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EXPERIMENTAL ARTICLES

The Dependence of the Antibacterial Effect of the Polycationic Peptide Warnerin on the Energy State of Target Cells

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Abstract—The bactericidal effect of the polycationic peptide warnerin, produced by *Staphylococcus warneri* IEGM KL-1, was found to depend on the energy state of susceptible *Staphylococcus epidermidis* cells. The pretreatment of these cells with compounds that diminish the proton-motive force of plasma membranes enhanced cell tolerance to warnerin. The components $\Delta\Psi$ and ΔpH of the membrane proton potential influenced the antibacterial activity of warnerin in different ways. In particular, the antibacterial activity of warnerin decreased when the electric component of the proton-motive force of target membranes declined.

Key words: *Staphylococcus*, membrane potential, cationic peptides, antibacterial effect.

The antibacterial polycationic peptides produced by some gram-positive and gram-negative bacteria belong to a numerous group of low-molecular-weight bacteriocins with molecular masses less than 10 kDa. Most bacteriocins contain cysteine residues, which are able to form disulfide bridges. Some low-molecular-weight bacterial peptides contain uncommon amino acid residues, which are responsible for the unique molecular properties of these peptides [1]. In particular, some gram-positive bacteria synthesize peptides, known as lantibiotics, that contain didehydroamino acids and the thioether amino acids lanthionine and 3-methyllanthionine [2]. Many antibacterial peptides contain hydrophobic amino acid residues. The small amount of neutral and negatively charged amino acid residues and the large amount of positively charged amino acid residues give rise to a net positive charge of these peptides, while the clusterization of nonpolar residues in their hydrophobic domain and cationic groups in the charged zone make them amphipathic [3].

The primary target of most polycationic peptides is cytoplasmic membrane. Attacking the membrane with these peptides results in the formation of transmembrane pores and the release of intracellular ions and low-molecular-weight metabolites into the medium [4]. The pore-forming ability of some cationic peptides and, hence, their efficiency were found to depend on the transmembrane potential of target cells [5, 6].

The aim of this work was to study the role of the energy state of target cells in the antibacterial efficiency of the low-molecular-weight extracellular cationic peptide produced by the rare species *Staphylococcus warneri* grown on nutrient-rich media [7].

MATERIALS AND METHODS

The peptide was isolated from the strain *Staphylococcus warneri* IEGM KL-1 [8]. The strain was grown at 37°C in 250-ml flasks with 50 ml of LB medium on a UVMT-12-250 shaker (180 rpm) to the late exponential growth phase. The culture was centrifuged at 3000 *g* for 30 min. The supernatant was subjected to ultrafiltration [7] and concentrated by using a Centriplus YM-3 system (Millipore, United States). The concentrate was applied onto a column with Heparin-agarose (Type 1, Sigma), which was equilibrated with 10 mM potassium phosphate buffer (pH 7.2). The column was washed with ten volumes (with respect to the volume of the applied preparation) of the equilibrating buffer. The peptide was eluted with a gradient of 0–0.5 M NaCl in the same buffer. Fractions with antibacterial activity were pooled and desalted on a PD-10 column (Amersham Biosciences). The desalted preparation was lyophilized and stored at –18°C until further use.

The peptide was analyzed by mass spectrometry in a Voyager-DE STR Biospectrometry Workstation (Pepseptive Biosystems).

The antibacterial activity of the peptide was studied with the test culture *Staphylococcus epidermidis* 33, which was obtained from the Tarasevich State Institute of Standardization and Control of Medical and Biological Preparations, Moscow, Russia. The test culture was

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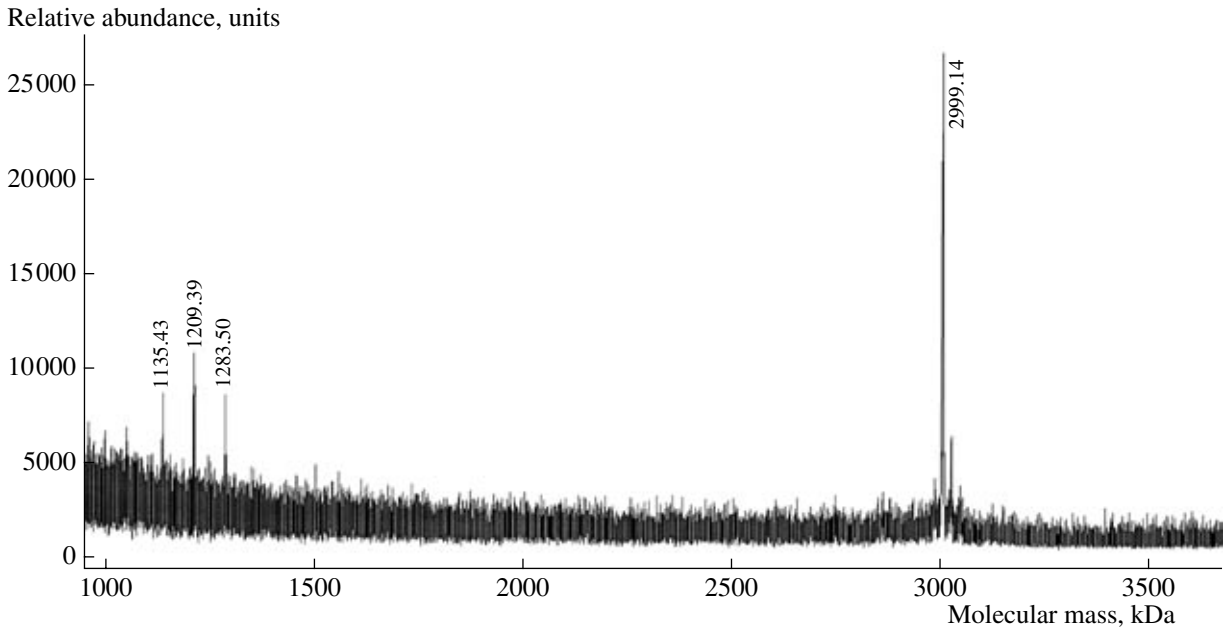

Fig. 1. Mass spectrometry of the antibacterial peptide produced by *S. warneri* IEGM KL-1.

grown in a liquid LB medium under the same conditions as in the case of the producing strain. The test cells were harvested by centrifugation at 6000 *g* for 5 min, washed twice, and resuspended in 0.14 M NaCl to a concentration of (1.5–2) × 10^8 cells/ml. The minimal inhibitory concentration (MIC) of the peptide was determined by the method of twofold dilutions on immunological plates [9]. The peptide was quantified by comparing the MICs of the standard and investigated peptide preparations. The number of colony-forming units (CFUs) was determined by plating the tenfold serial dilutions of bacterial cultures on plates with 1.4% LB agar. Before colony counting, the plates were incubated at 37°C for 1–2 days.

In experiments with compounds that diminish the membrane potential of cells, NaCl in LB medium was replaced with 0.64% KCl. In addition, the medium was supplemented with one of the ionophores valinomycin, nigericin, pentachlorophenol, or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at subbactericidal concentrations (5, 50, 50, and 200 μM, respectively). These ionophore concentrations were chosen such that the initial cell concentration (2 × 10^6 CFU/ml) decreased by no more than 40% after 30 min of incubation at 37°C. After incubation, bacterial cells were collected by centrifugation at 6000 *g* for 5 min, washed in 0.14 M NaCl, and resuspended in fresh medium to a concentration of 2 × 10^5 CFU/ml. The suspension was dispensed into the plate wells containing the twofold dilutions of the peptide in LB medium. The final concentration of bacterial cells in the wells was 2 × 10^4 CFU/ml. The plates were incubated at 37°C for 60 min. Then the contents of the wells were plated, in 5-μl aliquots, onto LB agar to determine the number of surviving bacterial cells [10].

All the experiments were performed at least in triplicate. The results were processed with the aid of the Statistica for Windows program by using Student's *t*-test. The error bars in Fig. 2 show the standard deviations.

RESULTS AND DISCUSSION

Our earlier studies showed that the species *S. warneri* produces an antibacterial low-molecular-weight peptide factor [7]. The use of the additional ultrafiltration step allowed us to obtain a preparation containing a biologically active compound with a molecular mass of below 3 kDa. Two further chromatographic steps yielded an apparently homogeneous peptide with a molecular mass of 2999 kDa, as estimated by mass spectrometry (Fig. 1).

The high degree of purification on Heparin-agarose suggests that the peptide produced by *S. warneri* IEGM KL-1 possesses cationic properties. A comparison of the molecular mass of this peptide with those of the known lantibiotics [11] showed that it may be considered a new member of the family of cationic antibacterial peptides. By analogy with other compounds of such type, the peptide produced by *S. warneri* was called warnerin.

It is known that the cationic properties of peptides largely determine the first stage of their interaction with bacterial targets, namely, the stage of their sorption on the charged surface of the cell wall. The further advance of the cationic peptides into the bacterial cell and their interaction with the cytoplasmic membrane consider-

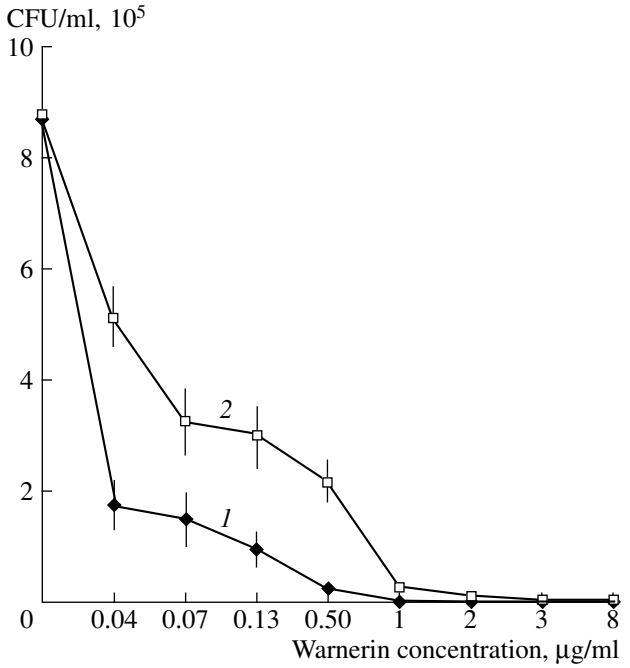

Fig. 2. The antibacterial effect of warnerin on (*1*) exponential-phase and (*2*) stationary-phase *S. epidermidis* cells.

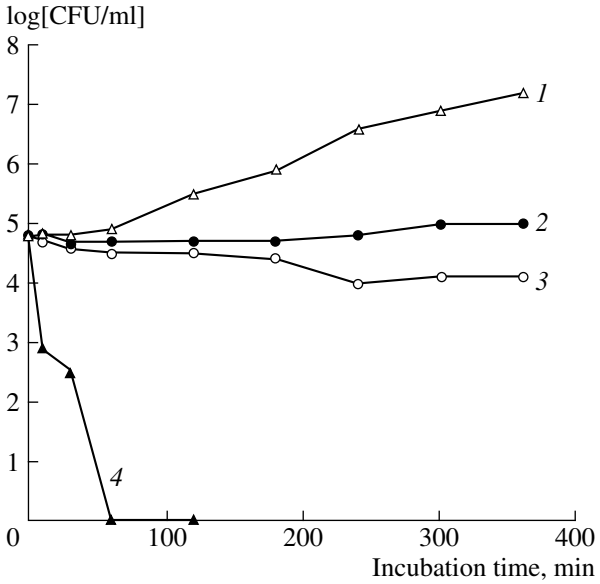

Fig. 3. The effect of incubation temperature on antibacterial activity of 4 μg/ml warnerin: (*1*) incubation at 37°C without warnerin, (*2*) incubation at 4°C without warnerin, (*3*) incubation at 4°C with warnerin, and (*4*) incubation at 37°C with warnerin.

ably depend on the electric component of the proton potential of the membrane, Δψ, which is an integral indicator of the intensity of energy processes in the cell [6].

The role of the energy state of target bacterial cells in the bactericidal activity of warnerin was studied in experiments of two types. The first type of experiment was carried out with bacterial cells with different levels of physiological activity (logarithmic-phase cells, stationary-phase cells, and cells whose metabolism was diminished by incubation at low temperatures).

As is evident from Fig. 2, the bactericidal effect of the peptide taken at low concentrations was maximal with respect to the exponential-phase bacterial cells, which are distinguished by intense energy processes. For instance, the bactericidal effect of the peptide at a concentration of 0.04 μg/ml (six times below MIC) was three times more efficient with respect to the exponential-phase cells than with respect to the stationary-phase cells. At the same time, peptide concentrations higher than MIC showed close bactericidal activities with respect to these two types of bacterial cells.

Similar data were obtained by Koo *et al.*, who found that stationary-phase *S. aureus* 502A cells were 25 times more resistant to the thrombocyte cationic peptide tPMP than exponential-phase cells [12]. The lower peptide susceptibility of stationary-phase bacterial cells may be related to a decrease in the intensity of energy processes in the stationary growth phase. It is known that the proton potential of bacterial cells, Δρ, decreases in the stationary growth phase due to a decline in the potential Δψ, which strongly depends on the growth rate of cells [13, 14]. The decrease in Δψ may considerably diminish the sorption of positively charged peptide molecules on the target bacterial cells, thereby reducing the antibacterial activity of the peptide.

It was found that there is a threshold level of the membrane potential of bacterial cells for the antibacterial activity of peptides [4–6]. For instance, a decrease in the Δψ of menadione-auxotrophic respiratory mutant cells of *S. aureus* 6850 from –143 to –97 mV led to a considerable increase in the tolerance of the mutant cells to antibacterial peptides [14]. Sahl *et al.* showed that a value of Δψ no less than –80 mV is a necessary condition for the bactericidal effect of the cationic peptide nisin [16].

The dependence of the antibacterial effect of warnerin on the intensity of energy processes in the target bacterial cells was clearly demonstrated in low-temperature experiments (Fig. 3). During the incubation of *S. epidermidis* cells at 37°C, their deaths were observed as soon as 60 min after the addition of the peptide to the medium, whereas the same concentration of warnerin induced only a slight decrease in the number of viable bacterial cells even after several hours of incubation at 4°C. Similar results were obtained with two other staphylococcal strains, *S. aureus* 502A [12] and *S. aureus* 209P [17].

Similar changes in the warnerin susceptibility of *S. epidermidis* cells whose energy state was modified in different ways suggest that the antibacterial efficiency of warnerin may depend on the Δρ of the target cells. This suggestion was confirmed in experiments with ionophores that differ in the mechanism of action on the proton potential of membranes.

As is evident from Fig. 4, the preincubation of the target bacterial cells with nigericin diminished the antibacterial effect of warnerin, but only slightly (by about two times). This was likely related to a small decrease in $\Delta\rho$ due to the H^+/K^+ antiport and, to a lesser degree, the transport of Na^+ ions, which promoted the active import of protons inside the bacterial cells until the osmotic component ΔpH of the membrane proton potential was completely eliminated [18]. At external pH values close to neutral, ΔpH in *S. aureus* cells comprises about one-third of the proton-motive force [19]. For this reason, the aforementioned elimination of the transmembrane pH gradient does not play an important role in the antibacterial efficiency of warnerin. Under these conditions, the negative charge of the inner membrane surface, which is necessary for the peptide to bind to the membrane, may be provided by $\Delta\psi$, due to which warnerin would be able to partially retain its antibacterial activity.

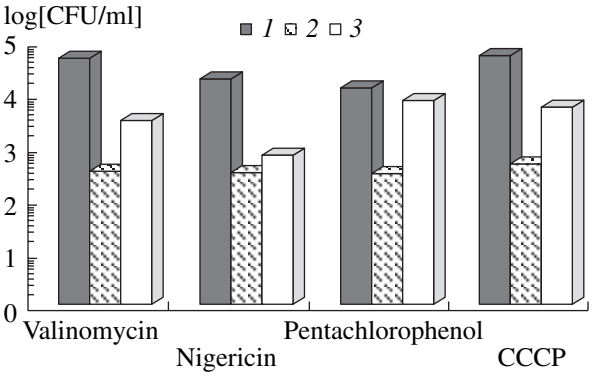

Fig. 4. The effect of pretreatment of *S. epidermidis* cells with ionophores on the antibacterial activity of warnerin: (*1*) cells incubated with ionophore, (*2*) cells incubated with warnerin, and (*3*) cells pretreated with ionophore and incubated with warnerin.

This suggestion was confirmed by experiments with valinomycin, which considerably decreased the susceptibility of bacterial cells to warnerin (Fig. 4). At a warnerin concentration of 2 μg/ml, valinomycin increased the number of surviving target cells by about nine times, which can be explained by the high activity of valinomycin as a selective transmembrane carrier of K^+ ions. If ΔpH exists on the membrane, valinomycin is able to carry K^+ ions inside the cell even when an equilibrium is attained (i.e., against the concentration gradient), due to which $\Delta\psi$ rapidly falls against the background of the nearly constant transmembrane pH gradient [18].

It is known that the membrane apparatus of bacterial cells is highly susceptible to valinomycin [20]. $\Delta\psi$ tends to drop as soon as valinomycin begins to exert its action on the *S. epidermidis* cells that oxidize glucose or endogenous substrates [21]. Valinomycin-induced elimination of $\Delta\psi$ was found to prevent consumption of cationic compounds by *S. aureus* 86W cells [6]. An analysis of the effect of valinomycin on the energy metabolism of staphylococci showed that $\Delta\psi$ may considerably determine the electrostatic migration of warnerin to the cytoplasmic membrane from the sites of its primary attachment to the cell wall and, hence, may play an important role in the implementation of the biological activity of warnerin.

Investigations showed that CCCP and pentachlorophenol are the most efficient antagonists of warnerin, as is evident from the fact that pretreatment of the target bacterial cells with these uncouplers increased the number of surviving cells by 12 and 20 times, respectively (Fig. 4). CCCP and pentachlorophenol are weak acids (pK_a 6.0 and 4.89, respectively) that possess high mobility in the lipid zone of the membrane. Being protonated under acidic conditions (outside the cell) and deprotonated under alkaline conditions (inside the cell), these uncouplers equalize pH on either side of the cell membrane, causing its complete deenergization and inhibiting almost all energy processes in the bacterial cell, except for the substrate phosphorylation [20]. At concentrations higher than 5 μM, pentachlorophenol is a potent inhibitor of electron transport via the respiratory chain [22]. Consequently, the observed protective effect of the uncouplers may be related to the complete dissipation of $\Delta\rho$, which substantially restricts the interaction of warnerin with the membrane structures of the target bacterial cells and thus increases their survival in the presence of warnerin.

To conclude, the antibacterial effect of warnerin depends on the energy state of the target bacterial cells. The electric component $\Delta\psi$ of the electrochemical transmembrane potential of hydrogen ions influences the antibacterial efficiency of warnerin to a greater degree than the osmotic component ΔpH.

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