Change in membrane permeability induced by protegrin 1: implication of disulphide bridges for pore formation

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Abstract Protegrin 1 (PG-1) is a naturally occurring cationic antimicrobial peptide that is 18 residues long, has an aminated carboxy terminus and contains two disulphide bridges. Here, we investigated the antimicrobial activity of PG-1 and three linear analogues. Then, the membrane permeabilisation induced by these peptides was studied upon *Xenopus laevis* oocytes by electrophysiological methods. From the results obtained, we concluded that protegrin is able to form anion channels. Moreover, it seems clear that the presence of disulphide bridges is a prerequisite for the pore formation at the membrane level and not for the antimicrobial activity.

Key words: Protegrin; Antimicrobial peptide; Disulphide bond; Membrane permeabilisation; Ionic channel

1. Introduction

Protegrins are five antimicrobial peptides (PG-1 to PG-5, Table 1) isolated from porcine leukocyte cells [1–3]. These short and cationic peptides present two disulphide bridges (Cys^6-Cys^{15} and Cys^8-Cys^{13}) [3], and share some sequence similarities with mammalian defensins and tachyplesins (Table 1) [4,5]. We recently reported the solid-phase synthesis and the solution structure of PG-1, which consists of two antiparallel strands ($Leu^5 \rightarrow Arg^9$ and $Phe^{12} \leftarrow Val^{16}$) linked by a distorted β-turn ($Arg^9 \rightarrow Phe^{12}$) (Aumelas et al., in press). Like all other antibiotic peptides, protegrins possess an amphipathic structure in which the positively charged residues are spatially separated from the hydrophobic ones. Several studies indicate that host defence peptides, whatever may be their structure (amphipathic helix, β-sheet or a mixture of these two motifs), act by direct lysis of the pathogenic cell membranes. As pointed out by Maloy and Kari [5], their basic nature facilitates their interaction with the cell membrane and their amphipathic character allows them to be incorporated into the membrane, ultimately disrupting its structure either by formation of pores or by alteration of the bilayer fluidity [6,7].

In the present study, we examine the antimicrobial activity of several synthetic protegrin analogues. Using *Xenopus laevis* oocytes, we show that PG-1 is able to alter the cellular membrane permeability by forming ionic pores that present some specific properties in common with human defensin-induced channels [8]. Then, using a set of analogues, we demonstrated cysteine bridges to be an absolute prerequisite for membrane permeability alteration but not for antimicrobial activity.

2. Materials and methods

2.1. Peptide synthesis

The sequence of the protegrin synthesised corresponds to Protegin-1 [2]. Peptide synthesis was carried out on a fluorenemethoxycarbonyl (Fmoc) PAL-PEG resin, or a Fmoc Arg(Pmc)-PEG resin (Millipore), by Fmoc strategy on an automatic apparatus (9050 Pepsynthesiser, Milligen). Peptide synthesis was performed as described elsewhere [9]. Then, the crude peptide-amide was precipitated with ether, filtered and dried in a desiccator.

2.2. Disulphide bridge formation and peptide purification

Each crude peptide was purified by reversed-phase high performance liquid chromatography (HPLC, Dpack C18 10 μm, 20 mm × 250 mm) using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (TFA). The disulphide bridge formation was performed on PG-1 and PG-1OH as described by Tam et al. [10]. This protocol yielded almost 100% of peptide preparation oxidised in the correct configuration. The disulphide pattern was resolved by NMR in H_2O and dimethyl sulphoxide (DMSO), and the Cys^8-Cys^{13} and Cys^6-Cys^{15} bonds were clearly identified (Aumelas et al., in press). Briefly, 120 mg of crude peptide was diluted in 150 ml of dissolving buffer (50% H_2O, 50% 2-propanol, pH 5.5). Then, the peptide solution was added slowly (1 ml/min) by peristaltic pump into a 0.1 M Tris(hydroxymethyl)aminomethane (Tris) solution (400 ml) containing 25% DMSO, and 10% 2-propanol at pH 6.85. At the completion of the reaction, the oxidation mixture was acidified with TFA to pH 3, and then folded peptides were purified as described above. Treatment of purified folded peptide by Ellman's reagent showed the absence of free SH groups. After hydrolysis with HCl, 6 N in evacuated sealed tubes at 110°C for 24 h, the amino acid analysis confirmed the good composition of the peptide. The mass spectrometry (Electrospray ionisation on a Fisons Instrument) of unfolded or oxidated protegrin analogues was in agreement with the expected structure, 2159 ± 1.75 Da and 2155 ± 1.89 Da, respectively.

2.3. Biological assays

Antimicrobial activities of synthetic PG-1 analogues were determined by microdilution technique against one Gram-negative bacillus (*E. coli*, ATCC: 25922) and two Gram-positive cocci (*S. aureus*, ATCC: 25923 and *S. epidermidis*, ATCC: 12228). Peptide stock solutions were made in water containing 0.01% acetic acid and then filter sterilised. For microdilution technique, wells of a sterilised tray were incubated with a final volume of 100 μl of 10^6 CFU/ml organism suspensions in Mueller-Hinton broth (Sanofi, France) and serial 2-fold dilutions of peptide (from 256 μg to 0.125 μg). The minimal inhibitory concentration (MIC) was determined after 24 h incubation at 37°C by measuring absorbance. The MIC is defined as the dose at which 100% inhibition of growth was observed. Then, the minimal concentration of peptide showing 100% bactericidal activity (MBC) was determined by incubating a 10 μl aliquot from each well onto a Mueller-Hinton plate. Results were interpreted after 24 h incubation at 37°C. Each MIC and MBC was determined from two independent experiments performed in triplicate.

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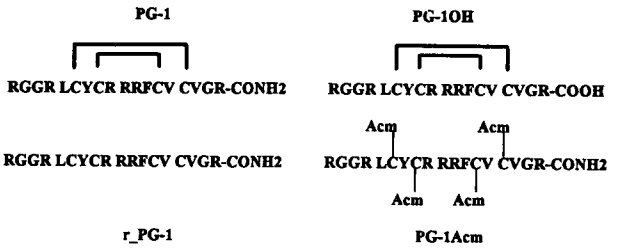
Table 1
Sequence alignment of protegrins (PG-1–5), rabbit defensin (NP-3a), human defensin (HNP-1) and tachyplesin (TP-1) as proposed by Kokryakov et al. [1]

PG-1	RGGR**LCYCR**RRFCVCVG**R**
PG-2	RGGR**LCYCR**RRFCICV
PG-3	RGGG**LCYCR**RRFCVCVG**R**
PG-4	RGGR**LCYCR**GWICFCVG**R**
PG-5	RGGR**LCYCR**PRFCVCVG**R**
NP-3a	GI**C**A**CR**RRF**C**PNSE**R**FSGYCRVNGARYVRCCS
HNP-1	A**CYCR**IPA**C**IAGE**R**RYGTCIYQGRLWAFCC
TP-1	KWCFRV**CY**RGICYRRCR

Table 2
Chemical structures of protegrin analogues used in this study



2.4. Oocyte preparation and electrophysiological recordings

Oocytes (stage V–VI) were removed from mature *Xenopus laevis* females (Elevage de Lavalette, Montpellier, France), and separated as described elsewhere [11]. After complete separation, oocytes were extensively washed in a recovery solution (ND96) containing (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH = 7.6. Complete defolliculation was verified by visual inspection under a stereomicroscope. Double-electrode voltage clamp experiments were performed employing the Gene Clamp amplifier (Axon, Burlingame). Data were collected using a TL-1 DMA interface, and stored in an IPC 486 personal computer. Off-line data analysis was carried out with version 6 of pCLAMP software. We used standard intracellular electrodes with a resistance of 0.5 MΩ, when filled with a solution containing (in mM): 2800, CsCl; 10, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA); 10, Hepes; adjusted to pH = 7.2, with CsOH. Oocytes were then bathed in a 50 μl recording chamber. When testing peptides, the perfusion device was arrested, and the peptide-containing solution added to the bath. Protegrin-induced changes in membrane conductance were measured as the ratio between the conductance in control condition (g_0), and its maximal value during protegrin superfusion (g_1). In order to characterise the protegrin-induced membrane permeability, we used a set of solutions with different ionic composition. We started from a basic solution containing (in mM): Hepes 10, $MgCl_2$ 1, pH = 7.2. 100 mM of either NaCl, KCl, LiCl, CsCl, tetraethylammoniumchloride (TEA-Cl), or choline-chloride (ChCl) was then added as required. Intracellular injection of BAPTA was performed to ensure blockade of the endogenous Ca^{2+}-activated Cl^- current [11]. The BAPTA-containing solution had the following composition (mM): BAPTA free acid 100, CsOH 10, Hepes 10, pH = 7.2. Intracellular concentration of BAPTA was estimated to be about 2–5 mM. All experiments were performed at room temperature (20–22°C).

3. Results and discussion

3.1. Peptide synthesis and antibacterial activities

In order to determine the structure–function relations of protegrins, we synthesised an aminated peptide corresponding to PG-1, an analogue with a COOH-terminal arginine α-carboxylated (PG-1OH), a linear analogue (r_PG-1) with reduced cysteines, and one derivative (PG-1Acm) in which the four SH groups were protected with acetamidomethyl (Acm) groups (Table 2). Cysteine oxidation of PG-1 and PG-1OH was performed in the presence of DMSO using the protocol developed by Tam et al. [10], which allowed us to produce synthetic protegrins with a good yield (>59%). It is worth noting that in the absence of DMSO no disulphide bridge formation was observed after a few days, for a pH range from 6 to 7.5. As determined by NMR studies, the PG-1 and PG-1OH showed only one type of cysteine connection, which corresponds to the pattern in the natural product ($Cys^6 \rightarrow Cys^{15}$, $Cys^8 \rightarrow Cys^{13}$) (Aumelas et al., in press). Using a microdilution technique, the antimicrobial activity of peptide analogues was evaluated with *S. aureus* (ATCC: 25923), *S. epidermidis* (ATCC: 12228), and *E. coli* (ATCC: 25922) as test organisms, and the MIC and the MBC of each peptide were determined (see section 2.3). As shown in Table 3, PG-1 and PG-1OH present the same potency to inhibit bacterial proliferation as judged by their MIC values. However, PG-1OH is less bactericidal (2-fold) than PG-1 against all types of bacteria tested (MBC values). r_PG-1 has a decreased potency against the Gram-positive *S. epidermidis,* the most sensitive bacteria used in this work, but this analogue was as efficient as PG-1OH against the Gram-positive *S. aureus,* and most powerful against the Gram-negative *E. coli.*

By contrast, PG-1Acm shows a very low antimicrobial activity against the two Gram-positive bacteria tested. Interestingly, this analogue is still as bactericidal as PG-1 against the Gram-negative *E. coli.* Taken together, these data indicate that modification of the C-terminal CONH2 group for a neg-

Table 3
Antibacterial activity of synthetic PG-1 analogues against Gram-negative and Gram-positive bacteria tested with the microdilution technique (n = 3 from two independent experiments)

	E. coli (Gram-negative) MCI (μg/ml) MCB (μg/ml)	*S. aureus* (Gram-positive) MCI (μg/ml) MCB (μg/ml)	*S. epidermidis* (Gram-positive) MCI (μg/ml) MCB (μg/ml)
PG-1	64 128	64 128	2 2
PG-1OH	64 256	64 256	2 4
r_PG-1	32 64	64 256	16 32
PG-1Acm	32 128	512 >512	128 128

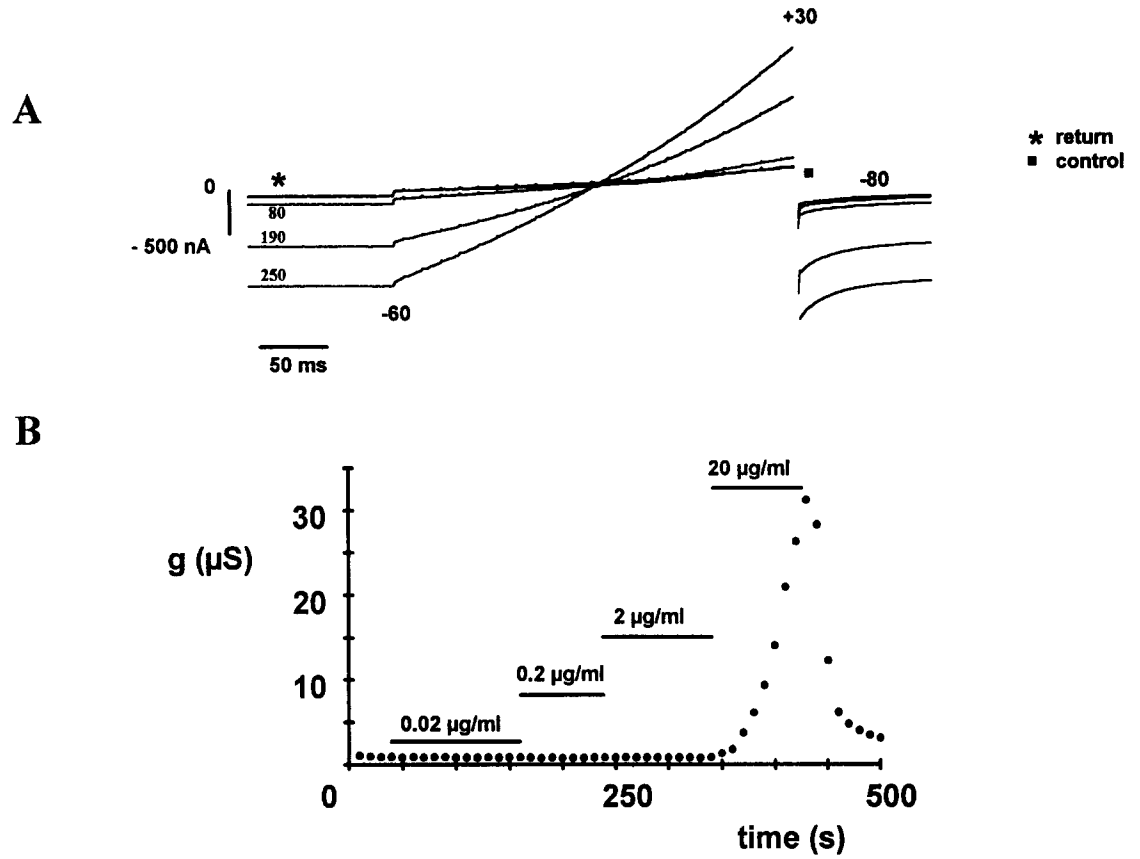


Fig. 1. A: Time course and reversibility of membrane permeability change in an oocyte superfused with PG-1 20 µg/ml. Membrane permeability was studied by application of a 375 ms voltage ramp from −60 to +30 mV, from an holding potential of −80 mV. Numbers on traces indicate the time (in seconds) after start of protegrin perfusion. Current reversal potential was −16 mV. B: Membrane conductance increment for an oocyte that has been superfused with incremental concentrations of PG-1.

atively charged group (COOH) slightly (2-fold) decreases the antimicrobial activity of PG-1. This observation is in good accordance with a study dealing with the antimicrobial activity of dermastin peptides [12]. On the other hand, the capability of r_PG-1, but not PG-1Acm, to kill Gram-positive bacteria suggests that free SH group cysteines, possibly through disulphide bridge formation during the experiment, are necessary to exert antimicrobial activity against such Gram-positive bacteria. Interestingly, disulphide bridges seem not to play a crucial role against Gram-negative bacteria, because both r-PG-1 and PG-1Acm were highly active against *E. coli*.

3.2. Change in membrane current induced by synthetic PG-1 in *Xenopus laevis* oocytes

The effect of synthetic protegrins on membrane permeability was investigated in a set of pilot experiments carried out in ND96 extracellular solution. As shown in Fig. 1A, PG-1 application induced a continuous increase in membrane permeability following superfusion at 20 µg/ml. For the example shown in Fig. 1A, the total oocyte membrane conductance was increased almost 10-fold (g_1/g_0 ratio was 9.67, as measured 10 s before protegrin application, and at 250 s during protegrin superfusion). Typical recordings (see Fig. 1A) showed a non-linear, slightly outward-rectifying current wave-

Table 4
Summary of the action of synthetic protegrins on the oocyte membrane conductance at different doses

	PG-1	PG-1OH	r_PG1	PG-1Acm	PG-1 + DTT(1.5 mM)
10 µg/ml	+	+			
	n = 4/4	n = 8/8			
20 µg/ml	+	+	0		
	n = 5/5	n = 8/8	n = 6/6		
50 µg/ml	++		0	0	
	n = 3/3		n = 3/3	n = 3/3	
100 µg/ml			+	0	0
			n = 7/7	n = 5/5	n=6/6
200 µg/ml			++	0	
			n = 5/5	n = 6/6	
E_{rev} (mV)	−18.1 ± 0.68	−16 ± 0.77	−15.9 ± 0.4		
	n = 8	n = 13	n = 8		

Symbols (0, +, ++) indicate conductance ratios (g_1/g_0, see section 2) of <2, 25–50, >50.

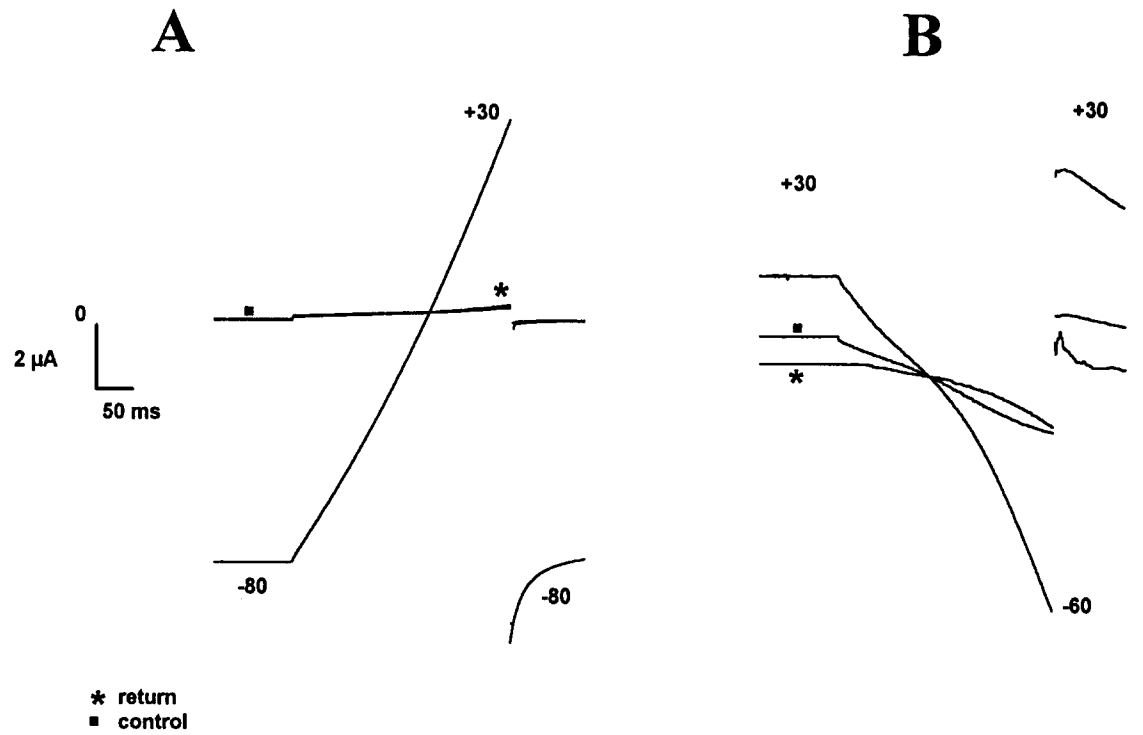


Fig. 2. Lack of voltage dependence for the change in membrane conductance induced by PG-1 20 μg/ml with respect to ramp polarity. The figures shows an experiment performed on an oocyte that had been bathed in the PG-1-containing solution, rinsed and subsequently exposed to a new round of peptide perfusion while applying a voltage ramp of inverted polarity. The protegrin-induced current reversal potentials were −11 and −9 mV in (A) and (B) respectively.

form during ramp application, and the presence of an inward tail current following return to −80 mV. Current reversal potential was constant after the onset of PG-1 effect (see Table 4), and independent of the membrane current amplitude. The PG-1 effect was always reversible for all doses and extracellular ionic conditions tested. When long PG-1 superfusion was applied, no steady-state PG-1 effect could be reached, and protegrin-induced changes in membrane conductance rapidly overcame the compliance of the voltage clamp amplifier (see for example Fig. 1B). Fig. 1B shows the change in membrane conductance of a test oocyte during applications of increasing concentrations of PG-1. In this case, no effect was observed at 2 μg/ml, and a full effect was observed at 20 μg/ml. We then studied the change in membrane conductance induced by PG-1OH. Only a slight difference was observed between the two folded protegrin analogues, with respect to the minimal peptide quantity able to induce a significant change in membrane conductance. Indeed, PG-1 gave a detectable effect at 3 μg/ml (n = 3), and PG-1OH showed a concentration threshold at about 5 μg/ml. The membrane conductance increment induced by PG-1 at 3 μg/ml showed slower kinetics of onset, but the time course of membrane recovery upon washing did not seem to be altered compared to that observed at 20 μg/ml (data not shown).

These observations suggest that synthetic protegrins are capable of changing membrane permeability by forming pores. In light of previous reports dealing with natural defensins [8], where voltage-dependent ionic channel formation has been described, we checked for the effect of membrane voltage on protegrin-induced membrane conductance. In particular, we were interested to see if folded protegrins are able to form functional channels in the oocyte membrane at positive holding potentials. Fig. 2 shows an example of an experiment where the voltage protocol has been inverted in polarity. Even when held at +30 mV, PG-1 (20 μg/ml) was still able to increase membrane permeability (Fig. 2B, see legend). Current reversal potential did not significantly change upon inversion of the ramp polarity, thus confirming that the same type of conductance is in fact responsible for the protegrin-induced change in membrane permeability observed using the two different protocols. After inversion of the ramp polarity, partial reversibility was still observed after peptide washing, and a subsequent round of protegrin perfusion could be successfully performed.

3.3. Role of disulphide bridges

To investigate the influence of protegrin folding on its biophysical action, we carried out experiments, where the complete set of folded and linear peptides was used (Table 2). The results are summarised in Table 4. In a first batch of oocytes, the two folded peptides were able to induce an increment in membrane conductance in the concentration range between 5 and 20 μg/ml. In contrast, the linear peptide (r_PG-1) showed a concentration threshold about 20 times higher (see Table 4). Indeed, a significant change in membrane conductance was observed only for concentrations around 100 μg/ml, although a slight effect was sometimes observable at 50 μg/ml (Table 4). These observations strongly suggest that disulphide bridges are a prerequisite for pore formation. The effect of the linear peptide at high concentrations could be related to slight spontaneous cysteine oxidation close to the oocyte membrane. To test this hypothesis, in a separate batch of oocytes, we compared the action of r_PG-1 to that of PG-1Acm. In this case, we were unable to find a significant increase in mem-

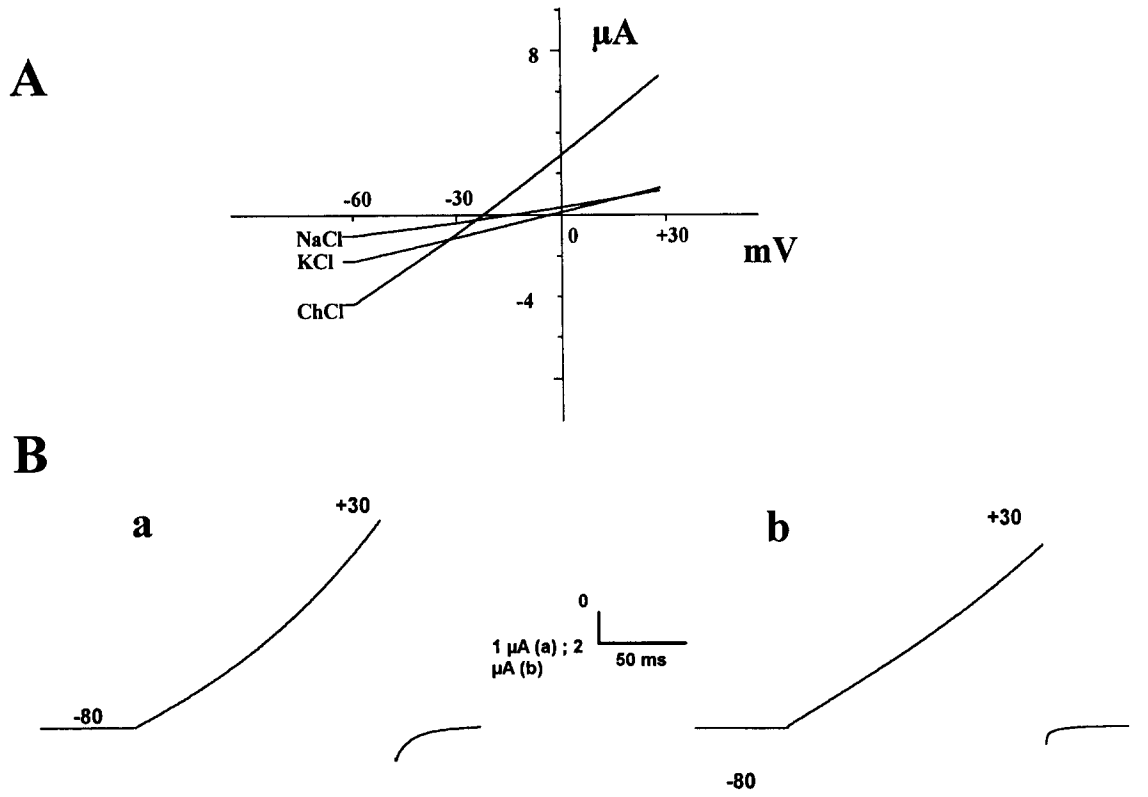


Fig. 3. A: Current–voltage relations for the PG-1-induced (20 μg/ml) membrane current for the voltage range from −60 to +30 mV, in an oocyte bathed in different extracellular solutions (see section 2). Current reversal potentials were −15.9, −3 and −21 mV for the NaCl, KCl and ChCl solutions respectively. B: Ca^{2+} permeation in an oocyte superfused with PG-1. a: Protegrin-induced current in control condition. b: Same oocyte after intracellular injection of a BAPTA-containing solution (see section 2).

brane conductance up to 200 μg/ml upon perfusion of PG-1Acm (see Table 4). Moreover, the activity of PG-1 (100 μg/ml) was completely blocked in the presence of 1.5 mM of dithiothreitol (DTT), which confirms the necessity of disulphide bridges in membrane permeability activity of the protegrins.

3.4. Ionic characteristic of protegrin-induced membrane current

Upon superfusion with PG-1 in extracellular ND96 solution, current reversal potential is close to the equilibrium potential for Cl^- ions ([13], see Table 4). This led us to hypothesise that PG-1 can form anionic channels. However, even classical anionic channels can also pass small cations moderately well [14]. This is the case, for example, for insect defensin-induced channels [8]. Fig. 3 shows an experiment where an oocyte has been challenged with three rounds of PG-1 superfusion, in three different extracellular cation-containing solutions, while keeping the chloride concentration constant. Upon superfusion with PG-1, current reversal potentials were −16, −5.5 ± 1.8, and −21 mV, for the NaCl, KCl, and ChCl solutions in $n = 3$ oocytes, respectively. We were able to confirm these observations in another set of experiments, employing other cations. Current reversal potentials were: −6 ± 3 ($n = 3$), −8 ± 1 ($n = 4$), 0 ± 1 ($n = 3$), −9 ± 1 ($n = 3$), and −11 ± 1 mV ($n = 3$) for the Li^+-, Na^+-, K^+-, Cs^+- and TEA^+-containing solutions respectively. In contrast, currents recorded in the presence of extracellular choline show a reversal potential that almost overlaps that of Cl^- which suggests that choline ions are probably too large to permeate the channel. These results confirm that small cations, like Na^+ and K^+, are indeed able to permeate the pore formed by PG-1, together with Cl^- ions. The consequence is a shift of the current reversal potential toward more positive potentials.

3.5. Calcium permeation through the protegrin-induced conductance

Ca^{2+} is an important intracellular messenger, involved in many regulatory processes. However, Ca^{2-} may also induce cell death, through cytotoxic actions, as well as apoptosis (see for example [15]). In our conditions, two observations suggest that calcium permeates the protegrin-induced conductance: (1) the reversal potential of the PG-1-induced current shifted toward a more positive potential upon switching from extracellular 1.8 mM Ca^{2+} (ND96 solution) to the NaCl solution that lacks extracellular Ca^{2+}, and (2) PG-1-induced current has a linear current-to-voltage relation in the NaCl-, Ca^{2+}-free, extracellular solution. A simple way to account for these observations, is that Ca^{2+} permeation activates the endogenous Ca^{2+}-dependent Cl^- current [16] during PG-1 application, thus leading to the appearance of apparent outward rectification, and the tail current. Fig. 3B shows an experiment where PG-1 (20 μg/ml) has been perfused in normal conditions (a), and after intracellular injection of BAPTA to block the activation of the endogenous Cl^- current (b). In control conditions, protegrin-induced current shows the typical outward rectification, and a prominent tail current upon return to −80 mV. In contrast, after intracellular BAPTA injection, the current-to-voltage relation became linear, and the tail current was almost completely suppressed. These observations constitute a clear evidence that Ca^{2+} ions are in fact able to

permeate the protegrin-induced pore, thus activating the endogenous Cl^- current. Accordingly, current reversal potential slightly shifted, upon injection of BAPTA, toward the positive direction (−8 and −5 mV in (a) and (b) respectively).

4. Conclusion

A common feature of the defensin or tachyplesin family is to present an amphipathic structure constrained by two or three disulphide bridges with basic hydrophilic clusters due to the presence of cationic side chains [5,6]. Several studies indicate that these peptides act by altering the permeability of biological membranes. However, the mechanism of the membrane permeabilisation is not yet well understood. For human defensin, it has been suggested that membrane alteration occurs through peptide aggregation with a solvent channel between them. We found that synthetic protegrins form weakly selective pores in *Xenopus laevis* oocytes. Small monovalent cations (especially K^+) as well as Ca^{2+} permeate the protegrin-induced pore together with Cl^-. Despite their size, K^+ ions were particularly favoured, so that in their presence the pore became completely unselective. This effect could be explained by the existence of steric, K^+-specific, rearrangements induced by permeation of this ion in the channel pore. Our data thus suggest that protegrins and defensins present a similar activity toward the membrane, in the sense that they both form weakly selective ionic channels, where anions, as well as small cations, are able to permeate ([8,17], this report). However, contrary to human and insect defensins [8,17], the protegrin-induced membrane conductance does not show voltage dependence in the voltage range between −100 and +30. In particular, pore formation is not blocked at positive holding potentials. The linearity of the current waveform during ramp application further suggests that channel permeation is voltage-independent. We also find that protegrin folding induced by disulphide bridges is essential for membrane permeabilisation, but not for bactericidal activity (see Table 3). In particular, connectivity of disulphide bridges seems not to be required for activity against Gram-negative bacteria. Taken together, these data suggest that antimicrobial activity could exist independently from membrane pore formation. Experiments are in progress to study the cytotoxic mechanism of linear protegrin analogues.

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References

[1] Kokryakof, V.N., Harwig, S.S.L., Panyutich, E.A., Shevchenko, A.A., Aleshina, G.M., Shamova, O.V., Korneva, H.A. and Lehrer, R.I. (1993) FEBS Lett. 327, 231–236.
[2] Migorodskaya, O.A., Shevchenko, A.A., Abdalla, K.O., Chernushevich, I.V., Egorov, T.A., Musoliamov, A.X., Kokryakov, V.N. and Shamova, O.V. (1993) FEBS Lett. 330, 339–342.
[3] Harwig, S., Swiderek, K.L.T. and Lehrer, R.I. (1995) J. Peptide Sci. 3, 207–215.
[4] White, S.H., Wimley, W.C. and Selsted, M.E. (1995) Curr. Opin. Struct. Biol. 5, 521–527.
[5] Maloy, L.W. and Kari, U.P. (1995) Biopolymers 37, 105–122.
[6] Matsuzaki, K., Nakayama, M., Fukui, M., Otaka, A., Funakoshi, S., Nobutaka, F., Bessho, K. and Miyajima, K. (1993) Biochemistry 32, 11704–11710.
[7] Wimley, W.C., Selsted, M.E. and White, S.H. (1994) Protein Sci. 3, 1362–1373.
[8] Kagan, B.L., Selsted, M.E., Ganz, T. and Lehrer, R.I. (1990) Proc. Natl. Acad. Sci. USA 87, 210–214.
[9] Chavanieu, A., Calas, B., Vaglio, P. and Grigorescu, F. (1992) Eur. J. Biochem. 208, 367–373.
[10] Tam, J.P., Wu, C.R., Lui, W. and Zhang, J.W. (1991) J. Am. Chem. Soc. 113, 6657–6662.
[11] Charnet, P., Bourinet, E., Dubel, S. J., Snutch, T.P. and Nargeot, J. (1994) FEBS Lett. 344, 87–90.
[12] Mor, A., Hani, K. and Nicolas, P. (1994) J. Biol. Chem. 269, 31635–31641.
[13] Dascal, N. (1987) CRC Crit. Rev. Biochem. 22, 317–387.
[14] Hille, B. (1992) Ionic Channels of Excitable Membranes. Sinauer Inc., Sunderland, MA.
[15] Ghosh, A. and Greenberg, M.E. (1995) Science 268, 239–247.
[16] Barish, M.E. (1983) J. Physiol. 342, 309–325.
[17] Cociancich, S., Ghazi, A., Hetru, C., Hoffmann, J.A. and Letellier, L. (1993) J. Biol. Chem. 268, 19239–19245.