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Membrane channel formation by antimicrobial protegrins

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Abstract

Protegrins are small, arginine- and cysteine-rich, β-sheet peptides with potent activity against bacteria, fungi, and certain enveloped viruses. We report that protegrins form weakly anion-selective channels in planar phospholipid bilayers, induce potassium leakage from liposomes and form moderately cation-selective channels in planar lipid membranes that contain bacterial lipopolysaccharide. The disruption of microbial membranes may be a central attribute related to the host defense properties of protegrins. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Protegrins are small (2 kDa), cysteine- and arginine-rich antimicrobial peptides that were originally isolated from porcine leukocytes [1]. In their general structure, protegrins resemble the tachyplesins found in horseshoe crab hemocytes [2,3], and the 4 kDa defensins present in certain leukocytes, epithelial cells and secretions of humans and other vertebrates [4,5]. Protegrin monomers manifest a β-hairpin structure in solution [6,7], and form dimers in dodecylphosphocholine phospholipid micelles [8].

Tachyplesins form anion-selective pores in lipid bilayers, and this process is facilitated by applying a *cis*-negative transmembrane potential [9]. We previously reported that defensins form voltage-dependent, ion permeable channels in planar lipid bilayer membranes [10]. Although several studies [11,12] indicate that protegrins permeabilize membranes, their activity on planar lipid bilayers has not been reported previously.

2. Materials and methods

2.1. Planar membrane experiments

Planar lipid bilayer membranes were formed from a 15 mg/ml solution of lipid in *n*-heptane, using 250

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or 500 μm diameter Teflon tubing, as described previously [13]. The chamber's construction allowed substitution of the solution in the *cis* compartment (i.e., the one to which peptides were added) within several seconds. After the membrane had turned black, the solution in the *cis* side was replaced with protegrin-containing solution. Usually, after initial incorporation of protegrin, unbound peptide in the aqueous solution was washed out. Solvent-free membranes were formed as described previously [14]. Membranes used in experiments were stable, with a conductance of < 10 pS, up to voltages of ± 100 mV for at least 10 min prior to protegrin addition. The usual aqueous solution included 100 mM KCl and 10 mM buffer (Tris or HEPES, pH 7.4). Any other solutions used in the experiments are described in the text. Lipopolysaccharide prepared from *Escherichia coli* 0111:B4 by trichloroacetic acid extraction, and diphosphoryl lipid A from *Salmonella minnesota* Re-595 and azolectin (type II-S, soybean lecithin) were obtained from Sigma.

2.2. Recording equipment

Voltage clamp conditions were employed. Contact with the aqueous phases was made using Ag/AgCl electrodes with agar salt bridges. Electrode asymmetry was always less than 1 mV. Membrane formation was verified by monitoring membrane capacitance and resistance. Data were digitized and stored on VHS tape and played back for later analysis. An Axopatch 1C amplifier with CV-3B headstage was used for measuring membrane current. A digital tape and videocassette recorder allowed us to record large amounts of data. A storage oscilloscope was used to monitor membrane capacitance and to obtain single-channel recordings. The '*cis*' solution (to which protegrin was added) was taken as virtual ground. The sign of the membrane voltage corresponded to the *trans* membrane side.

2.3. Liposome studies

Liposomes were made by ultrasonicating and then freeze-thawing a lipid/water mixture, as follows. 1-Palmitoyl, 2-oleoylphosphatidylcholine (POPC) and 1-palmitoyl, 2-oleoylphosphatidylglycerol (POPG) were prepared as 10 mg/ml solutions in chloroform. One ml of each solution was mixed, and then dried onto the walls of a 10 ml glass vial. After residual chloroform was removed under vacuum for 30 min, 2 ml of salt solution (100 mM KCl, 10 mM Tris-HCl, pH 7.5) was added, and the vial was closed in a nitrogen atmosphere. After hydrating the lipids for 30 min at 0°C, the lipid/water mixture (final lipid concentration = 10 mg/ml) was sonicated at 4°C in a Branson B-2 water bath sonicator until it clarified. The resulting liposomal solution was fast frozen in isopropanol/dry ice and slowly thawed at room temperature over 20–30 min. This freezing-thawing procedure was repeated three times. To exchange the external salt solution, the liposomes were passed through a Sephadex G-25M column (bed height, 5 cm) that was eluted with 100 mM NaCl, 10 mM Tris-HCl, pH 7.5. POPC and POPG were purchased from Avanti Polar Lipids.

2.4. Peptides

PG-1 is an amidated peptide with the following sequence: RGGRLCYCRRRFCVCVGR. PG-3 and PG-1 are identical except that PG-3 contains glycine (G) in place of the underlined arginine (R) residue in the PG-1 sequence. Native protegrins purified from porcine leukocytes as previously described [1], were used in our initial experiments. Later, we used synthetic protegrins made by solid phase synthesis with conventional Fmoc chemistry. The synthetic protegrins were purified, folded and characterized as previously described [11]. Native and synthetic protegrins showed comparable behavior in our experiments.

2.5. K^+ release

A potassium ion-selective microelectrode (Microelectrodes) was calibrated to reference K^+ standard solutions, and liposomes from the column (200 μl) were allowed to equilibrate with the electrode. Baseline K^+ leakage and electrode drift in this condition were minimal. If significant drift occurred, new liposomes were prepared.

3. Results

3.1. Liposomes

Fig. 1 demonstrates the leakage of potassium from POPC:POPG liposomes (3:1 ratio) that were treated with protegrin PG-1. K^+ efflux was generally completed within 30–60 s, and could be measured after treatment with as little as 1 μM protegrin. Efflux reached a maximal extent with about 30 μM protegrin, corresponding to ≈35% of the total K^+ released by Triton X-100. Most likely, the remaining K^+ was trapped in multilamellar liposome compartments that were inaccessible to protegrin. At intermediate concentrations between 1 and 30 μM, the final magnitude of K^+ efflux was roughly proportional to protegrin concentration. The rate of K^+ efflux increased steeply with protegrin concentration (Fig. 1, insert).

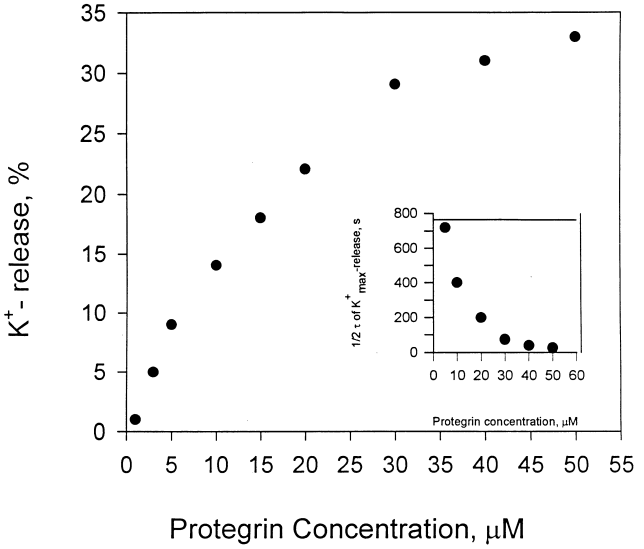


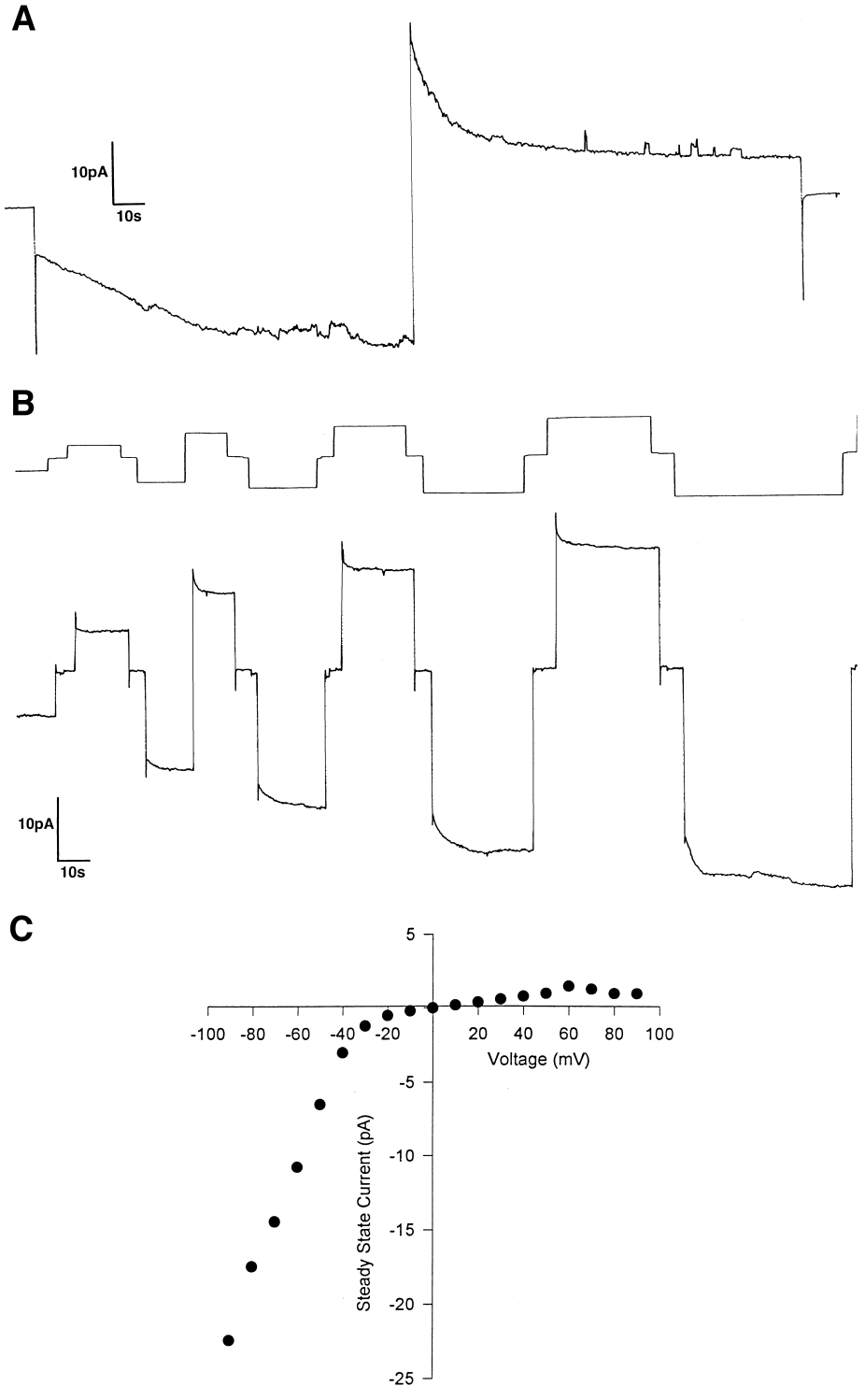
Fig. 1. Liposomal release of potassium induced by protegrins. Liposomes were composed of 1-palmitoyl, 2-oleoylphosphatidylcholine and 1-palmitoyl, 2-oleoylphosphatidylglycerol in a 3:1 molar ratio. The internal salt solution contained 100 mM KCl, 10 mM Tris pH 7.4, and the external salt solution was 100 mM NaCl, 10 mM Tris pH 7.4. Potassium release was monitored with a potassium electrode. Maximal release was determined by adding Triton X-100. The inset shows the kinetics of potassium release as a function of protegrin concentration. The figure shows a typical experiment, similar to more than 20 others done with protegrins on liposomes.

3.2. Planar bilayers

Fig. 2A shows the macroscopic conductance induced in a planar phospholipid bilayer by protegrin PG-3. Current (I) is shown as a function of time. The line begins at zero current and its first downward spike represents capacitive transient in response to the application of a −80 mV pulse. In response, a current through the membrane developed, increased, and reached a steady state within approx. 1 min. This current trace goes downward because current is in the negative direction. The vertical line in the middle of the figure represents a switch in the voltage polarity, from −80 to +80 mV. Instantaneously, the current reversed sign, but had equal magnitude and then rapidly decayed toward zero current. During its decay, occasional current flickers were detected, possibly representing transiently open single channels. Thus, conductance activated rapidly at *trans*-negative voltages, and partially turned off at *trans*-positive voltages. If conductance was allowed to activate for over 10 min at *trans*-negative voltages, the membranes usually broke.

In its initial stages, membrane conductance induced by protegrins exhibited voltage-dependent behavior. After 10–30 min time, a voltage-independent conductance sometimes developed, replacing some or all of the voltage-dependent conductance (Fig. 2B). This voltage independence may also have reflected a shift in protegrin channel conformation within the membrane. As the protegrin channels could not be removed by extensive washing, they were evidently associated intimately with the membrane.

Early in the onset of action of protegrins (Fig. 3) stable single channels were observed. 'Staircase laddering', a characteristic of channels induced by larger microbicidal proteins [15–17], including yeast killer toxin and colicins, was not observed. Single channel current jumps were not homogeneous, and appeared to increase in amplitude with time. Both protegrins PG-1 and PG-3 displayed similar channel forming activity on lipid bilayers. Although both cations and anions were freely permeable through protegrin channels, anions were slightly favored. Macroscopic reversal potential experiments indicated that both formed channels that were weakly anion selective. Reversal potentials in a 10-fold salt gradient ranged between 12 and 28 mV (PG-1) and from 8 to 12 mV (PG-3), dilute side negative. The range of reversal



potentials may have represented variable mixtures of protegrin conformations in the membrane.

Because protegrins are highly effective against Gram-negative bacteria, we examined the ability of protegrins to form pores in a membrane containing bacterial lipopolysaccharide (LPS), a principal component of the outer leaflet of their outer membranes. Incorporating either lipid A (Fig. 3) or lipopolysac-

Fig. 2. (A) Voltage dependence. The current through a planar phospholipid bilayer membrane treated with 4 μg/ml of PG-1 is shown as a function of time. The line begins at zero current and the first downward spike represents application of a voltage pulse of −80 mV. The vertical lines in the middle of the figure represent a polarity change, from −80 mV to +80 mV. Note that current instantaneously reverses sign, but shows equal magnitude before rapidly decaying toward zero. Occasional current flickers can be seen during this decay phase. (B) Development of voltage-independent current. Tracings of membrane voltage (above) and current (below) are shown vs. time, for a membrane containing PG-3. The membrane voltage is stepped back and forth between ±20, ±40 and ±60 mV. Although voltage-dependent channel opening is seen at negative voltages, a significant fraction of current is voltage-independent. Although not always observed this voltage-independent behavior of the channels sometimes became more prominent over time, and may have represented an altered conformation of the membrane-associated protegrin. (C) Steady state I-V. In this current vs. voltage plot, which was obtained with 4 μg/ml of PG-3, the sign of the voltage refers to the side *trans* (opposite) to the protegrin. Note the larger currents (and conductances) when these voltages are negative, as they would be in actively respiring target cells. Virtually all channels are closed at positive voltages. The aqueous solution contained 100 mM KCl and 10 mM Tris, pH 7.4.
←

charide significantly enhanced the formation and stability of single channels, and, rather remarkably, altered the ionic selectivity of PG-3 channels to cation selective (42 mV in 10-fold NaCl gradient). Additional properties of the channels formed by PG-1 or PG-3 in membranes with or without LPS are shown in Table 1. The threshold concentration is the minimal protein concentration needed to observe channel activity.

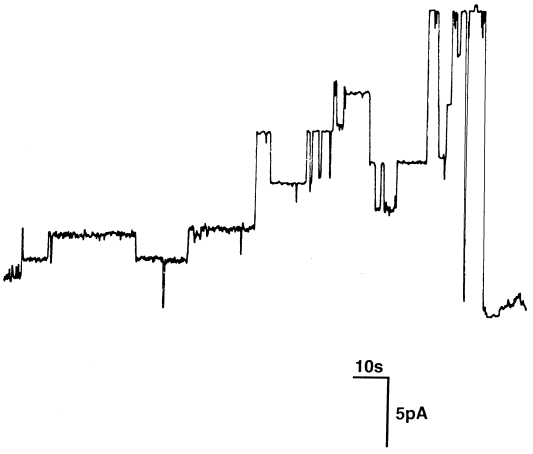


Fig. 3. The figure shows current fluctuations suggestive of individual ion channels induced by protegrin PG-3 (2 μM) in a planar phospholipid bilayer membrane. The membrane was held at −60 mV and was composed of azolectin to which 10% lipid A had been added. Salt solutions contained 100 mM KCl, 10 mM Tris, pH 7.4. Note that several different sizes of current fluctuations are present. These may represent unique molecular species or different conformations of a single molecular species. Such multi-level conductance fluctuations are typical of peptide channel formers which aggregate into oligomers to form channels. Note also the relatively long lifetimes lasting into seconds or even tens of seconds.

4. Discussion

We found that protegrins caused ionic leakage or channel formation at concentrations comparable to those at which they exert antimicrobial effects, and that channel formation proceeded to complete membrane disruption over a relatively narrow concentration range. These membrane effects correspond to several physiological observations related to the antimicrobial action of protegrins. First, the broad-spectrum activity of protegrins is entirely consistent with membrane lipid being the target of action, rather than any specific protein receptor. This is reinforced by the functional equivalence of PG-1 and *enantio* PG-1 (composed exclusively of D-amino acids) with respect to antimicrobial activity [18]. Second, channel formation was favored at a negative *trans* voltage, which mimicked the membrane potential of the microbial target cell. Third, the poor ionic selectivity of protegrin membrane pores was highly suggestive of unregulated toxic channels, as opposed to signaling or physiologic channels. Formation of non-selective, poorly regulated channels could cause microbial targets to depolarize, leak ions and micronutrients, and undergo disruption of transport and macromolecular synthesis.

The enhancement of channel activity and conductance by LPS suggests that bacterial membranes may be especially sensitive to protegrin action. The effects of LPS on protegrin pore selectivity indicate that LPS may play a key role in the structure of the pore.

Mangoni et al. [12] reported that protegrin PG-1 induced voltage-independent ionic conductances in *Xenopus* oocytes, whereas we noted both voltage-dependent and voltage-independent conductances in

Table 1
Properties of protegrin channels formed by PG-1 and PG-3 in the presence or absence of LPS are given

Peptide	LPS	Single channel conductance (pS)	Selectivity	Threshold conc. (μg/ml)
PG-1	No	50–100	Anion	4
PG-3	No	40–100	Anion	10
PG-3	Yes	80–360	Cation	2
PG-3	Lipid A	80–360		2

The polarity of ionic selectivity measured by reversal potential in a 10-fold KCl gradient is given, although all protegrin channels allow both cations and anions to permeate. Threshold concentration is defined as the minimal protegrin concentration which exhibits channel activity. LPS denotes the presence or absence of lipopolysaccharide in the membrane.

the membrane bilayers. This discrepancy may reflect differences in the lipid composition (oocyte membranes vs. phospholipid bilayers), salt solution (presence or absence of phosphates, divalent cations, etc.), or other factors present in oocytes but not in bilayers. Alternatively, a voltage-dependent conformation in oocytes may be short lived, and progress rapidly to a voltage-independent conductance.

The voltage dependence and ionic selectivity of protegrin pores are similar to those we previously described for defensins [10]. Although protegrins are considerably smaller than defensins, the net positive charges of PG-1 and PG-3 (+6 and +5, respectively) are intermediate between those of rabbit α-defensin NP-1 (net charge, +9) and human α-defensin HNP-1 (net charge, +3). Furthermore, although monomeric protegrin molecules are too small to span a membrane bilayer as monomers, they dimerize in dodecylphosphocholine micelles [8]. Whereas defensin dimers contain a six-stranded β-sheet [19], the comparable region in protegrin dimers is four-stranded [8]. Defensin pores are believed to result from a multimeric assembly of dimeric defensins [20,21]. Since protegrin dimers are even smaller than defensin dimers, the formation of protegrin pores is likely also to involve further oligomerization. While the precise structure of protegrin membrane pores remains to be determined, scanning electron micrographs have shown the presence of multiple, annular structures on the outer membranes of *Neisseria gonorrhoeae* [22] and other protegrin-treated Gram-negative bacteria.

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