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Antibacterial peptide pleurocidin forms ion channels in planar lipid bilayers

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Abstract

Pleurocidin, a 25-residue α helical cationic peptide, isolated from skin mucous secretions of the winter flounder, displays a strong anti-microbial activity and appears to play a role in innate host defence. This peptide would be responsible for pore formation in the membrane of bacteria leading to lysis and therefore death. In this study, we investigated the behaviour of pleurocidin in different planar lipid bilayers to determine its mechanism of membrane permeabilisation. Macroscopic conductance experiments showed that pleurocidin did not display a pore-forming activity in neutral phosphatidylcholine/phosphatidylethanolamine (PC/PE) lipid bilayers. However, in 7:3:1 PC/PE/phosphatidylserine (PS) lipid bilayers, pleurocidin showed reproducible *I*/*V* curves at different peptide concentrations. This activity is confirmed by single-channel experiments since well-defined ion channels were obtained if the lipid mixture was containing an anionic lipid (PS). The ion channel characteristics such as—no voltage dependence, only one unitary conductance, linear relation ship current–voltage—, are not in favour of the membrane permeabilisation according to the barrel model but rather by the toroidal pore formation.

Keywords: Amphipathy; Pore-forming; α Helix; Skin mucus; Fish

1. Introduction

During the past two decades, many antibacterial peptides have been isolated and characterised from a broad variety of animals and plants [1,2]. For more information, see the databank of antimicrobial peptides and proteins: http://www.bbcm.univ.trieste.it/~tossi/search.htm. Many of these peptides are involved in the innate immune response to bacterial aggressions and insure the first line of defence. Broad anti-microbial spectra and weak resistance developments by bacteria make these compounds potential alternatives for the classic anti-biotherapy which is increasingly confronted with the emergent problem of drug-resistant pathogenic bacteria.

In invertebrates, since the first cecropin from *Hyalophora* [3], many other anti-microbial peptides have been isolated from different insect families [4–6]. In vertebrates, bombinin was the first isolated peptide from skin secretions of frog [7] followed by several other peptides such as magainins [8], brevinins [9] or dermaseptins [10]. In higher vertebrates, including humans, defensins [11], cathelicidin [12], protegrins [13] were also isolated and more recently calcitermin [14] and hepcidin [15]. Although their primary sequences are very different and their secondary structure can be constituted of α-helices or β sheets or both, these peptides possess often several positive charges and an amphipathic character. These particularities seem to play an essential role in different action mechanisms described in the literature about the membrane permeabilisation involved in the cell death. Briefly, three mechanisms are proposed in the literature. In the first case, the membrane is permeabilised by the formation of transmembrane pores composed of a bundle of amphipathic helices according to the "barrel-stave model" as observed in alamethicin [16,17]. In the second case, lipids are inserted between helices to form a mixed pore according to the "toroidal model" as described for magainin [18,19]. In the last model, called "carpet-like mechanism" peptides act as detergent and disrupt the membrane via, eventually, the formation of transitory pores [20]. Nevertheless, whatever the mechanism involved, the first step is the peptide adsorption at the membrane surface.

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The interaction between peptide and bilayer is submitted to important parameters such as charge, structure and amphipathicity of peptides but also to the chemical composition of lipids. Thus, the high proportion of negative charges in gram-negative outer membrane would favour the electrostatic interaction with cationic peptides contrarily to erythrocytes lipids which are mainly neutral or zwitterionic.

Recently, pleurocidin, a new peptide isolated from skin mucosa of the winter flounder (*Pseudopleuronectes americanus*), was isolated and characterised [21]. This 25-amino-acid-residue peptide presents a strong antibacterial activity against gram-negative and gram-positive bacteria. Pleurocidin shows primary sequence homology with dermaseptins [22,23] and ceratatoxins [24,25] and like those possesses an amphipathic α helical structure. More recently, CD measurements and experiments of dye-leakage activity showed that pleurocidin, like magainin, was able to form pores in the lipid membrane [26]. However, there has been no direct investigation to determine (i) the ability of pleurocidin to form ion channels in lipid membranes and (ii) the action mechanism used to permeabilise the bacterial membrane leading to the death of bacteria. In this paper, we study the behaviour of pleurocidin in neutral planar lipid bilayers as well as in bilayers containing an acidic phospholipid, by measuring its pore-forming activity and by determining its single-channel characteristics. The results obtained allow us to propose a mechanism of action involving the "toroidal pore model" [19].

2. Materials and methods

Pleurocidin, a native peptide from skin secretions of winter flounder, is a generous gift of G. Diamond (New Jersey, USA). The sequence of this 25-residue peptide is GWGSFFKKAAHVGKHVGKAALTHYL.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) grade 1 from egg yolk and phosphatidylserine (PS) grade 1 from bovine spinal cord were purchased from Lipid Products (Surrey, UK).

In macroscopic conductance experiments, virtually solvent-free planar lipid bilayers were formed by the Montal and Mueller technique [27]. The membrane was formed over a 100- to 150-μm hole in a Teflon film (10 μm thick), pre-treated with a mixture of 1:40 (v/v) hexadecane/hexane, separating two half glass cells. Lipid monolayers were spread on top of electrolyte solution (1 M KCl, 10 mM Tris, pH 7.4) in both compartments. Bilayer formation was achieved by lowering and raising the electrolyte level in one or both sides and monitoring by capacity responses. The lipid solution at 5 mg/ml was either a mixture of PC/PE 7:3 (w/w) or PC/PE/PS 7:3:1 (w/w). Voltage was applied through an Ag/AgCl electrode in the *cis* side. Pleurocidin wad added from a stock solution in MeOH (10^{-5} M). The doped membranes were subjected to slow voltage ramps (6.6 mV/s) and transmembrane currents were fed to a Keitley amplifier (model 427). Current–voltage curves were recorded on an X–Y plotter.

In single-channel recordings, lipid bilayers were formed at the tip of fire-polished patch clamp pipettes pulled in two steps by a Narishige pipette-puller (Tokyo, Japan) from GB150 glass capillaries (Bio-Logic, Claix, France). Planar lipid bilayers were formed according to the tip-dip technique [28] with the same mixtures of lipids used in macroscopic experiments but at 1 mg/ml.

Currents were amplified and potentials were applied simultaneously by a patch-clamp amplifier (RK 300, Bio-Logic). Single-channel currents were monitored on an oscilloscope (R5103N, Tektronix, Beaverton, OR, USA) and stored on a DAT recorder (DTR 1202, Bio-Logic) for off-line analysis. For further analysis, Satori (V3.1, Intracel Software, Royston, UK) and Sigma Plot (SPSS, Chicago, IL, USA) were used. All experiments were performed at

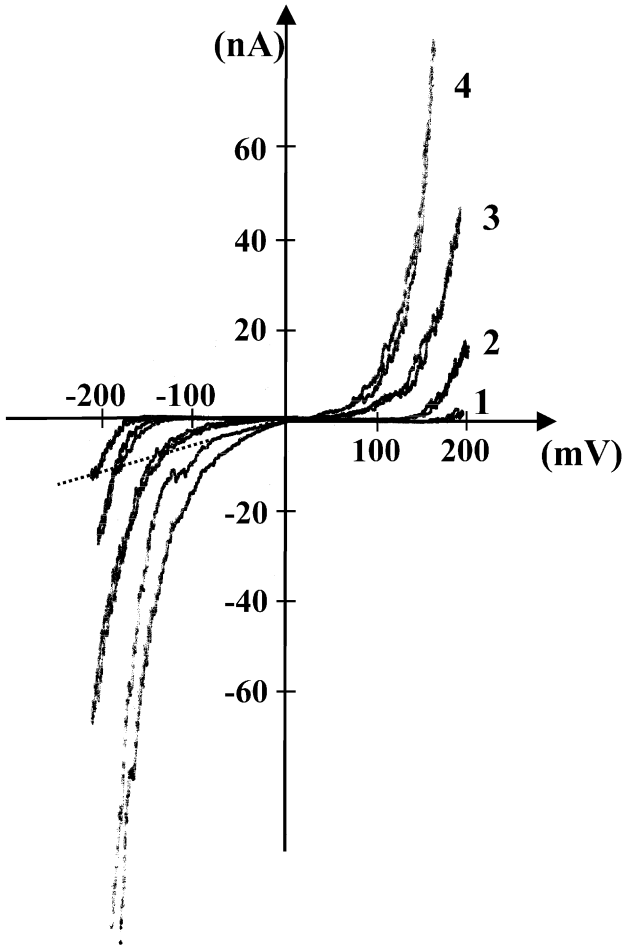

Fig. 1. Macroscopic current–voltage curves (*I*/*V*) for pleurocidin at different concentrations: (1) 5×10^{-8} M, (2) 10^{-7} M, (3) 6×10^{-7} M, (4) 4×10^{-6} M. Lipids used were a 7:3:1 mixture of PC/PE/PS and the electrolyte solution was 1 M KCl, 10 mM Tris, pH 7.4. The characteristic voltages Vc were determined from crossings of traces with a reference conductance which is represented by a dashed line. The macroscopic current measurements were performed at room temperature and the *I*/*V* curves were recorded at a potential sweep rate of 6.66 mV/s.

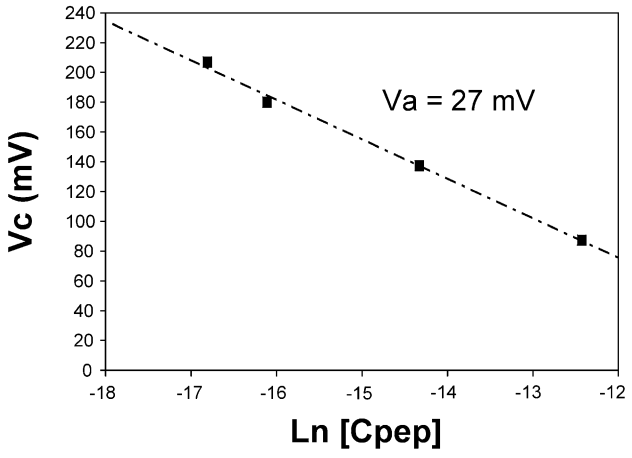

Fig. 2. Concentration dependence of *I*/*V* curves for pleurocidin. Lipids used were a 7:3:1 mixture of PC/PE/PS. Electrolyte solution was 1 M KCl buffered with 10 mM Tris at pH 7.4. The slope (Va) reflects the voltage threshold shift resulting from an *e*-fold change in bath concentration.

room temperature. Data are presented as mean ± S.E. for *n* experiments. Data were filtered at 1 kHz before digitising at 4 kHz for analysis and traces were filtered at 300 Hz for figures.

3. Results

3.1. Macroscopic current measurements

Pleurocidin was incorporated into PC/PE (7:3) planar lipid bilayers formed according to the Montal–Mueller technique [27]. Typically, after 30 min, the bilayer was submitted to repetitive triangular voltage ramps. Despite numerous experiments, no reproducible current voltage (*I*/*V*) curves could be recorded and superimposed, ensuring that partitioning equilibrium between membrane and peptide was reached. It was necessary to impose high voltages to incorporate peptide in bilayers and when incorporation occurred, the bilayers broke. In a previous paper [26], the authors showed by fluorescent measurements that the interaction between lipid and peptide is strongly increased if the membrane contains a proportion of negative charges. For that reason, new experiments were performed into PC/PE/PS (7:3:1) lipid bilayers. In this case, *I*/*V* curves were obtained at different concentrations of pleurocidin in 1 M KCl electrolyte (Fig. 1). An exponential development of membrane current above a voltage threshold was observed for each concentration. As previously demonstrated for alamethicin [29], the evolution of the *I*/*V* curves with the peptide concentration allows to determine different threshold voltages Vc. The graph Vc versus Ln[C] (Fig. 2) gave a

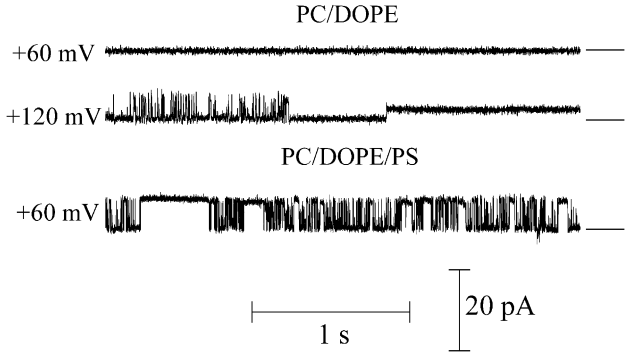

Fig. 3. Single-channel recordings for pleurocidin in different lipid bilayers formed at the tip of patch-clamp pipettes. Current measurements were performed at room temperature in 1 M KCl electrolyte buffered with 10 mM Tris at pH 7.4. Pleurocidin concentration was 2×10^{-8} M. The close state was represented by a horizontal bar.

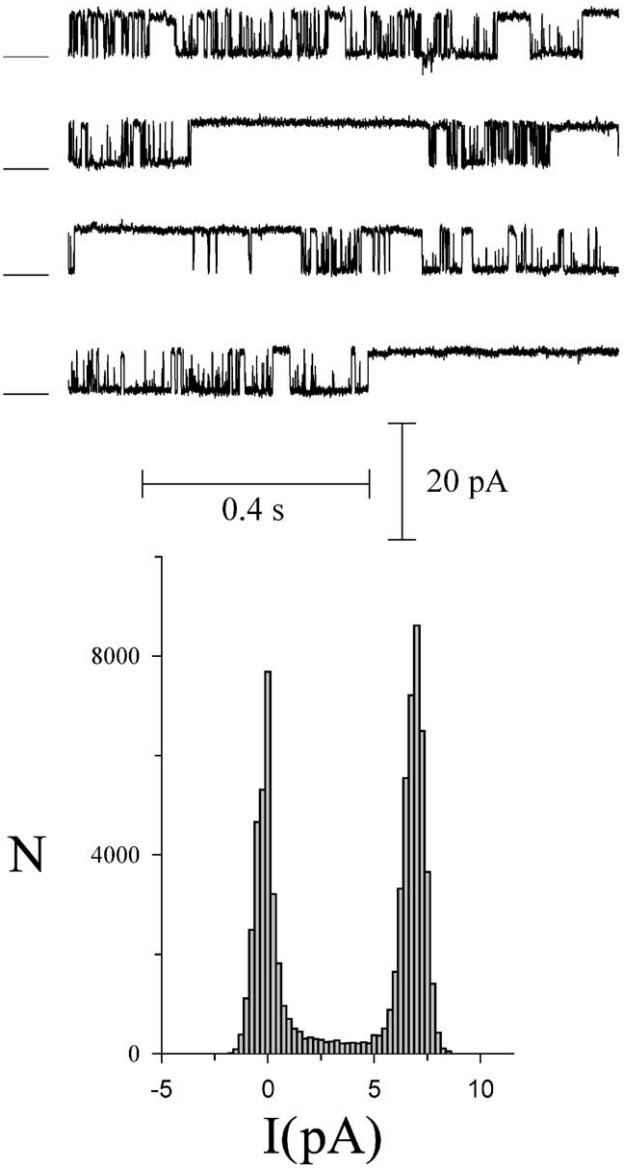

Fig. 4. Single-channel current fluctuations induced by pleurocidin in a 7:3:1 PC/PE/PS bilayer at 60 mV with the associated amplitude histogram. Same conditions as that of Fig. 3.

straight line with a slope, − Va, which is the concentration dependence factor. The Va value of about 27 mV was indicative of a high dependence concentration. In the same way, the voltage increment resulting in an *e*-fold change in conductance on the exponential branch of *I*/*V* curve is characteristic of the voltage dependence. The found value of 32 mV seems to indicate a weak voltage dependence in contrast to the 6-mV value found for alamethicin for instance [29]. Thus, the Va/Ve ratio called N_{app},—which represents the apparent number of monomers constituting the pore-forming peptide aggregate according to the "barrel-stave model"—is not significant.

3.2. Single-channel recordings

The trends revealed in the macroscopic analysis in PC/PE were confirmed at the single-channel level. In fact, when bilayers were formed at the tip of patch pipettes from this lipid mixture, no discrete current fluctuations were observed in a voltage range of − 100 to + 100 mV (e.g. at 60 mV in Fig. 3). If the voltage is increased to 120 mV, noisy current fluctuations could be observed but in that case, membranes became very fragile and broke rapidly (Fig. 3). However, when a PC/PE/PS membrane was used, numerous well-defined single channels were observed at 60 mV (Fig. 3).

For several current traces at 60 mV in a PC/PE/PS mixture (Fig. 4), amplitude histograms were obtained and allowed us to estimate at 100 pS the mean conductance value in 1 M KCl (Fig. 4). Very rarely it was possible to see a second level of same conductance. For five experiments at 60 mV, an opening channel probability of 0.59 was calculated and a mean lifetime value of channels could be estimated at 14 ± 4 ms. These high values agree with a good incorporation of peptides through the membrane and their ability to form stable ion channels.

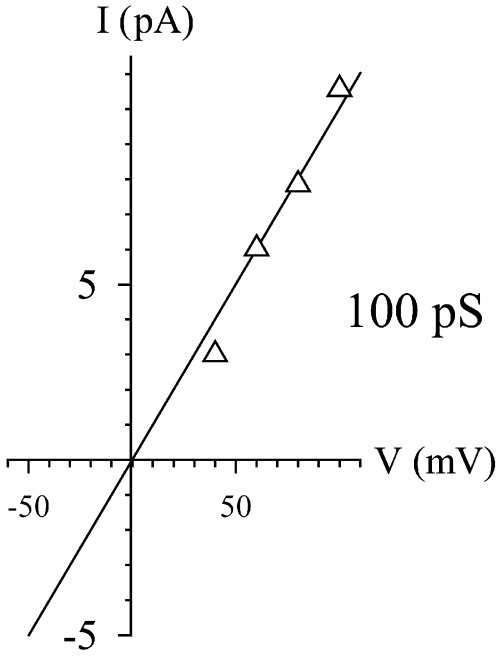

Fig. 5. Single current–voltage relations displayed by pleurocidin. Electrolyte was 1 M KCl buffered at pH 7.4 by 10 mM Tris. Room temperature.

To confirm the weak voltage dependence observed in macroscopic recordings, different experiments were performed at several voltages. The current voltage relationship for the open channel (Fig. 5) was linear and the average slope conductance of unitary currents gave 100 ± 5 pS, a close value of the one found for an alamethicin tetramer (120 pS) [30].

4. Discussion

Pleurocidin, a 25-mer cationic peptide isolated from the winter flounder, presents a broad antibacterial activity [21] which could be due to its ability to permeabilise bacterial membranes. The helical wheel diagram of pleurocidin shows an amphipathic α helical conformation confirmed by CD measurements in DOPC/DOPG (3:1) vesicles [26]. Moreover, by using spectrometry of tryptophan fluorescence and dye-leakage experiments, the authors suggested that pleurocidin, like magainin, was able to form pores in the lipid membrane. However, no direct evidences showed that pleurocidin is capable of forming ion channels in reconstitution experiments. In addition, the different experiments described about this peptide in the literature are not sufficient to decide among the several proposed mechanisms which is the one used by pleurocidin to permeabilise the bacterial membranes. Consequently, the behaviour of pleurocidin was investigated by conducting different reconstitution experiments in artificial planar lipid bilayers.

The *I*/*V* curves and single channel experiments showed a weak incorporation of pleurocidin in neutral bilayers since no ion channels were observed in a bilayer composed of a 7:3 mixture of PC/PE. In fact, if the voltage is strongly increased, the incorporation would occur but the membrane then becomes leaky and unstable. This behaviour is reminiscent of an amphipathic vector peptide whose incorporation was voltage dependent [31]. On the contrary, when the used planar bilayer contains a proportion of anionic lipid (PS), pleurocidin shows (i) reproducible *I*/*V* curves at different concentrations and (ii) at different voltages, well-defined single channels characterised by a high open channel probability and long open state lifetimes. This behaviour agrees with tryptophan fluorescence shift measurements which demonstrated that pleurocidin interacts weakly with a neutral phospholipid but strongly with an anionic phospholipid [26]. Two peptides, dermaseptin from frog skin [32] and ceratotoxin from medfly [33] which present 68% and 50% of similarities with pleurocidin, respectively, also presented a strong affinity for anionic lipids. Thus, the presence of anionic lipid seems important to favour the interaction between lipid and peptide, as already observed with other anti-microbial cationic peptides [34]. This would explain the strong interaction generally observed between

these cationic peptides and the anionic outer membrane of gram-negative bacteria.

If the first step is common to the three mechanisms of membrane permeabilisation mentioned previously [20,35], the second step can occur (i) by formation of helical bundle according to the barrel stave model, (ii) by formation of toroidal pore including lipid molecules between the helices [19], and (iii) by disruption of the membrane according to the "carpet-like" mechanism. The *I*/*V* curves obtained with pleurocidin allowed us to determine the characteristic Va and Ve values [29]. The 27-mV value calculated for Va indicates a high concentration dependence while the 32-mV Ve value (compared to 6-mV of alamethicin [29]) shows that pleurocidin does not induce voltage-dependent channels. This behaviour is confirmed by the applied voltage values requested to induce ion channels since we were able to observe pore-forming activity under a 60-mV voltage. Moreover, the linear relationship *I*/*V* and the unique conductance value of 100 pS observed from single-channel experiments do not support a barrel stave model. Firstly, in this model, the uptake and release of peptides in the conducting aggregate modify the pore size and thus induce different levels of conductance. Secondly, the graph *I*/*V* in this case is not linear because the increments between the different conductance values are not proportional to the increase of the pore diameter. In a previous study, we observed a similar behaviour with magainin [36]. We showed that magainin presented only two main conductance levels occurring independently in separate trials. We assumed that pre-aggregates lying on the membrane surface at rest and drawn into the bilayer upon voltage application in a behaviour contrasting with the classical multi-states displayed by alamethicin. Another common point between magainin and pleurocidin is the large polar sector developed in their helical wheel projection. In contrast, alamethicin, the archetype of the barrel stave model, presents a narrow polar sector which forms the hydrophilic pore after association of several molecules by strong hydrophobic interactions. It seems that the amphipathic character of helices is an important factor to explain the auto association of peptides before embedment in the membrane according to one or other mechanism. When this hydrophilic sector increases, the ion channel characteristics of the barrel stave model would disappear. This conclusion is supported by a recent study [37] where the authors, from two synthetic peptides possessing polar angles of 100 and 180°, showed that this parameter was essential in determining peptide–lipid interactions. Thus, like magainin, the toroidal model seems more appropriate to explain the pore-forming properties of pleurocidin.

In this study, we concluded that antibacterial properties of pleurocidin are likely due to the formation of ion pathways in bacterial membrane. From our experiments, we ruled out the "barrel-stave" model and deduced that the toroidal model was the most appropriate one. Recent studies emphasise that there is no contradiction between the carpet and the toroidal models since the second could occur complementary to the first one [38,39]. The different characteristics of the ionophoric behaviour described in this paper suggest that the action mechanism of membrane permeabilisation by pleurocidin could occur by the "carpet" mechanism via the formation of toroidal pores.

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