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The Bacterial Peptide Pheromone Plantaricin A Permeabilizes Cancerous, but not Normal, Rat Pituitary Cells and Differentiates between the Outer and Inner Membrane Leaflet

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Abstract Plantaricin A (PlnA) is a 26-mer peptide pheromone with membrane-permeabilizing, strain-specific antibacterial activity, produced by *Lactobacillus plantarum* C11. We investigated the membrane-permeabilizing effects of PlnA on cultured cancerous and normal rat anterior pituitary cells using patch-clamp techniques and microfluorometry (fura-2). Cancerous cells displayed massive permeabilization within 5 s after exposure to 10–100 μM PlnA. The membrane depolarized to nearly 0 m*V*, and the membrane resistance decreased to a mere fraction of the initial value after less than 1 min. In outside-out membrane patches, 10 μM PlnA induced membrane currents reversing at 0 m*V*, which is compatible with an unspecific conductance increase. The D and L forms of the peptide had similar potency, indicating a nonchiral mechanism for the membrane-permeabilizing effect. Surprisingly, inside-out patches were insensitive to 1 mM PlnA. Primary cultures of normal rat anterior pituitary cells were also insensitive to the peptide. Thus, PlnA differentiates between plasma membranes and membrane leaflets. Microfluorometric recordings of $[Ca^{2+}]_i$ and cytosolic concentration of fluorochrome verified the rapid permeabilizing effect of PlnA on cancerous cells and the insensitivity of normal pituitary cells.

Keywords Antimicrobial peptide · Plantaricin A · *Lactobacillus plantarum* · Anterior pituitary cells · Membrane permeabilization · Patch clamp

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Introduction

Antimicrobial peptides are produced by nearly all organisms: bacteria, plants, invertebrates and vertebrates (Zasloff, 1987; Nissen-Meyer & Nes, 1997; Chen et al., 2003; Fimland et al., 2005). The function of such peptides is protection from competing or pathogenic microorganisms. In bacteria, production of ribosomally synthesized antimicrobial peptides provides an ecological advantage over competitors. Antimicrobial peptides are essential for immune defense in plants and invertebrates, which lack the adaptive immune system of higher animals. In vertebrates, antimicrobial peptides are important parts of the innate immune system.

Antimicrobial peptides have diverse primary structures but are often cationic and amphiphilic or hydrophobic. Their positive charge presumably facilitates interactions with negatively charged membrane components, whereas their amphiphilic character enables membrane insertion. The antimicrobial effect of these peptides is mainly due to membrane permeabilization. Several mechanisms of action have been suggested, ranging from formation of distinct transmembrane pores and less defined, variable pores to a detergent-like effect on the membrane (Matsuzaki, 1998; Oren & Shai, 1998; Hancock & Chapple, 1999; Tossi, Sandri & Giangaspero, 2000; Shai, 2002; Zelezetsky et al., 2005). However, the molecular mechanisms are still not completely understood.

It is an intriguing finding that certain antimicrobial peptides from insects (Chen et al., 1997; Ye et al., 2004), frogs (Cruciani et al., 1991; Jacob & Zasloff, 1994) and mammals (Lichtenstein, 1991; Lehrer, Lichtenstein & Ganz, 1993) also kill a variety of tumor cells at concentrations that do not affect normal eukaryotic cells. Numerous studies have recently been conducted to

determine how lytic peptides selectively permeabilize tumor cells, and several synthetic peptides have been designed to optimize their antitumor properties and improve their therapeutic potential (*for review, see* Leuschner & Hansel, 2004).

The antimicrobial peptides produced by bacteria (often termed "peptide bacteriocins") seem to be more potent toward bacteria than antimicrobial peptides produced by higher organisms, although the bacterial peptides usually show narrower target cell specificities. Bacterial peptides have not been reported to permeabilize normal or cancerous eukaryotic cells.

Lactic acid bacteria, like virtually all bacteria, produce antimicrobial peptides, usually containing between 25 and 60 residues (Nissen-Meyer & Nes, 1997; Sablon, Contreras & Vandamme, 2000). The peptides from lactic acid bacteria are of particular interest because of the "food-grade quality" of the bacteria. These bacteria are used in food production, they are part of the natural microbial flora in food humans have consumed for centuries and they constitute a significant part of the indigenous flora of mammals, including humans. Thus, peptides from lactic acid bacteria are considered nontoxic and relatively safe agents for use as food preservatives and drugs.

The peptide pheromone plantaricin A (PlnA) controls the production of antimicrobial peptides in *Lactobacillus plantarum* C11 (Diep et al., 1994; Hauge et al., 1998) and has in addition strain-specific antimicrobial activity (Anderssen et al., 1998). Upon interaction with membrane lipids, PlnA adopts a membrane-induced α-helical structure that enables nonchiral interaction with the target cell membrane, where it subsequently binds to the receptor mediating the pheromone effect (Kristiansen et al., 2005). The membrane-interacting mode of action may explain why PlnA displays antibacterial activity against some sensitive strains in addition to its pheromone activity.

Three PlnA variants are produced by *L. plantarum* C11: a 26-residue full-length peptide (PlnA-26) and two N-terminally truncated forms containing 23 (PlnA-23) and 22 (PlnA-22) residues. The three variants are all derived from a 48-residue precursor coded by the *plnA* gene (Diep et al., 1994), and they display identical antimicrobial (Anderssen et al., 1998) and pheromone (Diep, Havarstein & Nes, 1995) activities. Recently, it has been shown that PlnA-22 at micromolar concentrations kills human leukemic T cells by both necrosis and apoptosis (Zhao et al., 2006). However, the mechanism for this effect is not clear.

In the present study, we investigated the membrane-permeabilizing effects of PlnA-22 on cancerous (GH_4 cells) and normal (primary culture) rat anterior pituitary cells by means of patch-clamp recordings (whole-cell and excised patch) and microfluorometry (fura-2).

Materials and Methods

Synthesis and Purification of PlnA

The natural L-enantiomeric form and the D-enantiomeric form of PlnA-22 were synthesized according to the sequence reported previously (Diep et al., 1994; Hauge et al., 1998). For purification, the peptides were dissolved in 0.1% (v/v) trifluoroacetic acid and applied to a 3-ml RESOURCE RPC reverse-phase column (GE Healthcare, Milwaukee, WI) equilibrated with 0.1% (v/v) trifluoroacetic acid. The peptides were eluted from the reverse-phase column with a linear 0–60% (v/v) 2-propanol gradient containing 0.1% (v/v) trifluoroacetic acid. The fractions containing peptides were collected, diluted five times with water containing 0.1% (v/v) trifluoroacetic acid and rechromatographed on the reverse-phase column. The primary structure and purity of the peptides were confirmed by mass spectrometry using a Voyager-DE RP matrix-assisted laser desorption ionization time-of-flight mass spectrometer (PerSeptive Biosystems, Cambridge, MA) and analytical reverse-phase chromatography. Peptide concentrations were determined by measuring the absorbance at 280 nm and using the molar extinction coefficient deduced from the amino acid composition.

Culture of Cancerous Rat Anterior Pituitary Cells

The GH clonal strains of rat pituitary cancer cells were established by Tashjian et al. (1968). These cells spontaneously synthesize and secrete prolactin and/or growth hormone and are electrically excitable. In the present study, we used the GH_4C_1 subclone, which mainly produces prolactin. The cells were grown as monolayer cultures in plastic tissue culture flasks containing Ham's F-10 medium supplemented with horse serum (7.5%), fetal calf serum (2.5%), penicillin (50 U · ml^{-1}) and streptomycin (100 μg · ml^{-1}) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were seeded in 35-mm dishes 3–7 days prior to recording.

Primary Culture of Normal Rat Anterior Pituitary Cells

Anterior pituitaries of Wistar rats (300 g) were removed immediately after decapitation and placed in Dulbecco's modified Eagle's medium (DMEM) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer on ice. The pituitaries were then washed with ice-cold phosphate-buffered saline (PBS), chopped into 1-mm^3 pieces and washed again. The tissue fragments were treated with trypsin (type II-S, 2.5 mg · ml^{-1} PBS) for 40 min in a shaker water bath at 37°C. The trypsin solution was then replaced with PBS containing trypsin inhibitor (type I-S, 2.5 mg · ml^{-1}) and the tissue incubated for another 5 min in the water bath.

The tissue fragments were then mechanically dissociated in ice-cold PBS using a plastic pipette. The cell suspension was filtered through a nylon mesh and centrifuged for 10 min at 100 *g*. Cells were then resuspended in growth medium (DMEM) with hydrogen carbonate buffer supplemented with fetal calf serum (5%), penicillin (50 $U \cdot ml^{-1}$) and streptomycin (100 $mg \cdot ml^{-1}$) and seeded in 35-mm plastic dishes coated with poly-L-lysine. The dishes were kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air until the start of experiments. DMEM was purchased from Invitrogen (Carlsbad, CA); other chemicals were from Sigma (St. Louis, MO).

Solutions

The recordings were performed in the following extracellular solution (mM): 150 NaCl, 5 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 10 glucose, 10 HEPES/NaOH (pH 7.4). The patch electrodes were filled with the following solution (mM): 120 CH_3O_3SK, 20 KCl, 10 HEPES/NaOH, 20 sucrose (pH 7.2).

Electrophysiology

Standard whole-cell and excised patch-clamp recordings were used to measure membrane potential and membrane currents (Hamill et al., 1981). The patch-clamp recordings were carried out at room temperature. The patch electrodes were made from Na-glass with filament and fire-polished before use, and the electrode resistance was 4–10 MΩ. The electrodes were connected to an EPC-7 patch-clamp amplifier (Heka, Lambrecht/Pfaltz, Germany) controlled by the software PClamp 9.2 (Axon Instruments, Union City, CA). The recorded signals were digitized at 4–10 kHz, filtered at one-third of the sampling rate and stored on a computer. The recordings were not adjusted for the electrode junction potentials. Membrane currents flowing from the cytoplasmic to the extracellular side were defined as positive and displayed as upward deflections in the current traces. Data analysis was performed using PClamp 9.2 and Origin 7.0 (OriginLab, Northampton, MA). The cells and excised patches were exposed to various concentrations of PlnA by pressure ejection (about 10 kPa) from a micropipette (inner tip diameter 1–2 μm) placed about three cell diameters from the cell or excised patch or by leakage from the pipette at a distance of about one cell diameter. No artifacts were observed when ejecting or leaking normal extracellular solution onto cells from these distances.

Microfluorometry of Fura-2-Loaded Cells

The cells were loaded with the fluorescent Ca^{2+} indicator fura-2 by exposure to 5 μm fura-2/AM (Molecular Probes, Eugene, OR) in extracellular solution for 40 min at 37°C, followed by washout of the fura-2 ester and further 30 min incubation at room temperature. The cells were then mounted on an Olympus inverted microscope, forming the central part of the Olympus OSP-3 system for dual excitation fluorometry (Olympus, Tokyo, Japan). The excitation light was switched at 200 Hz between 360 and 380 nm using a rotating mirror. The emitted fluorescence was recorded at 510 nm with a photomultiplier, and the measurements were restricted to single cells by a pinhole diaphragm. The ratio between emissions at the two different excitation wavelengths (F360/F380) reflects the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). The emission at the isosbestic wavelength 360 nm is independent of $[Ca^{2+}]_i$ and, thus, monitors the cytosolic concentration of the fluorochrome. Cells were exposed to PlnA by pressure ejection or leakage from a micropipette, as described above.

Results

PlnA Permeabilizes Cancerous Rat Anterior Pituitary Cells

In our initial experiments, the effects of PlnA on GH_4 cells were studied by whole-cell patch-clamp recordings in the current clamp and voltage clamp modes. Figure 1A shows a representative current clamp recording from a cell exposed to 100 μM PlnA by pressure ejection directly on to the cell from the time indicated by the arrow. Hyperpolarizing current pulses (20 pA, 500 ms) were injected into the cell at regular intervals (0.5 Hz) in order to monitor the membrane resistance, which is proportional to the resulting membrane potential deflections. The depolarizing spike at the termination of the second current injection subsequent to the PlnA exposure is a Ca^{2+}-dependent action potential. Within 5 s the membrane started to depolarize and the membrane resistance to decrease. After about 30 s the membrane potential approached 0 m*V* and the membrane resistance was reduced to a mere fraction of the initial value, indicating massive membrane permeabilization.

Whole-cell recordings were also performed in the voltage clamp mode, and one of these experiments is presented in Figure 1B. In these recordings, membrane conductance was monitored by applying hyperpolarizing steps (20 m*V*, 250 ms) at regular intervals (0.25 Hz) from the holding potential of –60 m*V*. The resulting current deflections are directly proportional to the membrane conductance. From the inset at higher amplification, it is evident that the membrane conductance was about tripled already 6 s after exposure to 100 μM PlnA by pressure ejection close to the cell. Within 40 s following the start of exposure, the

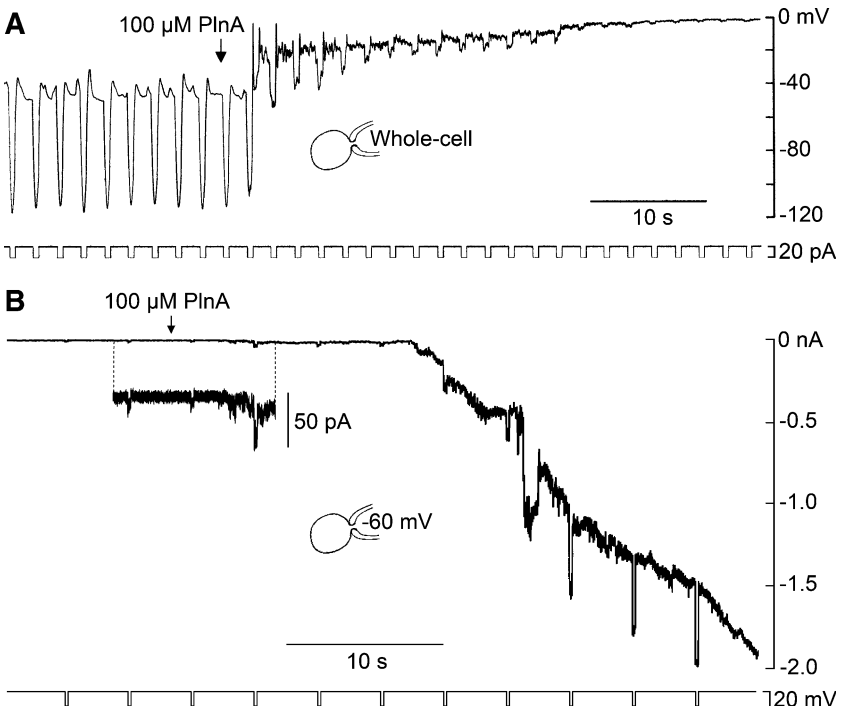

Fig. 1 PlnA reduces membrane resistance and induces inward current in cancerous rat anterior pituitary cells. (**A**) *Upper trace* shows a representative whole-cell current clamp recording of the membrane potential of a GH_4 cell. Current pulses (*lower trace*: 20 pA, 500 ms) were injected at a frequency of 0.5 Hz during the recording in order to monitor the membrane resistance. *Arrow* indicates start of continuous pressure ejection of 100 μM PlnA onto the cell. PlnA depolarized the cell toward 0 m*V* concomitant with a dramatic reduction of the membrane resistance, indicating massive membrane permeabilization. (**B**) *Upper trace* shows a representative whole-cell voltage clamp recording of the membrane current of a GH_4 cell. Hyperpolarizing voltage steps (*lower trace*: 20 m*V*, 250 ms) were applied from the holding potential of –60 m*V* at a frequency of 0.25 Hz during the recording in order to monitor the membrane conductance. *Arrow* indicates start of continuous pressure ejection of 100 μM PlnA onto the cell. A part of the recording is displayed at higher amplification. PlnA induced a large inward current concomitant with a dramatic increase of the membrane conductance, indicating massive membrane permeabilization. *Insets* in this and the following figures indicate the recording mode

holding current increased to about 2 nA and the membrane conductance increased from about 0.3 to about 25 nS.

In total, 29 cells were exposed to 100 μM PlnA during whole-cell patch-clamp recordings. In 27 of these, the peptide induced dramatic membrane permeabilization within seconds. Three cells were exposed to 1 mM PlnA, and all were instantly permeabilized. At a concentration of 10 μM, PlnA permeabilized 12 of the 25 cells tested. At a concentration of 1 μM, PlnA had no detectable effect on membrane conductance. Thus, PlnA has a dose-dependent, permeabilizing effect on GH_4 cells.

The PlnA-Induced Conductance Increase Is Unspecific

As shown in Figure 1A, PlnA depolarized the cells toward 0 m*V*, indicating that the induced ion conductance increase is unspecific. In order to study the specificity of the membrane conductance increase in more detail, we recorded from excised, outside-out patches voltage-clamped at different membrane potentials during exposure to PlnA. Figure 2 shows superimposed recordings from three voltage-clamped membrane patches at holding potentials of +5, 0 and –20 m*V*. Less than 5 s after exposure to 10 μM PlnA, the membrane patches turned leaky, and for the patches clamped at +5 and –20 m*V*, the holding current steadily increased. The time course of the induced current reflects the conductance increase linked to the membrane permeabilization. The membrane current induced by PlnA evidently reversed around 0 m*V*. In three outside-out patches, the reversal potential of the induced current was determined by tuning the holding potential to the level requiring zero holding current. The reversal potential estimated from this series of recordings was –1.4 ± 1.6 m*V* (standard deviation [SD]), confirming that the conductance increase was not restricted to one particular ion species.

Curiously, excised outside-out patches seemed to be more sensitive to PlnA than whole cells. In 41 outside-out patches voltage-clamped at –40 m*V*, 36 were rapidly permeabilized by 10 μM PlnA, revealed by increased negative holding current. Exposure to 100 μM PlnA permeabilized all of the 26 outside-out patches tested, whereas 5 μM PlnA

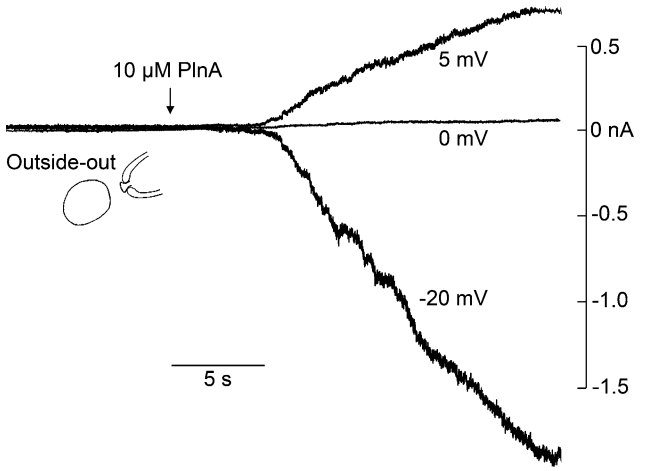

Fig. 2 PlnA induces membrane current with reversal potential close to 0 m*V* in cancerous rat anterior pituitary cells. Three superimposed current recordings from different outside-out patches excised from GH_4 cells are presented. The patches were voltage-clamped at the holding potentials indicated on the figure. *Arrow* indicates start of continuous pressure ejection of 10 μM PlnA onto the patches. The peptide made the membrane patches leaky, as shown by the induced membrane currents. The membrane current reversed around 0 m*V*, which is compatible with an unspecific conductance increase

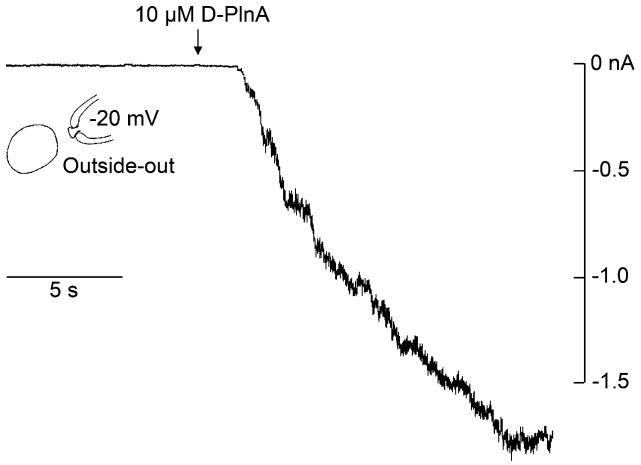

Fig. 3 D-PlnA is as potent as L-PlnA at permeabilizing cancerous rat anterior pituitary cells. The representative current recording is from an outside-out patch excised from a GH_4 cell. The patch was voltage-clamped at –20 m*V*. *Arrow* indicates start of continuous pressure ejection of 10 μM D-PlnA (composed of only D-amino acids) onto the patch. The D form of the peptide permeabilized the cell membrane as effectively as the L form, apparent from the rapid induction of a large inward current. Thus, the permeabilizing effect of PlnA seems to depend on nonchiral interactions

had no evident effect on the nine outside-out patches examined. A likely explanation for the discrepancy in sensitivity between whole cells and excised outside-out patches may be that the latter are more exposed to the peptide. In cells growing as a monolayer, parts of the membrane are less accessible.

Numerous bacteriocins form discrete ion channels in the cytoplasmic membrane of bacteria, as do several bacterial toxins targeting eukaryotic cells (*reviewed in* Parker & Feil, 2005). Therefore, we examined if the membrane permeabilization induced by PlnA was associated with discrete, unitary conductance steps. Rapid conductance steps were occasionally observed in recordings from outside-out patches, but abrupt steps were not a striking feature of most recordings. In order to reveal the possible existence of pores with a unitary conductance, 84 selected steps were analyzed. The step amplitudes showed a continuum between about 0.4 and 28 pS, averaging 7.7 ± 5.7 pS, suggesting that the permeabilization induced by PlnA is not linked to insertion of unitary pores in the membrane.

The Permeabilizing Effect of PlnA Is Mediated through a Nonchiral Interaction with the Membrane

If the permeabilizing effect of PlnA is mediated through ligand-receptor interactions, the D-enantiomeric form of the peptide would be expected to have little or no effect. We therefore tested the effect of the D-enantiomeric form of the peptide, composed of only D-amino acids (Hauge et al., 1998). Figure 3 shows a recording from an outside-out patch voltage-clamped at –20 m*V* and exposed to 10 μM D-PlnA. Within seconds the peptide induced pronounced membrane permeabilization and a large inward current. Similar experiments were carried out on outside-out patches from 30 additional cells, of which 21 were exposed to 10 μM and 9 were exposed to 100 μM D-PlnA. All the recordings showed massive membrane permeabilization within seconds after peptide ejection. We therefore concluded that the D-enantiomeric form is as effective as the L form of PlnA at permeabilizing GH_4 cells. This strongly suggests that the interaction between PlnA and the plasma membrane is nonchiral.

PlnA Differentiates Between the Two Leaflets of the Membrane Bilayer

The inside-out recording mode of the patch-clamp technique offers a powerful method for selectively exposing the inner leaflet of the membrane to the peptide. Figure 4 presents an inside-out recording from a patch voltage-clamped at –40 m*V*. The inner leaflet of the membrane was exposed to 1 mM PlnA, which is 100 times above the concentration that permeabilizes about 90% of the outside-out patches. Surprisingly, there was no detectable effect on the membrane conductance in spite of this very high concentration of PlnA.

The expanded part of the recording shows single-channel currents through two active BK channels (big Ca^{2+}-activated K^+ channels). The peptide had no effect on channel activity, which is likely to be very sensitive to membrane

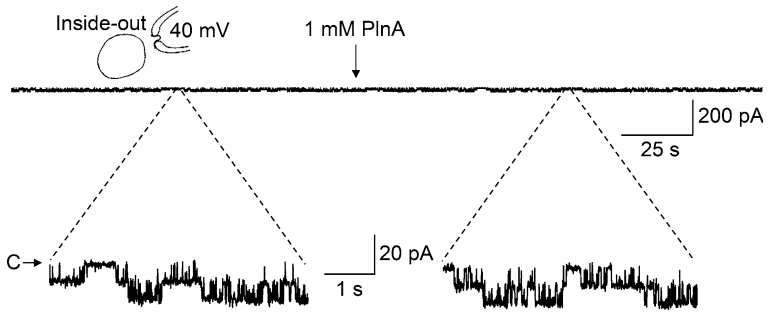

Fig. 4 The inner leaflet of the membrane of rat anterior pituitary cells is insensitive to PlnA. The representative current recording is from an inside-out patch excised from a GH_4 cell. The patch was voltage-clamped at –40 m*V* (cytosolic side). *Arrow* indicates start of continuous pressure ejection of 1 mM PlnA onto the patch. No effect on membrane conductance was detected, even at this high concentration of the peptide. The expanded parts of the recording show single-channel currents through two active BK channels (big Ca^{2+}-activated K^+ channels). *C* indicates closure of both channels. The peptide had no effect on channel activity, indicating that the peptide does not interfere with membrane integrity when exposed to the inner leaflet. Comparable recordings from normal rat anterior pituitary cells gave identical results

disruption, indicating that the peptide does not interfere with membrane integrity when exposed to the inner leaflet.

These findings were supported by 10 additional recordings from patches exposed to 1 mM PlnA, of which eight remained stable during the whole recording period of several minutes. The two remaining patches were permeabilized within 30 s after exposure to the peptide. When exposed to 100 μM PlnA, which permeabilized 100% of the outside-out patches, 17 out of 20 inside-out patches in this series of experiments were unaffected. Based on these experiments, we conclude that PlnA hardly interferes with membrane integrity when exposed to the inner leaflet.

Normal Rat Anterior Pituitary Cells Are Insensitive to PlnA

Several membrane-disrupting lytic peptides with antibacterial activity also permeabilize eukaryotic tumor cells, whereas their normal analogues are insensitive (*reviewed in* Leuschner & Hansel, 2004). Hence, we also studied the effects of PlnA on primary cultured rat anterior pituitary cells, which are the normal counterparts of GH_4 cells. Figure 5A demonstrates the relative insensitivity of normal rat anterior pituitary cells to PlnA. The recording is from an outside-out patch voltage-clamped at –60 m*V* during exposure to 1 mM PlnA. Even at this high concentration, the peptide did not induce any detectable membrane current. Figure 5B presents a comparable recording from an outside-out patch excised from a GH_4 cell. At a holding potential of –60 m*V*, exposure to 10 μM of the peptide (1:100 of the concentration in Fig. 5A) induced a large inward current within seconds, as previously described.

In total, 10 outside-out patches from normal cells were exposed to 1 mM PlnA, and only two of these displayed a detectable conductance increase. At a concentration of 100 μM, PlnA had no effect on five of the six outside-out patches tested. At both these concentrations, 100% of the outside-out patches from GH_4 cells were rapidly disrupted by the peptide. In agreement with the results from GH_4 cells, inside-out patches from normal cells were insensitive to PlnA ($n = 6$).

Whole-cell recordings confirmed the relative insensitivity of normal rat anterior pituitary cells compared to GH_4 cells. In total, 23 normal cells were exposed to 1 mM PlnA, and only four of these displayed a detectable conductance increase. At a concentration of 100 μM, PlnA had no effect on 23 of the 27 normal cells tested. For comparison, 93% of the exposed GH_4 cells were rapidly permeabilized by the peptide at a concentration of 100 μM.

Microfluorometric Recordings Confirm the Membrane-Permeabilizing Effect of PlnA

Among the inorganic ions in physiological solutions, Ca^{2+} has the greatest electrochemical gradient across the cell membrane. Thus, measurement of the elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is a sensitive method for detecting unspecific membrane permeabilization. To verify the results obtained by the patch-clamp method, we investigated the effect of PlnA by means of microfluorometry based on the Ca^{2+}-sensitive fluorochrome fura-2. $[Ca^{2+}]_i$ was monitored by recording the ratio between emissions at excitation wavelengths of 360 and 380 nm (F360/F380), whereas the cytosolic concentration of the fluorochrome was monitored by detecting emission at the isosbestic wavelength of 360 nm.

Figure 6A shows a microfluorometric recording from a normal rat anterior pituitary cell exposed to 1 mM PlnA by pressure ejection close to the cell. Evidently, the cell was insensitive to the peptide. A total of 19 normal cells were

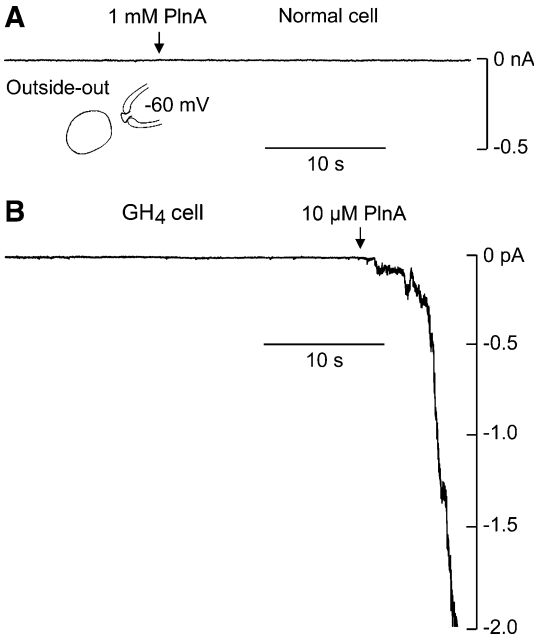

Fig. 5 Normal rat anterior pituitary cells are insensitive to PlnA. (**A**) Representative current recording from an outside-out patch excised from a normal rat anterior pituitary cell (primary culture). The patch was voltage-clamped at –60 m*V*. *Arrow* indicates start of continuous pressure ejection of 1 mM PlnA onto the patch. Even at this high concentration, the peptide did not induce any detectable membrane current. (**B**) Comparable recording from an outside-out patch excised from a GH_4 cell. At a holding potential of –60 m*V*, exposure to 10 μM of the peptide rapidly induced a large inward current. The time course of the induced current reflects the conductance increase linked to the membrane permeabilization

exposed to 1 mM PlnA, and only two were affected by this very high peptide concentration.

Figure 6B presents a comparable recording from a GH_4 cell exposed to 10 μM PlnA. The peptide caused an instant elevation of $[Ca^{2+}]_i$ to saturating levels, followed by a delayed loss of fluorochrome. The instant elevation of $[Ca^{2+}]_i$ confirms the rapid effect of PlnA, whereas the loss of the relatively large fura-2 molecules demonstrates an extensive, unspecific membrane permeabilization. Five GH_4 cells were examined at a concentration of 10 μM PlnA, and three of these were permeabilized. At a concentration of 100 μM PlnA, all of the five cells tested were instantly permeabilized. Hence, the results from the microfluorometric experiments are in close agreement with the data obtained using the patch-clamp methods.

Discussion

Patch-Clamp Recordings for Studies of Membrane Permeabilization

Traditionally, the effects of antimicrobial peptides on eukaryotic cells have been studied using markers for necrosis and apoptosis or biochemical methods for recording changes in transmembrane gradients. Such methods do not reveal direct effects on the membrane and have inherently poor time resolution. Furthermore, the ion specificity of possible membrane-permeabilizing effects is not easily determined. In contrast, the various patch-clamp methods allow direct control and monitoring of the membrane potential and current with time resolution in the millisecond range. The time course of an induced membrane conductance increase is directly linked to membrane permeabilization. The ion specificity of the conductance increase is indicated by the reversal potential of evoked membrane currents. By using excised outside-out and inside-out patches, the two leaflets of the cell membrane can be selectively exposed to the peptides. Finally, patch-clamp methods are well suited for distinguishing between permeabilization caused by insertion of defined pores and general disruption of the membrane. Hence, in the present study, we employed patch-clamp techniques to investigate the effects of PlnA on rat anterior pituitary cells.

PlnA Permeabilizes Cancerous Rat Anterior Pituitary Cells by General Disruption of the Membrane

Some permeabilizing, cationic bacteriocins form defined ion channels, usually subsequent to specific binding to docking molecules in the bacterial membrane (Brotz et al., 1998; Breukink & de Kruijff, 1999; Wiedemann et al., 2001; Bauer & Dicks, 2005; Sobko et al., 2006). These peptides may be effective against bacteria at nanomolar concentrations and display high target-cell specificity, with no effect on eukaryotic cells. The peptides may also permeabilize artificial bilayers lacking docking molecules but at much higher peptide concentrations. Numerous bacterial toxins targeting eukaryotic cells are also pore-forming and effective at very low concentrations (*for review, see* Parker & Feil, 2005).

Other cationic antimicrobial peptides permeabilize their target cells by a mechanism commonly termed the "carpet model" (Oren & Shai, 1998; Huang, 2000; Yang et al., 2001; Shai, 2002; Thennarasu et al., 2005). Once these peptides reach the negatively charged plasma membrane, they lie parallel to the surface of the outer leaflet, where they accumulate until a threshold concentration is reached. The membrane is then permeabilized by a general, detergent-like action or by formation of variably sized areas of increased permeability. It is currently not possible to assess the likely mechanism of membrane permeabilization of a given peptide simply on the basis of its structure (Chen et al., 2003).

PlnA induced extensive leakage of the relatively large fura-2 molecules from GH_4 cells preloaded with the fluorochrome. Therefore, if PlnA causes insertion of distinct channels in the membrane, these would be expected to

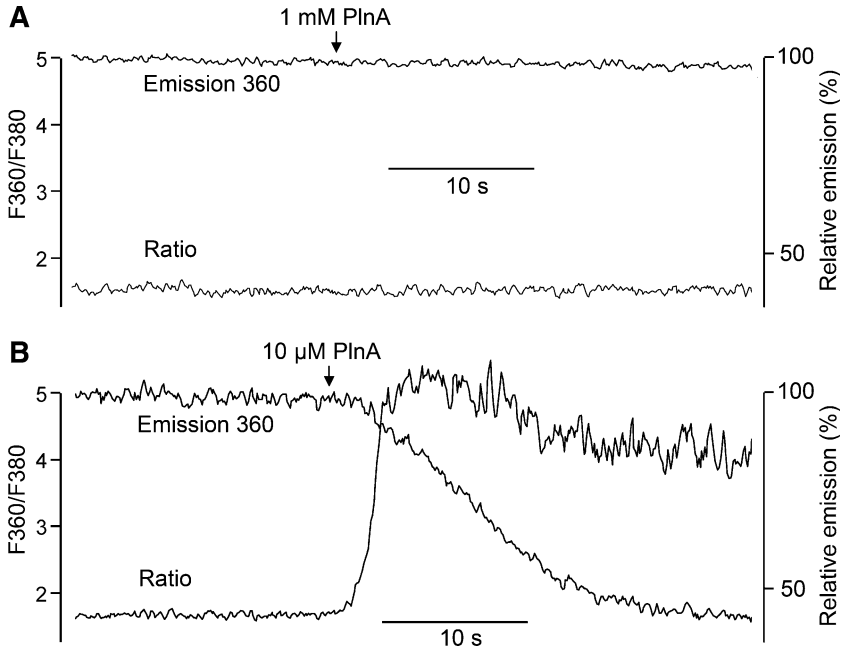

Fig. 6 Microfluorometric recordings verify the relative insensitivity of normal rat anterior pituitary cells compared to the cancerous GH_4 cell line. $[Ca^{2+}]_i$ was monitored by microfluorometric recordings based on the Ca^{2+}-sensitive fluorochrome fura-2. The ratio between emissions at the excitation wavelengths 360 and 380 nm (F360/F380) reflects $[Ca^{2+}]_i$, whereas emission at the isosbestic excitation wavelength 360 nm reflects [Fura-2]$_i$. (**A**) Representative recording from a normal rat anterior pituitary cell loaded with fura-2. *Arrow* indicates start of continuous pressure ejection of 1 mM PlnA onto the cell. Evidently, the cell was insensitive even to this high concentration of the peptide. (**B**) Comparable recording from a GH_4 cell exposed to 10 μM PlnA. PlnA evoked instant elevation of $[Ca^{2+}]_i$ to saturating levels, followed by delayed loss of fluorochrome, indicating massive membrane permeabilization

have large unitary conductance and should thus be easily detected by excised patch-clamp recordings. However, the outside-out patch recordings from GH_4 cells showed that PlnA induced a gradual conductance increase, with only occasional conductance steps of varying amplitudes. These results exclude the formation of defined ion channels of unitary conductance.

The extremely steep dose-response relationship, with full effect at 10 μM and no detectable permeabilization at 5 μM, supports the notion that a threshold concentration of PlnA in the membrane is required for permeabilization to occur. The fast time course of the permeabilizing effect, which is initiated after only a few seconds of exposure to sufficiently high concentrations of PlnA, was a striking finding in our study. Furthermore, it appears that PlnA exerts its effect on GH_4 cells through nonchiral interactions with membrane molecules because the natural L form of the peptide and its mirror image, the D-enantiomeric form, have similar potency. Finally, both the whole-cell depolarization toward 0 m*V* and the reversal potential of about 0 m*V* for the induced transmembrane currents show that the conductance increase was rather unspecific and not limited to one particular ion species. The carpet model, or a related mechanism, thus seems to be most applicable for the permeabilizing effect of PlnA on GH_4 cells.

Permeabilization of Cancerous Cells by PlnA May not Depend on Increased Level of Phosphatidylserine in the Outer Membrane Leaflet

We have shown that normal rat anterior pituitary cells were insensitive to PlnA at a concentration 100 times above that required to permeabilize the cancerous GH_4 cells. To our knowledge, this is the first report of a bacterial peptide that preferentially permeabilizes cancerous cells relative to their normal counterparts. In all cells, the distribution of phospholipids in the two membrane leaflets is asymmetrical (*for review, see* Balasubramanian & Schroit, 2003). As opposed to bacteria, eukaryotic cells keep most of their negatively charged phospholipids, particularly phosphatidylserine, in the inner membrane leaflet by means of specific, energy-requiring transporters. Cationic antimicrobial peptides bind much better to the negatively charged outer leaflet of bacterial membranes than to the predominantly zwitterionic outer leaflet of normal eukaryotic cell membranes, which are usually not permeabilized by such peptides (Matsuzaki et al., 1995; Matsuzaki, 1999; Shai, 1999; Castano et al., 2005). In senescent, apoptotic and damaged eukaryotic cells, an increasing amount of phosphatidylserine is exposed on the outer surface, where phosphatidylserine acts as a determinant for recognition and capture

by phagocytes (Fadok et al., 1992; Martin et al., 1995; Zwaal & Schroit, 1997; Zwaal, Comfurius & Bevers, 2005). A slight increase of phophatidylserine in the outer leaflet of the cell membrane has been reported in several types of cancer cells (Utsugi et al., 1991; Rao, Tait & Hoang, 1992; Sugimura et al., 1994; Williamson & Schlegel, 1994). Therefore, the ability of some cationic membrane-permeabilizing peptides to differentiate between cancer cells and normal cells may be due to the increase of phophatidylserine in the outer leaflet of the cell membrane of cancer cells compared to their normal counterparts. However, the difference in membrane lipid composition between cancer cells and normal cells is still small. Therefore, the question arises of whether this difference is sufficient to explain the ability of some cationic antimicrobial peptides to preferentially kill cancer cells, and it has been suggested that other membrane components bind and bring these peptides close to the cancer cell membrane (Papo & Shai, 2003). This hypothesis is based on the fact that not only phosphatidylserine but also other negatively charged molecules, such as lipoproteins, *O*-glycosylated mucins and sialic acid, are present at relatively high levels on the outer membrane leaflet of several tumor cells (Utsugi et al., 1991; Williamson & Schlegel, 1994; Cappelli, Paladini & D'Agata, 1999; Raval et al., 2003; Miyagi et al., 2004).

Using the outside-out and inside-out configurations of the patch-clamp technique, we examined the importance of negatively charged phospholipids for the permeabilizing effect of PlnA. This is the first time the outer and inner leaflets of the plasma membrane have been selectively exposed to a permeabilizing peptide. Neither in GH_4 cells nor in normal rat anterior pituitary cells were the membrane patches permeabilized by 1 mM PlnA when the inner leaflet was exposed to the peptide. This finding was contrary to our prediction since negatively charged phospholipids are much more abundant in the inner than in the outer leaflet (Balasubramanian & Schroit, 2003). Our data, showing that PlnA only permebilizes membrane patches from GH_4 cells if the outer leaflet is exposed, thus support the hypothesis that membrane components other than negatively charged phospholipids are essential for the membrane-permeabilizing effect of PlnA on tumor cells.

Conclusion and Future Studies

We have shown that PlnA preferentially permeabilizes cancerous GH_4 cells compared with normal rat anterior pituitary cells. The peptide is active only at the outer membrane leaflet of GH_4 cells, and the permeabilizing effect is dependent on nonchiral interactions with membrane components. Thus, PlnA differentiates between plasma membranes and membrane leaflets, which makes PlnA particularly interesting for further studies. PlnA and variants of this peptide may, for instance, be developed into new chemotherapeutic drugs or into probes that may be used to analyze the molecular characteristics of membranes. In this context, it is significant that the protease-resistant D-enantiomeric form has the same potency as the natural L form. A likely direction for future research is modification of the peptide in order to obtain simpler, shorter and protease-resistant variants with retained or enhanced activity and reduced antigenicity. The latter may be achieved by constructing diastereomeric peptides, which contain both D and L forms of amino acids (Benkirane et al., 1993). Future experiments are, of course, also needed to examine if the preferential effect on cancerous cells is a general feature of PlnA or restricted to specific cell types.

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