation

Trp3 was synthesized manually according to the standard *N*^α-*tert*-butoxycarbonyl (Boc) protecting group strategy [14,15] using *p*-toluenesulfonyl (Tos) and formyl (For) Boc-amino acid derivatives of Arg and Trp, respectively. The synthesis was performed as described in [16]. Analytical high performance liquid chromatography (HPLC) (Waters), LC/MS (electrospray–Micromass)–mass spectrometry (theoretical *m/z* = 1902.28; found: 1902) and amino acid analysis (Biochrom 20 Plus, from Amersham Biosciences) were used to check the homogeneity of purified Trp3.

The concentration of 25 μ M peptide stock solutions was determined by UV–visible absorption in a Hitachi U-2010 spectrophotometer using a calculated molar extinction coefficient (at 280 nm) of 16 500 $M^{-1} cm^{-1}$. Stock solutions were frozen immediately after preparation and stored protected from light as small aliquots at $-20^{\circ}C$. Thawed stock solutions were never frozen and used again [17].

2.3. Planar lipid bilayer preparation and electrical measurements

Planar lipid bilayers (or black lipid membranes – BLMs) were formed from azolectin or from DPhPC. The approximate phospholipid composition of azolectin is phosphatidyl choline (PC), 29%; phosphatidyl ethanolamine (PE), 30%; phosphatidyl inositol (PI), 26%; phosphatidic acid (PA), 14%; phosphatidyl serine (PS), 1% [18]. Azolectin or DPhPC were dissolved in *n*-decane to yield a 2.5% solution. Planar lipid bilayers were formed across a 0.2–1-mm hole in a polypropylene cylindrical cup inserted in a special acrylic chamber. The cup separated two solution-filled compartments designated *cis* and *trans* [19]. The *cis* compartment (to which the peptide was added) was held at ground (0 mV) and the *trans* compartment was clamped to a range of potentials. The sign of the membrane potential refers to the *trans* compartment. Both compartments (*cis* and *trans*) contained the same volume (about 3 ml) and concentration of salt in buffer solution (100 mM KCl in 5 mM PBC, pH 7.2, symmetrical conditions). Three microliters of 25 μ M Trp3 stock solution were added to the *cis* side which was stirred for 2 min using a stream of nitrogen obliquely directed over the surface.

The transmembrane current (I_m), under different clamping potentials (V_{clamp}), was monitored using a patch-clamp amplifier (Dagan 8900) configured in voltage-clamp mode. Membrane conductance (G_m) was obtained as $G_m = I_m/V_{clamp}$. Data were acquired using the Axotape 2.0.2 software and analyzed with AxoScope 8.0 (Axon Instruments).

The channel ion selectivity was determined by measuring the open-circuit (spontaneous) transmembrane voltage under a KCl concentration difference imposed across the membrane. For this procedure a high-impedance digital electrometer (Keithley Instruments model 616) was used.

3. Results

3.1. Trp3 channel-like activity

The ability of the antimicrobial peptide Trp3 to induce ion conductance was tested in azolectin planar lipid bilayers. This soy phospholipid mixture consists of zwitterionic PC and PE and negatively charged PI, PA, and PS (see Section 2). Channel-like activity, due to the addition of an aqueous solution of the peptide to the BLM bath, was observed as a sudden appearance of discrete current fluctuations when a constant voltage (V) was applied across the membrane.

Trp3 formed channels at both positive and negative potentials. V_{clamp} corresponds to the potential difference between the *trans*- and the *cis*-side (ground). By convention, a positive current reflects the flow of cations from the *cis*- to the *trans*-compartment or the flow of anions in the opposite direction.

Fig. 1 shows the peptide's channel activity in azolectin BLM. The traces present stochastic transitions between conducting (open) and non-conducting (closed) states. The predominant ion-channel activity is characterized by very regular and discrete current steps (corresponding to openings) of uniform amplitude and relatively long (of the order of seconds) residence times. Occasionally, multiple conductance steps were observed, indicating the simultaneous opening of more than one pore. A second type of channel event was also observed exhibiting shorter dwell open times. Fig. 2 shows the voltage–current relationship for the open channel and indicates a non-linear behavior of I – V relationships for high positive or negative voltage values.

Trp3 generates ion-channel activity with a predominant conductive state with conductance $G_1 = 1054 \pm 108$ pS ($n = 15$) and less predominant conductance values of $G_2 = 1638 \pm 96$ pS ($n = 6$); $G_3 = 2532 \pm 322$ pS ($n = 3$); $G_4 = 508 \pm 56$ pS ($n = 2$). n is the number of regular opening events.

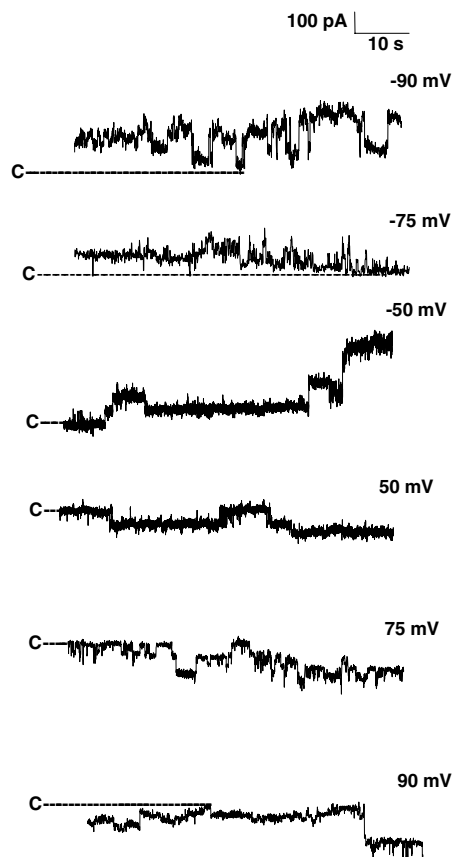


Fig. 1. Trp3 channels in azolectin BLM. Single-channel current–time traces under symmetric conditions: 100 mM KCl, 5 mM PBC, pH 7.2, at different applied voltages. Upward open levels occur at negative applied voltages and downward open levels occur at positive applied voltages, closed levels are indicated by dashed lines.

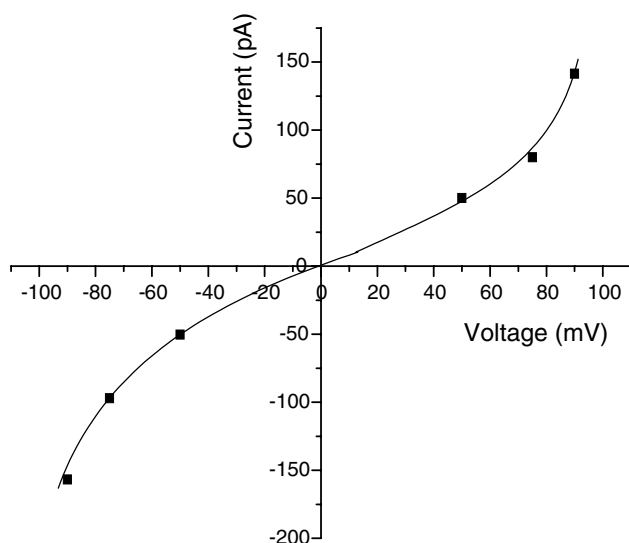


Fig. 2. Current–voltage relationship of the Trp3 channel formed in azolectin BLM.

We have also tested the ion-channel activity of Trp3 in planar bilayers of zwitterionic DPhPC (Fig. 3). In this case, the ion-channel activity was less frequent and less evident. The number of regular events did not allow a statistical treatment and G was equal to 445 pS.

3.2. Trp3 channel ion selectivity

The ion selectivity of the pores formed by Trp3 was investigated by establishing a KCl gradient across the membrane and measuring the open-circuit voltage (reversal potential) across the bilayer, using a pair of Ag–AgCl electrodes. After the increase of membrane conductance induced by addition of the peptide, successive 100 μ l aliquots of 2 M KCl were introduced in the *cis* side under stirring and the same volume was taken out in order to increase the KCl concentration while keeping constant the solution level so as to preserve membrane integrity. The open-circuit voltage was measured continuously by means of a high-impedance digital electrometer until a stable trans-membrane voltage of 20–30 mV was obtained. At this moment the membrane was broken and the voltage reading immediately recorded (post-breaking voltage). Invariably, the post-breaking voltage was half the total measured voltage, indicating 100% selectivity of K^+ over Cl^- . Since the total measured voltage across the membrane represents the sum of two electrode potentials plus a membrane potential, the finding of a post-breaking voltage equalling half the total voltage indicates that the electrode potential is equal to the membrane potential and both are Nernst equilibrium potentials.

4. Discussion

Measurements of conductance in planar lipid bilayer are considered a sensitive tool to study channel formation. One main advantage is the remarkable sensitivity and specificity of the assay. Many antimicrobial peptides as well as rationally designed channel-forming peptides interact with BLM at very low concentrations (10^{-6} – 10^{-12} g/ml), inducing ion currents [20–23].

In this study, incorporation of the antimicrobial peptide Trp3 into azolectin planar lipid bilayers (which carry a net negative surface charge) induced the appearance of a predominantly rectangular, stable, and regular current fluctuation, as well as much less frequent and irregular current fluctuations (Fig. 1). This finding indicates that Trp3 is a pore-forming peptide, displaying ion channel-like activity with very high single-channel conductance, ranging from a few hundreds to about two thousand pS. Other relatively small peptides form channels: the cationic A β amyloid peptides form large conductance multistate channels (10–2000 pS [23,24]); the rabbit neutrophil peptide (NP-1) gives rise to channels whose conductance varies from 10 to 1000 pS [25], magainin 1 originates channels with two levels of conductance, 366 and 683 pS [26], and the antimicrobial peptide indolicidin, a 13-residue Trp-rich peptide, forms discrete channels with conductance values ranging from 50 to 150 pS [27].

It should be noticed that azolectin contains a considerable amount of negatively charged head groups, as usually found on the bacterial cell surface (PI, 26%; PA, 14%; PS, 1% [18]). However, preliminary data for the zwitterionic phospholipid DPhPC indicate pronounced, but less well-defined and less frequent, ion channel activity (Fig. 3). This is in agreement with the lesser leakage effect of Trp3 in zwitterionic dioleoylphosphatidyl choline:dioleoylphosphatidyl ethanolamine (DOPC:DOPE) vesicles [11].

Schibli et al. [11] have reported studies of the interaction between Trp3 and other Trp-rich antimicrobial peptides with membranes of variable lipid composition. Fluorescence studies of maximum emission wavelengths, red-edge effects, quenching by water-soluble and membrane-embedded quenchers showed that Trp3 binds preferentially to membranes containing negatively charged dioleoylphosphatidyl glycerol (DOPG), and, to a lesser extent, to DOPC:DOPE. Moreover, peptide-induced leakage from the inner aqueous compartment of large unilamellar vesicles indicated that the effect followed the order: DOPE:DOPG > DOPC:DOPG \gg DOPC:DOPE. In addition, Trp3 had the largest effect among the studied peptides, including indolicidin. These data are in agreement with our observation of the pronounced channel activity of Trp3 in negatively charged azolectin BLM and with the fact that Trp3 ion channels display larger conductance (this work) than those

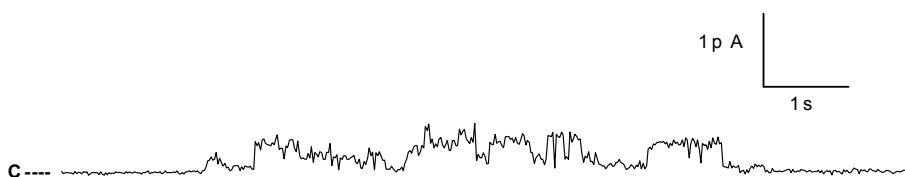


Fig. 3. Trp3 channels in DPhPC BLM. Single-channel current–time traces under symmetric conditions: 100 mM KCl, 5 mM PBC, pH 7.2, at –80 mV. Upward open levels occur at negative applied voltage, closed level is indicated by dashed line.

formed by indolicidin [27]. Interestingly, preliminary data in [11] showed that Trp3 has an approximately twofold higher antimicrobial activity than indolicidin. Furthermore, the very low leakage induced by Trp3 in DOPC:DOPE membranes [11] correlates well with the presently found less frequent and less pronounced ion-channel activity of the peptide in DPhPC (Fig. 3).

In the work by Schibli et al. [11], the fact that Trp3 displayed the largest red-edge effect was interpreted as reflecting the peptide more stable insertion in the interface, which resulted in it being more motionally restricted. This behavior was proposed as the explanation for Trp3 being the most lytic among the Trp-rich peptides. This fact could also account for the stable and regular ion-channel activity of the Trp3 pore (Fig. 1), suggesting a fixed stoichiometry (i.e., a fixed number of peptides per pore). The symmetric supralinear current-voltage relationship of the open-channel (Fig. 2) remains to be explained. Nevertheless, it should be noticed that this is a common finding in many types of ion channels [28].

The ion-selectivity assay indicates that Trp3 forms a cation-selective pore. This is an unexpected finding, considering the cationic nature of the peptide. Ion selectivity is frequently found but not always straightforwardly correlated with the sign of charged sites in the channel. This lack of correlation is probably due to the fact that channels use a variety of mechanisms to discriminate between ions, and the selectivity often reflects different aspects of the channel structure [29]. Several positively charged peptides form cation-selective channels: a segment of the sodium channel polypeptide [30], synthetic α -helical peptides containing Arg as the main charged amino acid residue [31], magainin 2, a natural antibiotic [32]. The high Trp3-induced conductance suggests a large pore diameter and a low interionic discrimination based on the small difference of the Pauling radii of the conducting ions (K^+ 1.33 Å; Cl^- 1.81 Å). The selectivity would not be determined by the dehydration energy of the ions [33] either, since the hydration energies [34] of chloride ($\Delta H_{\text{hydration}} = -82$ kcal/mol) and potassium ($\Delta H_{\text{hydration}} = -85$ kcal/mol) are very similar.

The present results bring about several questions: (1) What is the pore structure and dynamics? (2) What is the molecular mechanism of pore formation? (3) What is the molecular basis of ion selectivity? Several models have been proposed for the mechanism of action of antimicrobial peptides in lipid membranes [35–39]. The barrel-stave model involves a bundle of membrane-spanning amphipathic secondary structures (usually α -helices) with their polar faces lining the pore lumen. This model is illustrated by alamethicin, whose self-assembly in lipid bilayers generates ion channels with reproducible discrete multiple conductance states [40]. Trp3 is a 13-residue peptide not expected to span the lipid bilayer as an α -helix. In an extended conformation (as a β -sheet) its length would be ca. 4.55 nm, enough to span the bilayer, whose hydrocarbon core thickness is approximately 3.5 nm. However, due to its sizeable dipole and quadrupole moments, as well as hydrogen-bonding potential [41], the tryptophan indole moiety displays a preference for the membrane interface rather than a more hydrophobic environment, which would be energetically unfavorable [42]. The organization of Trp3 molecules in a bilayer-spanning form would require the tryptophan residues to be located in a more hydrophobic environment, therefore being energetically unfavorable.

Indeed, spectroscopic data do not provide evidence for β -sheet conformation for Trp3 bound to SDS micelles [9]. NMR studies [9] showed that the peptide forms two adjacent turns around the two Pro residues; the first turn involves residues 4–7, followed by a second 3_{10} -helical turn involving residues 8–11. The hydrophobic residues are clustered together and are clearly separated from the basic Arg residues, forming an amphipathic structure. NMR measurements in the presence of a spin label evinced that Trp3 lies near the micelle surface [9]. A similar location was also determined in lipid bilayers by fluorescence quenching [11].

Nevertheless, Trp3 is able to induce leakage of solutes located in the aqueous inner compartment of liposomes [11] and the present results clearly demonstrate that the peptide can form ion channels in negatively charged and zwitterionic lipid bilayers. A second model of membrane permeabilization has been proposed for several α -helical peptides. According to this model, known as the Shai–Matsuzaki–Huang (SMH) model [1], initially the peptides bind at the membrane interface, with the helix axis approximately parallel to the bilayer surface. As discussed by Matsuzaki [43], this location pushes the lipid polar head groups aside, leading to the local induction of positive curvature strain. The unfavorable membrane-deformation energy reaches a critical value and several peptide molecules with surrounding lipids form a dynamic, peptide-lipid supramolecular complex pore [44] or a toroidal pore [35,45] whose polar surface is formed by both peptides and lipid. In this model the participation of negatively charged phospholipid head groups in toroidal pore formation might contribute to the pore cation selectivity.

Although this mechanism has not been verified for non- α -helical peptides yet, Schibli et al. [11] suggested that this could be the mechanism for Trp3-induced membrane permeabilization, especially in view of its Trp residues being located mostly at the membrane interface. However, the authors do point out that the leakage efficiency is higher for DOPE:DOPG than for DOPC:DOPG membranes. This observation is not in agreement with the notion that toroidal pore formation would be favored by positive curvature-inducing PC and inhibited by negative curvature-inducing PE [37,43].

Clearly, the lipid composition of bacterial membranes is complex and variable. Since a large number of bacterial species contain zwitterionic PE and negatively charged PG as major phospholipids, these lipids have been often used in model membranes for studies with antimicrobial peptides. However, in view of the current ideas about the requirement of lipids that favor positive curvature in order to form a toroidal pore, the latter have also been used in model membrane systems (e.g., PC, as in the work by Schibli et al. [11]). Azolectin contains lipids found in bacterial membranes, albeit sometimes in smaller amounts. Both positive (PC, 29%; PI, 26%; PA, 14%; PS, 1%, at the pH and ionic strength of the present experiments) and negative (PE, 30%) curvature-inducing lipids are present in azolectin [46]. Thus, although it does not contain PG, the negatively charged lipids found in azolectin (PI, PA, and PS, a total of 41%) favor positive curvature, like PG, under physiological conditions [46]. It should also be pointed out that Trp3 has been proposed to induce membrane positive curvature [41].

Electrostatic interactions between positively charged Trp3 and negatively charged phospholipid head groups would favor the displacement of these lipids to the conducting pathway

and, consequently, contribute to attract cations over anions to the formed pore. The high stability of the pores formed by Trp3 would be favored by a peptide–lipid composite pore with the peptide located at the interface as in the SMH toroidal pore model.

Correlating structure and function of peptides is crucial for understanding the underlying molecular and physicochemical basis of their mode of action. This work demonstrates that Trp3 can act as a pore-forming peptide. However, in the light of the existing data, a complete model cannot be proposed for the Trp3 pore structure and dynamics. Aiming at elucidating these questions, we are presently conducting detailed spectroscopic and electrophysiological studies of Trp3 conformational and ion-channel properties.

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