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Peptide Pheromone Plantaricin A Produced by *Lactobacillus plantarum* Permeabilizes Liver and Kidney Cells

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Abstract Certain antimicrobial peptides from multicellular animals kill a variety of tumor cells at concentrations not affecting normal eukaryotic cells. Recently, it was reported that also plantaricin A (PlnA), which is a peptide pheromone with strain-specific antibacterial activity produced by *Lactobacillus plantarum,* permeabilizes cancerous rat pituitary cells (GH_4 cells), whereas normal rat anterior pituitary cells are resistant to the peptide. To examine whether the preferential permeabilization of cancerous cells is a general feature of PlnA, we studied its effect on primary cultures of cells from rat liver (hepatocytes, endothelial, and Kupffer cells) and rat kidney cortex, as well as two epithelial cell lines of primate kidney origin (Vero cells from green monkey and human Caki-2 cells). The Vero cell line is derived from normal cells, whereas the Caki-2 cell line is derived from a cancerous tumor. The membrane effects were studied by patch clamp recordings and microfluorometric (fura-2) monitoring of the cytosolic concentrations of Ca^{2+} ($[Ca^{2+}]_i$) and fluorophore. In all the tested cell types except Kupffer cells, exposure to 10–100 μM PlnA induced a nearly instant permeabilization of the membrane, indicated by the following criteria: increased membrane conductance, membrane depolarization, increased $[Ca^{2+}]_i$, and diffusional loss of fluorophore from the cytosol. At a concentration of 5 μM, PlnA had no effect on any of the cell types. The Kupffer cells were permeabilized by 500 μM PlnA. We conclude that the permeabilizing effect of PlnA is not restricted to cancerous cells.

Keywords Antimicrobial peptide · Plantaracin A · *Lactobacillus plantarum* · Membrane permeabilization · Patch clamp · Cancer cells · Vero cells

Introduction

Antimicrobial peptides are produced by nearly all organisms: bacteria, plants, invertebrates, and vertebrates (reviewed in Chen et al. 2003; Fimland et al. 2005; Nissen-Meyer and Nes 1997; Zasloff 1987). In bacteria, antimicrobial peptides provide an ecological advantage over competitors. Antimicrobial peptides are essential for the immune defense in plants and invertebrates, which lack the adaptive immune system of higher animals. In vertebrates, antimicrobial peptides are an important part 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. Their antimicrobial effect is mainly as a result of 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 (Hancock and Chapple 1999; Matsuzaki 1998; Oren and Shai 1998; Shai 2002; Tossi et al. 2000; Zelezetsky et al. 2005). However, the molecular mechanisms are still not completely understood.

Intriguingly, certain antimicrobial peptides from insects (Chen et al. 1997; Ye et al. 2004), frog (Cruciani et al. 1991;

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Jacob and Zasloff 1994), and mammals (Lehrer et al. 1993; Lichtenstein 1991) also kill a variety of tumor cells at concentrations not affecting 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 a review, see Leuschner and Hansel 2004).

Antimicrobial peptides produced by bacteria generally show narrower target cell specificity than those produced by higher organisms and have been considered not to affect eukaryotic cells. However, the peptide pheromone plantaricin A (PlnA) produced by *Lactobacillus plantarum* also has strain-specific antimicrobial activity (Anderssen et al. 1998), and it has been reported that micromolar concentrations of PlnA kill human leukemic T cells by both necrosis and apoptosis (Zhao et al. 2006). Later, it was shown that PlnA permeabilizes the cell membrane of cancerous rat pituitary cells (GH_4 cells), whereas normal rat anterior pituitary cells are resistant to the peptide (Sand et al. 2007).

Lactic acid bacteria produce several antimicrobial peptides (Nissen-Meyer and Nes 1997; Sablon et al. 2000). 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. Consequently, the peptides from lactic acid bacteria are of particular interest because these peptides are considered nontoxic and relatively safe for use as food preservatives and drugs. Although PlnA kills certain bacterial strains by membrane permeabilization (Anderssen et al. 1998), its primary function is to control the production of antimicrobial peptides in *Lactobacillus plantarum* C11 (Diep et al. 1994; Hauge 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 et al. 1995) activities.

In the present study, we explored effects of PlnA-22 on normal and cancerous kidney cells, as well as normal liver cells, in order to clarify if its previously reported preferential effect on cancerous cells is a general feature of PlnA. Patch clamp recordings and Ca^{2+} microfluorometry were used to monitor membrane permeabilization. We report that the normal and cancerous cell types tested in this study show rather similar sensitivity to PlnA.

Materials and Methods

Synthesis and Purification of PlnA

PlnA-22 was synthesized according to the sequence reported previously (Diep et al. 1994; Hauge et al. 1998). The peptides were purified by Resource RPC reverse-phase columns (GE healthcare, Fairfield, CT, USA), as described previously (Sand et al. 2007). 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, Framingham, MA, USA) 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 Clonal Vero and Caki-2 Cells

The Vero cell line was derived from a normal, healthy kidney of African green monkey (*Cercopithecus aetiops*) in 1962 (Liebhaber et al. 1967). Consequently, the cells are not of cancerous origin. The Vero cells are epithelial cells, although their exact origin in the nephrone is not known. There are numerous subclones of the original Vero cell line, and in this study, Vero-CCL-81 was used. The cells were grown as described earlier (Hafting et al. 2006). The cells to be studied were seeded in 35-mm petri dishes 1–5 days before recording.

The epithelial Caki-2 cell line was established from a human primary kidney carcinoma in 1971 (Fogh et al. 1977). The cells were grown under the same conditions as Vero cells, but in McCoy 5A medium (Sigma, St. Lois, MO, USA) supplemented with newborn calf serum (NCS) (10%), penicillin (50 μg ml^{-1}), streptomycin (50 μg ml^{-1}) and L-glutamine (2 mM).

Primary Cultures of Normal Rat Liver Cells

A heterogenic cell suspension from liver was prepared from male Wistar rats in a two-step procedure (Blomhoff et al. 1984). The animal was anesthetized with pentobarbital injection (0.5 g/kg) before dissection. Briefly, the liver was initially perfused for 9 min with Ca^{2+}-free buffer prewarmed to 37°C, followed by perfusion with a prewarmed collagenase-containing (0.36 mg/ml) Hanks balanced salt solution until visible disintegration of the portal vein, i.e.,

after a further 5–10 min. The liver was then mechanically disintegrated, and connective and vascular tissue was removed. The cell suspension was sequentially filtered through 100 and 70-μm nylon meshes and kept at ≤4°C throughout the rest of the procedure. Hepatocytes sediment rapidly, and they were isolated from the suspension by centrifugation at 40×*g* for 2 min. This procedure was repeated once after resuspension of the cell pellet. Endothelial cells and Kupffer cells were isolated by 5-min centrifugation at 540×*g* of the supernatants from the previous centrifugations, followed by resuspension of the cell pellet and centrifugation at 760×*g* for 23 min on a Percoll gradient (GE Healthcare). Both cell types settled between the 50 and 25% Percoll layers. The cell suspension was extracted, diluted in phosphate-buffered saline, and centrifuged one last time at 680×*g* for 8 min at 4°C. The cell pellet was resuspended in Eagle minimal essential medium (Sigma). Kupffer cells bind to plastic, and they were isolated from endothelial cells by incubating the cell suspension in untreated plastic dishes for 6 min. Subsequently, the supernatant containing epithelial cells was seeded in dishes treated with fibronectin (1 mg/ml; Sigma). All cells were seeded at a density of 1,500,000 cells per 35-mm petri dish. The hepatocytes were also seeded in fibronectin-treated dishes and were grown in Dulbecco modified Eagle medium (Sigma) supplemented with penicillin (50 μg ml^{-1}), streptomycin (50 μg ml^{-1}), and L-glutamine (2 mM). Kupffer and endothelial cells were grown in Eagle minimal essential medium (Sigma) supplemented with penicillin (50 μg ml^{-1}) and streptomycin (50 μg ml^{-1}). The medium for the Kupffer cells also contained 10% NCS. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Primary Cultures of Normal Rat Kidney Cortex Cells

Kidneys were dissected from the same rats used for isolation of liver cells and kept on ice. The enveloping sheet of connective tissue was removed, and the outer layer of the cortex was dissected free. The cortex was cut in approximately 1-mm^3 pieces and washed in ice-cold Hanks balanced salt solution. The tissue fragments were then incubated in collagenase-containing Dulbecco phosphate-buffered saline solution (1 mg/ml) for 60 min in a shaker water bath at 37°C. Next, the tissue was transferred to prewarmed growth medium (Dulbecco modified Eagle medium/F-12, Sigma) containing 0.05 mg/ml DNAse (Sigma), and cells were partly dissociated mechanically using a fire-polished pipette. The cell suspension was kept on ice while the remaining tissue fragments were incubated in new collagenase solution for 20 min. This procedure was repeated three or four times until the fragments were completely disintegrated. The cell suspensions were then pooled and filtered through a 70-μm nylon mesh, followed by centrifugation at 100×*g* for 10 min. Finally, cells were seeded in 35-mm Petri dishes with growth medium containing 10% NCS, and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Experimental Solutions

Both the electrophysiological and the microfluorometric experiments were performed in normal 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 pipettes used for electrophysiological recordings were filled with the following solution (mM): 120 CH_3O_3SK, 20 KCl, 10 HEPES/NaOH, 20 sucrose, pH 7.2. All the experiments were conducted at room temperature.

Electrophysiology

Standard whole-cell and excised patch clamp recordings were used to measure membrane potential and membrane currents (Hamill et al. 1981). The recording conditions and equipment were the same as previously described (Sand et al. 2007). The cells and excised patches were exposed to various concentrations of PlnA by pressure ejection (about 1 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 artefacts 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 as previously described (Sand et al. 2007). 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 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

The membrane-permeabilizing effects of PlnA on rat liver cells in primary culture (hepatocytes, endothelial cells, and Kupffer cells), rat epithelial cells from the renal cortex in

primary culture, Vero cells (clonal epithelial renal cells derived from a normal kidney in green monkey), and human epithelial Caki-2 cells (derived from a cancerous renal tumor) were qualitatively similar. Therefore, the representative recordings displayed in the figures are restricted to Vero cells, while the results from all the six cell types are presented in the tables and graph.

Effects of PlnA on Electrical Membrane Properties

Cells in whole-cell configuration were voltage-clamped to a holding potential of −40 mV, which is close to their recorded resting membrane potential. In order to estimate membrane conductance, brief hyperpolarizing pulses were applied at a frequency of 0.5 Hz. In control tests ejecting normal extracellular solution, the membrane conductance varied less than 10% within the 2-min recording period after onset of ejection. Figure 1 shows representative recordings from Vero cells exposed to 10 μM PlnA and 500 μM PlnA, respectively. At a concentration of 10 μM, PlnA did not permeabilize the cell (Fig. 1a), whereas 500 μM PlnA dramatically increased the membrane conductance within seconds (Fig. 1b). After 12 s, the holding current was increased to about 2 nA, and the membrane conductance was increased from 4 to 39 nS, indicating a pronounced membrane permeabilization. A total of 46 Vero cells were exposed to PlnA at concentrations between 5 and 500 μM during voltage clamp recordings. The response to PlnA seemed to be of an all-or-nothing nature, i.e., either no response was observed, or the membrane conductance increased by several hundred percent within less than 30 s. At a concentration of 5 μM, PlnA had no permeabilizing effect ($n = 5$), while 6 of the 10 tested cells were permeabilized by 10 μM PlnA. At a concentration of 100–500 μM, PlnA permeabilized 29 of the 31 tested Vero cells.

To monitor changes in membrane potential during the PlnA-induced membrane permeabilization, we also performed current clamp recordings in the whole-cell configuration with zero holding current. Hyperpolarizing current pulses were injected at a frequency of 0.5 Hz in order to monitor the membrane resistance. Cells showing no evident response to PlnA (more than 10% reduction in membrane resistance) within 2 min after onset of exposure were defined as unresponsive to the applied peptide concentration. All responding cells started to depolarize within 30 s after onset of PlnA ejection, and the membrane resistance was dramatically reduced. Figure 2 shows typical recordings from Vero cells exposed to 10 and 500 μM

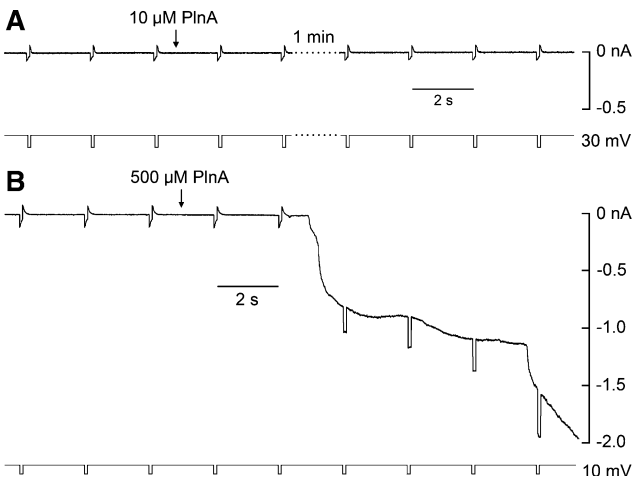

Fig. 1 Effect of plantaricin A (PlnA) on Vero cells voltage clamped in the whole-cell configuration. **a** Representative current recording (*upper trace*) from a Vero cell exposed to 10 μM PlnA from the time indicated by the *arrow*. Membrane conductance was monitored by applying hyperpolarizing voltage pulses (−30 mV, 100 ms) at 0.5 Hz from a holding potential of −40 mV (*lower trace*). At this concentration, PlnA had no visible effect on the membrane properties. **b** Representative current recording (*upper trace*) from a Vero cell exposed to 500 μM PlnA from the time indicated by the *arrow*. The other experimental conditions were similar to those in a, but the amplitude of the hyperpolarizing voltage pulses (*lower trace*) were −10 mV. PlnA induced a large inward holding current and increased the membrane conductance manyfold, indicating a dramatic permeabilization of the membrane

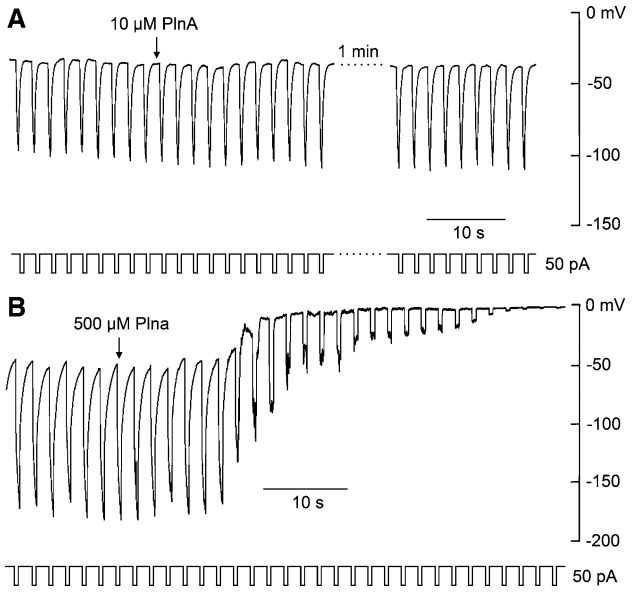

Fig. 2 Effect of plantaricin A (PlnA) on Vero cells current clamped in the whole-cell configuration. **a** Representative voltage recording (*upper trace*) from a Vero cell exposed to 10 μM PlnA from the time indicated by the *arrow*. Membrane resistance was monitored by applying hyperpolarizing current pulses (−50 pA, 500 ms) at 0.5 Hz (*lower trace*). At this concentration, PlnA had no visible effect on the membrane properties. **b** Representative voltage recording (*upper trace*) from a Vero cell exposed to 500 μM PlnA from the time indicated by the *arrow*. The other experimental conditions were identical to those in a. PlnA depolarized the cell membrane towards zero, and dramatically reduced the membrane resistance

PlnA, respectively. At a concentration of 10 μM, PlnA had no effect on the membrane potential or membrane resistance (Fig. 2a). After 13 s exposure to 500 μM PlnA, the cell started to depolarize from its resting membrane potential of about −45 mV, and the membrane potential approached 0 mV after further 10–20 s (Fig. 2b). Within 1 min after onset of ejection, the membrane resistance was reduced to less than 1% of the initial value, indicating severe membrane disruption. A total of 16 Vero cells were exposed to PlnA at concentrations between 5 and 500 μM during current clamp recordings. No cells were permeabilized by 5–10 μM PlnA ($n = 6$), while 9 of the 10 tested cells were permeabilized by 100–500 μM PlnA.

Corresponding experiments were also performed on the other cell types, and Table 1 summarizes the pooled data from all the voltage clamp and current clamp experiments in the whole cell configuration. With the exception of Kupffer cells, a major fraction of each cell type was permeabilized by 100 μM PlnA. None of the five Kupffer cells exposed to 100 μM PlnA was affected, whereas four of the five cells tested at 500 μM PlnA were permeabilized.

In the previous study of PlnA effects on clonal rat anterior pituitary cells (GH_4 cells), it was shown that the reversal potential of the PlnA-induced membrane current was close to 0 mV (Sand et al. 2007). It was also reported that that the inner leaflet of the membrane was insensitive even to 1 mM PlnA, whereas 10 μM PlnA readily permeabilized the membrane when the outer leaflet was exposed. In order to elucidate if these observations reflect general features of PlnA, we studied the effects of various concentrations of PlnA on excised membrane patches from Vero cells. None of the 3 outside-out patches exposed to 5 μM PlnA was affected by the peptide within the 5 min recording period, whereas 9 of the 12 outside-out patches exposed to 10 μM PlnA were permeabilized. At a concentration of 100 μM, PlnA permeabilized 19 of the 23 exposed patches. In contrast, none of the five inside-out patches exposed to 100 μM PlnA was affected by the peptide. At a concentration of 500 μM, all the 7 outside-out patches and the 10 inside out patches were permeabilized. Hence, also in Vero cells, the outer leaflet of the membrane is markedly more sensitive to PlnA than the inner membrane leaflet.

By tuning the holding potential of permeabilized outside-out patches to the potential giving zero membrane current, the reversal potential of the PlnA-induced conductance was estimated to -0.1 ± 2.4 mV ($n = 12$). This value is compatible with an unspecific conductance increase, and is in agreement with our previous study of clonal rat anterior pituitary cells (Sand et al. 2007).

Table 1 Summary of results from the patch clamp experiments on all cell types

Cell type	PlnA concentration				
	1 μM	5 μM	10 μM	100 μM	500 μM
Vero		7−	6+/8−	18+/2−	20+/1−
Caki-2		9−	4+/10−	16+/5−	12+/1−
Kidney cortex	5−	3+/5−	4+/1−	6+/3−	
Hepatocyte			7−	4+	
Endothelial			3−	9+	
Kupffer				5−	4+/1−

The number of plantaricin A (PlnA) exposures resulting in membrane permeabilization is marked with "+." The number of exposures with no measurable effect is marked with "−"

Effect of PlnA on Membrane Permeability Assessed by Microfluorometry

Elevation of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is a sensitive indicator of increased, unspecific membrane permeabilization due to the strong electrochemical gradient for Ca^{2+} into the cells. Therefore, the permeabilizing effect of PlnA was also studied by detecting changes of $[Ca^{2+}]_i$ in cells loaded with the fluorophore fura-2. $[Ca^{2+}]_i$ was monitored by measuring the ratio between emissions at the two excitation wavelengths (F360/F380). Because diffusional loss of fluorophore would be expected if the membrane was severely disrupted, the cytosolic concentration of fura-2 ($[fura\text{-}2]_i$) was monitored in parallel by measuring the emission at the isosbestic excitation wavelength 360 nm (F360). Figure 3 shows three representative microfluorometric recordings from different Vero cells exposed to 1, 10, and 100 μM PlnA, respectively. Each recording lasted 3 min, of which the first 1.5 min is displayed in the figure. At a concentration of 1 μM, PlnA had no effect on $[Ca^{2+}]_i$ during the recording period, indicating that the membrane remained intact. At a concentration of 10 μM, a small and transient increase in $[Ca^{2+}]_i$ was observed, but no leakage of fluorochrome occurred (Fig. 3b). In contrast, 100 μM PlnA induced a rapid and sustained increase in $[Ca^{2+}]_i$ after a delay of about 10 s, followed by loss of fluorochrome from the cell (Fig. 3c). Consequently, 100 μM PlnA caused a membrane permeabilization that not only allowed passage of inorganic ions, but also larger molecules like fura-2. A total of 84 Vero cells were exposed to varying concentrations of PlnA during microfluorometric recordings (Table 2). None of the cells was affected by 1–5 μM PlnA ($n = 20$), while 16 of the 30 tested cells were permeabilized by 10 μM PlnA. At a concentration of 100–500 μM, PlnA permeabilized 28 of the 34 tested cells. Interestingly, no intermediate responses between those represented by Fig. 3b and c were observed, confirming the all-or-nothing nature of the membrane permeabilization induced by PlnA.

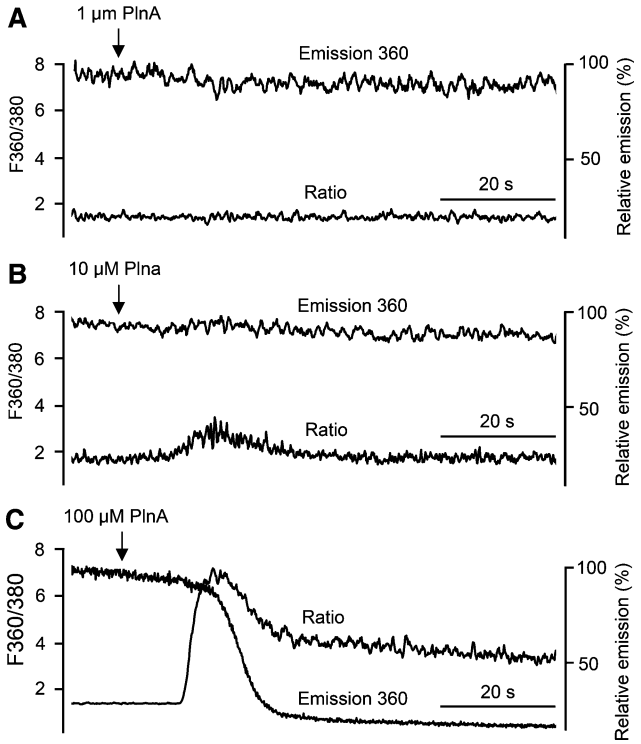

Fig. 3 Microfluorometric recordings verifying the membrane permeabilizing effect of plantaricin A (PlnA) on Vero cells. $[Ca^{2+}]_i$ was monitored in Vero cells loaded with fura-2. The ratio between emission at the two excitation wavelengths 360 and 380 nm (F360/380) reflects $[Ca^{2+}]_i$, while emission at the isosbestic excitation wavelength reflects $[fura\text{-}2]_i$. *Arrows* indicate start of continuous pressure ejection of PlnA onto the cell. **a** Representative recording demonstrating insensitivity to 1 μM PlnA. **b** Comparable recording from a cell exposed to 10 μM PlnA, which caused a brief and moderate increase of $[Ca^{2+}]_i$, but no leakage of fluorochrome. **c** Representative recording from a cell exposed to 100 μM PlnA. The cell displayed an immediate increase of $[Ca^{2+}]_i$ followed by a delayed loss of fluorochrome, indicating massive permeabilization of the membrane

Table 2 summarizes the results also for the other cell types tested. In agreement with the electrophysiological results, the microfluorometric data show that all the cell types were permeabilized by PlnA, and that the Kupffer cells were most resistant to the peptide.

Discussion

PlnA Permeabilizes Normal Liver Cells and Has No Preferential Effect on Cancerous Renal Cells

We have previously shown that normal anterior rat pituitary cells are resistant to PlnA, whereas the cancerous GH_4 cells derived from a rat anterior tumor are highly susceptible (Sand et al. 2007). The aim of the present project was to explore if the ability of PlnA to selectively permeabilize cancerous cells has general validity. Traditional methods for studying the cellular effects of antimicrobial peptides usually depend on markers for necrosis and apoptosis, or recordings of changes in transmembrane chemical gradients. Such methods have inherently poor time resolution, and possible membrane effects are measured indirectly. Therefore, in order to observe the membrane-permeabilizing effects of PlnA in more detail, we have recorded membrane conductance and $[Ca^{2+}]_i$ using patch clamp and microfluorometric techniques, respectively. Membrane conductance is a direct measure of membrane permeability, whereas elevation of $[Ca^{2+}]_i$ is an instant consequence of a general increase in membrane permeability.

Table 2 Summary of results from the microfluorometry experiments on all cell types

Cell type	PlnA concentration				
	1 μM	5 μM	10 μM	100 μM	500 μM
Vero	10−	10−	16+/14−	18+/6−	10+
Caki-2			10−	10+	
Kidney cortex	10−			8+/2−	
Hepatocyte			5−	5+	
Endothelial			5−	6+	
Kupffer				10−	10+

The number of plantaricin A (PlnA) exposures resulting in membrane permeabilization is marked with "+." The number of exposures with no measurable effect is marked with "−"

In all the normal cell types from rat liver and rat kidney, sufficiently high concentrations of PlnA induced rapid membrane permeabilization, reflected as increased membrane conductance and elevation of $[Ca^{2+}]_i$. The subsequent outward leak of fluorophore from the cells, observed in the microfluorometric experiments, indicates that the PlnA-induced membrane disruption was severe. In order to compare the susceptibility of the various cell types to PlnA, the electrophysiological and the microfluorometric data are displayed collectively in Fig. 4. With the exception of Kupffer cells, there are only minor differences in sensitivity among the normal cells studied. For each of these cell types, the major fraction of tested cells is permeabilized by 10–100 μM PlnA, whereas permeabilization of the Kupffer cells requires a PlnA concentration of 500 μM. Figure 4 also shows that the clonal renal epithelial cells of primate origin, i.e., Vero cells derived from a normal kidney and Caki-2 cells derived from a cancerous renal tumor, are definitely not more sensitive to PlnA than normal renal cells from the kidney cortex in rat. Our data thus demonstrate both that normal cells may be susceptible to PlnA, and that cancerous cells are not necessarily more sensitive to the peptide than normal cells. We have recently obtained similar results also for mammalian neuronal cells and lymphocytes (unpublished data).

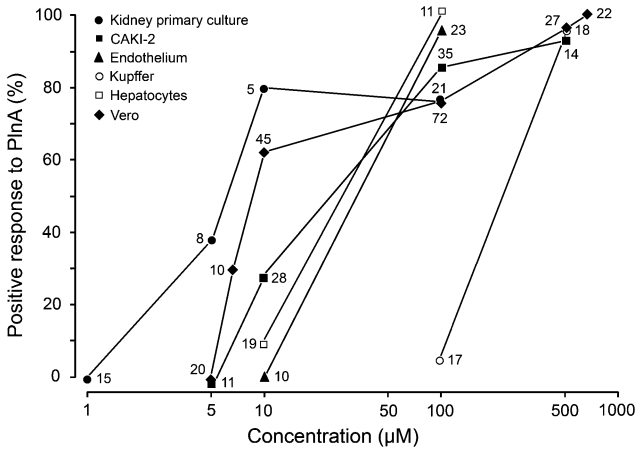

Fig. 4 Comparison of the sensitivity to plantaricin A (PlnA) for all tested cell types. Data from both the electrophysiological and the microfluorometric experiments are pooled. For each cell type and PlnA concentration, the percentage of cells that were permeabilized is plotted. The number at each data point represents the number of tested cells

PlnA Differentiates between Plasma Membranes and Membrane Leaflets

Regarding the potential use of PlnA for treatment of cancer, it is obviously disappointing that the previously reported strong selectivity of PlnA for cancer cells does not have general validity. However, the PlnA concentration required for membrane permeabilization was about 10 times higher for Kupffer cells than for the other cell types tested in the present study, and it is still intriguing that the potency of PlnA varies between different cell types. Even more striking is the observation that the outer membrane leaflet of Vero cells is markedly more sensitive to PlnA than the inner leaflet, in agreement with our previous comparison of the PlnA effects on the two membrane leaflets in GH_4 cells (Sand et al. 2007).

The distribution of phospholipids between the two leaflets of the plasma membrane is asymmetrical in all cells (for review, see Balasubramanian and Schroit 2003). Whereas bacteria keep most of their negatively charged phospholipids, particularly phosphatidylserine, in the outer membrane leaflet, the opposite is true for eukaryotic cells. Cationic antimicrobial peptides bind readily to the negatively charged outer leaflet of bacteria membranes, whereas the predominantly zwitterionic outer leaflet of normal eukaryotic cell membranes is usually not permeabilized by such peptides (Castano et al. 2005; Matsuzaki 1999; Matsuzaki et al. 1995; Shai 1999). In several types of cancer cells, the abundance of phophatidylserine in the outer leaflet of the cell membrane is somewhat increased (Rao et al. 1992; Sugimura et al. 1994; Utsugi et al. 1991; Williamson and Schlegel 1994), and this has been proposed as a possible explanation for the ability of some cationic membrane-permeabilizing peptides to preferentially affect cancer cells (for review, see Leuschner and Hansel 2004). Therefore, the finding that the inner leaflet of eukaryotic cell membranes is less sensitive to PlnA than the outer leaflet was unexpected, and indicates that factors in addition to negatively charged phospholipids may determine the permeabilizing effect of PlnA. We speculate that other negatively charged molecules associated with the outer membrane leaflet, such as lipoproteins, O-glycocylated mucines, and sialic acid, may be responsible for leading PlnA closer to the lipid membrane through electrostatic interactions, thus facilitating membrane interaction and permeabilization.

Conclusions and Future Studies

In the literature concerning antimicrobial peptides and their selectivity for cancer cells, several initial and optimistic reports were not followed up by subsequent publications. On the basis of our experience with PlnA, it is tempting to speculate that also other antimicrobial peptides may have turned out to be less specific for cancer cells than the initial studies indicated. However, we find it important to publish even such negative data, as a balance to the assumption that antimicrobial peptides able to permeabilize eukaryotic cells exhibit a general selectivity for cancer cells.

Although PlnA is not selective for cancer cells, it is still an interesting peptide, and its mode of action should be explored in more detail. The lack of distinct conductance steps in the voltage clamp recordings suggests that the peptide molecules probably do not assemble to form membrane channels, but rather permeabilize the membrane by a detergent-like action. The steep dose–response curves and the all-or-nothing nature of its permeabilizing effect indicate that PlnA must reach a threshold concentration in the cell membrane before a full-fledged response is elicited. The ability of PlnA to differentiate between cell membranes and membrane leaflets was confirmed in the present study, and the mechanism for this selectivity should be investigated in future experiments. A reasonable approach would be to study how modifications of the negatively charged macromolecules associated with the outer membrane leaflet affect the actions of PlnA. It should also be explored whether molecular modifications of PlnA may enhance its ability to differentiate between cell membranes.

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