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Potassium Efflux Induced by a New Lactoferrin-Derived Peptide Mimicking the Effect of Native Human Lactoferrin on the Bacterial Cytoplasmic Membrane

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Abstract—A 31-amino acid synthetic peptide (NH_2-FFSASCVPGADKGQFPNLCRLCAGTGENKCA-COOH) was chemically synthesized based on the amino acid sequence of a region of human lactoferrin homologous to other sequences present in the N- and C-lobes of all members of the transferrin family proteins. The peptide, termed kaliocin-1, and lactoferrin showed a bactericidal effect in assays performed in low-ionic-strength conditions. This is the first time that it is shown that the antimicrobial effect of lactoferrin depends on the extracellular cation concentration. The antimicrobial effect of kaliocin-1 was lower than that of human lactoferrin, but their activities were inhibited by Na^+ or K^+ in a cation concentration-dependent manner. In addition, the peptide was able to mimic native lactoferrin, inducing K^+-efflux and a selective dissipation of the transmembrane electrical potential of *Escherichia coli* cells without causing extensive damage to the outer and inner bacterial membranes. In contrast, the peptide, but not lactoferrin, was able to permeabilize different ions through liposomal membranes. The hypothetical interaction of kaliocin-1 with a bacterial membrane compound is discussed based in the different ion flux induced on cellular and artificial membranes as well as data from circular dichroism assays. Kaliocin-1 was not cytotoxic and could be a suitable model for the design of analogs able to mimic the antibacterial effect of human lactoferrin.

Key words: antimicrobial peptide, lactoferrin-derived peptide, lactoferrin, transferrin, lactoferricin, kaliocin, electrical potential, membrane permeabilization

Lactoferrin is a 77-kD glycoprotein belonging to the transferrin family proteins consisting of a 691-residue single polypeptide chain with N-linked oligosaccharides [1-3]. This protein is the most abundant iron-binding protein in mammalian mucosal fluids and is synthesized by neutrophilic leukocytes and acinar cells [4, 5]. Lactoferrin has pleiotropic functions on immune and non-immune cells and is often included as a component of the first-line of host defense against microbial invaders (reviewed in [6, 7]). However, despite the early known antimicrobial activity of this protein [8-10] and its relationship with phagocytic cells [11], the antimicrobial mechanism of action is poorly understood. The antimicrobial effect of lactoferrin has been related to its iron-chelating ability (reviewed in [12]) and to a direct effect on the bacterial surface [13, 14]. It is thought that bacterial surface damage induced by lactoferrin is due to a domain of the molecule that was first obtained by the pepsin-induced cleavage of the whole protein. This cationic sequence was designated lactoferricin (Lfcin), and it displayed a poor similarity with the corresponding domain of other transferrin family members but exhibited a higher antimicrobial activity than the com-

Abbreviations: hLf) human lactoferrin; hMf) human melanotransferrin; Of) ovotransferrin; hTf) human transferrin; Lfcin) lactoferricin; Lfpep) lactoferricin-derived peptide; BCECF) 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein; DiS-C_3-(5)) 3,3′-dipropylthiocarbocyanine; NPN) 1-*N*-phenylnaphthylamine; ONPG) *o*-nitrophenyl-β-D-galactopyranoside; PI) propidium iodide; pyranine) 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; TFE) trifluoroethanol; K-PIPES) potassium piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); PBS) phosphate-buffered saline; PPB) potassium phosphate buffer; BHI) brain heart infusion; MIC) minimal inhibitory concentration; OM) outer membrane; IM) inner membrane.

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plete lactoferrin molecule [15]. The human Lfcin peptide includes residues 1-47 of human lactoferrin (hLf) [15], but shorter human Lfcin-derived synthetic peptides (11 and 16 residues) retain a similar bactericidal effect [16, 17] causing a generalized permeabilization of the bacterial membrane [18-20].

The differences observed in the mode of action, antibacterial effect, and antimicrobial spectrum of lactoferrin and lactoferricin are compatible with different antimicrobial mechanisms of action for these molecules. This hypothesis was supported by recent evidence from our laboratory suggesting the concept that the antimicrobial activity of representative members of the transferrin family such as hLf, human transferrin (hTf), and ovotransferrin (Of) on *Escherichia coli* cells could be related to their ability to induce a K^+-efflux with selective dissipation of the transmembrane electrical potential ($\Delta\psi$) [21]. In contrast, we also demonstrated that a Lfcin-derived peptide (Lfpep; residues 18-40 of hLf) was able to dissipate the $\Delta\psi$ and the proton gradient (ΔpH) [20]. Electrical and proton gradients are the components of the protonmotive force (Δp), according to the chemiosmotic hypothesis of Mitchell [22] expressed in the abbreviated equation [23]: $\Delta p = \Delta\psi - 59\Delta\text{pH}$ at 20°C. The Δp energizes, either directly or indirectly, a wide variety of metabolic processes, including ATP synthesis, solute uptake, and active transport systems. Consequently, the loss of Δp induced by Lfpep on *E. coli* led to cell death [20].

The different effect on the bacterial cytoplasmic membrane exerted by lactoferrin and Lfpep prompt us to search for new sequences from the hLf molecule with antimicrobial activity. The comparison of the amino acid sequence of the transferrin family proteins revealed a common cysteine-motif (residues 153-183) on the molecule surface. A 31-residues peptide based in the deduced sequence, referred to here as kaliocin-1, was chemically synthesized and the antimicrobial activity and the effects on the *E. coli* cells and artificial membranes were analyzed. Interestingly, similar effects of kaliocin-1 and hLf on the bacterial cytoplasmic membrane were observed. Kaliocin-1 induced a K^+-efflux and selective dissipation of the electrical potential of *E. coli* cells in a similar way to the whole human lactoferrin molecule. In this study the native hLf and the cationic Lfcin-derived synthetic peptide Lfpep were included in control assays to compare their effects with that of the kaliocin-1 peptide.

MATERIALS AND METHODS

Materials. The iron non-saturated (apo-) forms of hLf, hTf, and Of were obtained from Sigma (USA). Purity of the proteins was assessed as described [24]. Total phospholipids of *E. coli* were obtained from Avanti Polar Lipids (USA). BCECF, DiS-C_3-(5), PI, and pyranine were purchased from Molecular Probes (USA). Gramicidin S, nigericin, nystatin, valinomycin, NPN, ONPG, and Dulbecco's modified Eagle's media were supplied by Sigma. Bactopeptone and BHI were purchased from Difco Laboratories (USA). All other materials were of reagent grade and were obtained from commercial sources.

Bacterial and eukaryotic cells. The strains included clinical isolates of *Morganella morganii*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Streptococcus mitis* (obtained from Central Hospital of Asturias, Oviedo), and *Kocuria rosea* ATCC 186 (formerly *Micrococcus roseus*). *E. coli* ML-35, a lactose permease-deficient strain with constitutive cytoplasmic β-galactosidase activity (*lac*I and *lac*Y mutations, *lac*Z^+) was kindly provided by Dr. R. Gennaro (University of Trieste, Italy). Human lymphocytes were isolated from the blood by centrifugation in a Lymphoprep (Nycomed Pharma AS, Norway) gradient at 400*g* for 30 min. Rat anterior pituitary GH3 cells (ATCC CCL-82.1) were obtained from Dr. F. Barros (University of Oviedo).

Sequence analysis. Similarities in the primary structure of representative members of the transferrin family proteins were determined using the program BLASTP 2.1.2 [25]. Sequence alignments were optimized using the MultAlin (v. 5.4.1) software [26].

Peptide synthesis. The peptide kaliocin-1 (NH_2-FFSASCVPGADKGQFPNLCRLCAGTGENKCA-COOH) and the lactoferricin-derived peptide (NH_2-TKCFQWQRNMRKVRGPPVSCIKR-COOH) termed Lfpep [27] were synthesized according to the Fmoc chemistry by Bethyl Laboratories (USA). Automated stepwise assembly was carried out on a Symphony peptide synthesizer (Protein Technologies, USA). The peptide kaliocin-1 was taken up in 0.1 M ammonium acetate buffer (pH 8.5) at a concentration of 75 μg/ml and was allowed to refold by air oxidation for 18 h at room temperature. The refolded peptide was purified by RP-HPLC (Aquapore RP300 column, 250 × 7 mm) with a linear gradient of 6-14% acetonitrile in 0.05% trifluoroacetic acid for 40 min at a flow rate of 1.5 ml/min. Composition and concentration of purified peptide was verified by mass spectrometry and amino acid analysis.

Antimicrobial assays. Determination of the growth inhibitory concentration was performed in sterile 96-well microtiter plates using a broth dilution method [28]. The incubation mixtures contained $1 \cdot 10^5$ colony forming units (CFU) per ml in 0.3% Bactopeptone and kaliocin-1 ranging from 0.016 to 200 μM, Lfpep (0.016-90 μM), hLf (0.2-50 μM), hTf (0.2-150 μM), or Of (0.2-100 μM) in a final volume of 100 μl. The plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of peptide or protein that completely inhibited visible growth after 24 h.

The bactericidal effect of inhibitory growth concentration of kaliocin-1 and hLf was monitored against *E. coli* and *K. rosea* as described [29]. An overnight culture

was diluted in fresh BHI and allowed to grow to logarithmic phase (A_{600} = 0.6), harvested by centrifugation, and washed twice in 5 mM PPB (pH 7.0). Then the bacterial suspension was diluted in the same buffer to obtain $1 \cdot 10^5$ CFU/ml. After the addition of an inhibitory concentration of kaliocin-1 or hLf to the cell suspension, aliquots were taken at intervals and plated in duplicate on BHI-agar. The plates were incubated at 37°C for 24 h and the grown colonies were counted. Cell survival was expressed as percentage of control and loss of viability was calculated as [1 – (colonies from kaliocin-1- or hLf-treated cells/colonies from control cells)] × 100%.

The influence of cations on the bactericidal activity of the synthetic peptide and hLf on *E. coli* cells was assessed as described above but the bacterial suspensions were prepared using different concentrations of sodium or potassium phosphate buffer (pH 7.0). The cell suspensions were incubated with kaliocin-1 or hLf at 37°C for 24 h. Then, aliquots were plated in duplicate onto BHI-agar to obtain a viable count.

Cell permeabilization assays. The outer membrane permeabilization activity on *E. coli* ML-35 was determined by the NPN assay [30]. Normally, NPN is excluded from Gram-negative bacteria, and its quantum yield increases when it is transferred from a hydrophilic to a hydrophobic environment. Logarithmic-phase bacteria were resuspended in buffer (5 mM Hepes, pH 7.2) and adjusted to $1 \cdot 10^6$ CFU/ml. Concentrations ranging from 75 to 175 μM of kaliocin-1, hLf (6-35 μM), or Lfpep (0.2-50 μM) were added to cell suspensions (1 ml) containing 10 μM NPN. Fluorescent changes were measured using a LS-50 spectrofluorometer (Perkin Elmer, USA) at excitation and emission wavelengths of 350 and 420 nm, respectively.

The inner membrane permeabilization of *E. coli* ML-35 was determined by measurement of the β-galactosidase activity using ONPG as substrate [31]. In this mutant strain extracellular ONPG is not accessible to the cytoplasmic β-galactosidase. The cleavage of ONPG is thus an indication of increased IM-permeabilization. Exponentially grown cells of *E. coli* were washed in 5 mM PPB (pH 7.0) and adjusted to $1 \cdot 10^6$ CFU/ml in the same buffer containing 1.5 mM ONPG. Several concentration ranges of kaliocin-1 (75-175 μM), hLf (6-35 μM), or Lfpep (50 μM) were assayed, and the ONPG-hydrolysis with production of *o*-nitrophenol was monitored spectrophotometrically at 420 nm. To determine the maximal rate of hydrolysis, the cells were ultrasonicated (Soniprep 150, U.K.) in an ice bath at 4 A for 30 sec cycles, and the β-galactosidase activity of the cell extract was tested.

Extracellular ion measurements. The cation efflux assays were performed in iso-osmotic conditions as previously described [32]. Cell suspensions of *E. coli* (A_{600} = 2.0) were incubated with kaliocin-1 (175 μM), hLf (25 μM), or Lfpep (50 μM). Samples (1 ml) were taken at intervals and centrifuged for 10 min at 4°C and 8,000*g*. The supernatant was immediately removed and the Na^+ or K^+ concentrations were quantified by atomic emission spectrometry (UNICAM 929, U.K.). The cation concentration was also determined in samples of non-treated cells and in protein or peptide solutions without cells.

Measurement of electrical potential and cytoplasmic pH in cells. The transmembrane electrical potential ($\Delta\psi$) of *E. coli* was monitored by the quenching of the potential-sensitive fluorescent probe DiS-C_3-(5) as described [33]. Cells were suspended (A_{600} = 0.05) in buffer (100 mM potassium phosphate, 20 mM glucose, and 5 mM Hepes, pH 7.0) and ranges of concentrations of kaliocin-1 (10-150 μM), hLf (3-25 μM), or Lfpep (10-25 μM) were tested. Fluorescent changes of DiS-C_3-(5) were measured using a spectrofluorometer at excitation and emission wavelengths of 643 and 666 nm, respectively. When required, cell permeabilization to the specific K^+-ionophore valinomycin was performed with 1 mM EDTA [34].

Changes in the intracellular pH of *E. coli* were determined by monitoring the fluorescence of the pH indicator BCECF as described [35]. The cells ($1 \cdot 10^6$ CFU/ml) were loaded with the probe by an acid shock treatment (50 mM HCl for 6 min). The fluorescence intensity was monitored at excitation and emission wavelengths of 502 and 525 nm, respectively. In control assays, Lfpep (50 μM) and the natural antimicrobial peptide gramicidin S (40 μM) were used as membrane permeabilizing agents.

Preparation of liposomes. Single bilayer *E. coli* phospholipid vesicles were prepared using total phospholipids from *E. coli* that were mixed in chloroform–methanol (9 : 1) and dried under a stream of N_2 gas [36]. Dried phospholipids were suspended in different buffers. For measurement of the $\Delta\psi$ and circular dichroism assays, the phospholipids were suspended in 50 mM PPB (pH 6.0). For determination of ΔpH changes, pyranine-containing liposomes were prepared as described [37] using a pyranine buffer solution (100 μM pyranine, 20 mM potassium phosphate, 100 mM potassium acetate, pH 6.0). The phospholipid suspensions were dispersed by sonication to obtain unilamellar vesicles. Strictly isotonic conditions were ensured by checking the osmotic pressure of intra- and extravesicular solutions in an osmometer (model 3300; Advanced Instruments, USA). Preparation of pyranine-containing liposomes included the separation of the non-encapsulated fluorescent indicator from the vesicle suspension by passage through a Sephadex G-50 gel filtration (Pharmacia, Sweden) column of 25 × 0.5 cm and eluted with buffer containing 20 mM potassium phosphate, 100 mM K-PIPES (pH 7.5). Phospholipid concentrations were determined by inorganic phosphorus analysis [38].

Measurement of electrical potential and pH gradient in liposomes. Changes in the transmembrane electrical potential of liposomes prepared from *E. coli* phospho-

lipids were monitored as described previously [39]. The reaction mixture (1 ml) contained 100-times diluted liposomes in a 50 mM sodium phosphate buffer (pH 6.0) and 1 μM of the fluorescent potential-sensitive indicator DiS-C_3-(5). A transmembrane electrical potential (negative inside) was generated in liposomal membranes by adding valinomycin (1 μM, final concentration). The fluorescence quenching of DiS-C_3-(5) in liposomes, treated and untreated with kaliocin-1 (0.02, 0.2, 2, 5, 10, 20, and 30 μM), hLf (0.25, 0.5, 1, 5, 10, and 25 μM), and Lfpep (0.1, 0.2, 0.5, 2, and 4 μM) was monitored. Nigericin (1 μM) was used as control of $\Delta\psi$ dissipation [39]. Experiments were performed at 25°C at wavelength of excitation and emission of 616 and 676 nm, respectively.

The internal pH of liposomes was monitored from the fluorescence of pyranine entrapped within the liposomes [39]. Liposomes were previously diluted 100-times in buffer (20 mM potassium phosphate, 100 mM K-PIPES, pH 7.5) for the generation of a pH gradient. Fluorescence of pyranine-containing liposomes treated or untreated with the above peptide or hLf concentrations was monitored at wavelength of excitation and emission of 450 and 508 nm, respectively. The initial ΔpH was estimated as 1.5 units using 1 μM nigericin.

Circular dichroism spectroscopy. Circular dichroism (CD) spectra of kaliocin-1 in 50 mM potassium phosphate buffer (pH 7.2), in the presence or absence of liposomes, or water–trifluoroethanol mixtures were obtained on a Jasco-715 spectropolarimeter fitted with a 150 W xenon lamp [40]. Quartz cells with a 1 mm path length were used, and the spectra were recorded in the far-UV region (195-250 nm) with a scanning speed of 50 nm/min, temperature 25°C. Four scans were accumulated and averaged for each spectrum. The acquired spectra were corrected by subtracting the appropriate blanks, subjected to noise-reduction analysis, and represented in terms of mean residue ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$).

Cytotoxic activity assays. Hemolytic assays were performed in a microtiter plate using human, mouse, or rabbit erythrocytes. The cells were washed three times with buffer (150 mM NaCl, 1 mM EDTA, 100 mM Hepes, pH 7.4), and then 10 μl of serial dilutions of the kaliocin-1 (6 to 150 μM) were added to the erythrocyte suspension (100 μl). As a positive control (100% lysis), a solution of 0.5% Triton X-100 was used. After incubation (4 h, 37°C), the plate was centrifuged and the absorbance of the supernatant was measured.

Permeabilization of human lymphocytes and rat GH3 cells induced by kaliocin-1 was determined by flow cytometry. The cells ($1 \cdot 10^6$) were incubated for 30 min with the DNA-staining fluorescent probe propidium iodide (5 μM, final concentration) in 350 μl of Dulbecco's modified Eagle's medium in the presence or absence of the peptide, washed with PBS (pH 7.2), and subsequently analyzed by flow cytometry. The fluorescence was recorded using a Cytoron Absolute (Ortho Diagnostics Systems Inc., USA) cytometer.

The GH3 cells were cultured and patch-clamped as usually [41] using an Axopatch-AD (Axon Instruments, USA) patch-clamp amplifier. The chamber was continuously perfused at 1 ml/min using an extracellular-like solution (140 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose, 5 mM $CaCl_2$, and 10 mM Hepes, pH 7.3). The electrodes (1-5 MΩ) were made from disposable micropipettes (Drummond Scientific, USA). The micropipette was backfilled with the pipette solution (10 mM NaCl, 65 mM KCl, 35 mM K_2SO_4, 1 mM $MgCl_2$, and 10 mM Hepes, pH 7.3) containing nystatin (0.25 mg/ml). Currents were filtered at 1 kHz and recorded onto videocassette tapes with a VR10 digital encoder (Instrutech Corp., USA), and replayed for illustration on a PC with a TL-1 interface (Axon Instruments). The records analysis and plots were performed using pCLAMP 6.0 software (Axon Instruments) and SigmaPlot v. 4.01, respectively.

RESULTS

Analysis of amino acid sequence similarity. The Cys-motif of the transferrin family proteins is defined by the position of two pairs of disulfide-linked cysteines that are strictly conserved. Homologous Cys-motifs are also present in the C-terminal domain of all transferrin family members (i.e., residues from 489 to 519 of hLf). Notably, the Cys-motif of the N-terminal domain of transferrin includes a cluster of three bridges joined by short connecting loops. The alignment of the amino acid sequence of the Cys-motif of representative members of the transferrin family is shown in Fig. 1. The sequence of the synthetic peptide kaliocin-1 (residues from 153 to 183) includes the amino acid sequence of the Cys-motif (Cys158–Cys174, Cys171–Cys182) of the N-terminal region of hLf.

Antimicrobial activity. The MICs of kaliocin-1 compared to that of hLf, hTf, Of, and Lfpep for different bacterial species are summarized in the table. Growth inhibition of the bacterial species by kaliocin-1 and hLf was observed only in assays performed in 0.3% Bactopeptone. Among the Gram-negative bacteria tested, the strain most sensitive to kaliocin-1 was *P. aeruginosa* (MIC = 75 μM), whereas *S. typhimurium* and *M. morganii* were only inhibited by the maximal concentrations assayed of kaliocin-1 (200 μM) and were resistant to hLf (>50 μM). In a similar way, hTf and Of inhibited the growth of the susceptible strains in the concentration range between 9 to 100 μM, and Of was the protein with lowest antimicrobial activity. According to previous reports [15, 19, 20] the bactericidal peptide Lfpep was also able to cause growth inhibition of all bacterial strains except *M. morganii* and *S. mitis*.

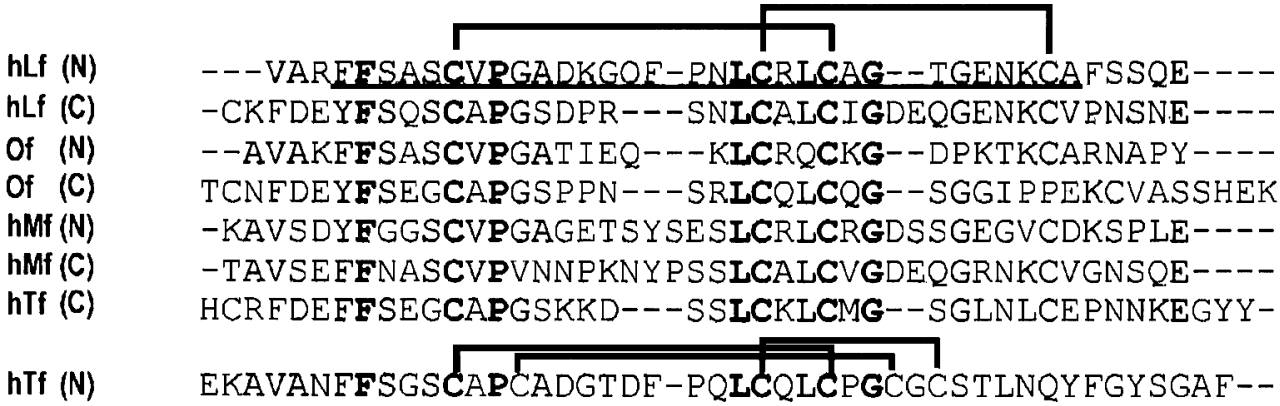

Fig. 1. Sequence alignment of the Cys-motif of representative transferrin family proteins. Amino acid sequences corresponding to the Cys-motif of N-terminal (N) and C-terminal (C) regions of human lactoferrin (hLf), human melanotransferrin (hMf), human transferrin (hTf), and chicken ovotransferrin (Of) were aligned by forcing the matching of cysteine residues. Sequence corresponding to the synthetic peptide kaliocin-1 is underlined. Bold residues are strictly conserved in more than 90% of the sequences. Shaded boxes indicate residues in positions conserved in more than 50% of the sequences.

The kinetics of killing by kaliocin-1 on *E. coli* cells and the Gram-positive species *K. rosea* showed a high reduction (>70%) of viable cells after 2 h of incubation (Fig. 2). Ninety percent of the cells treated with kaliocin-1 or hLf were killed after 4 h, and no living *E. coli* or *K. rosea* cells were detected after 24 h. However, the native protein exhibited a higher killing activity than that of the synthetic peptide. Progressive high concentrations of either Na^+ or K^+ caused a progressive loss of the hLf-candidacidal effect that was totally abolished when concentrations of $Na^+ \geq 40$ mM or $K^+ \geq 20$ mM were present in the buffer, whereas the Lfpep killing activity was not modified by the cation concentrations tested (Fig. 3).

Effect of kaliocin-1 on *E. coli* membranes. Previous reports have demonstrated OM damage of Gram-negative bacteria caused by hLf [13] and lactoferricin-like peptides [18, 19]. To determine the effect of kaliocin-1 on the OM of *E. coli*, we performed permeabilization assays

Antimicrobial activity of peptide kaliocin-1

Microorganism	Minimal inhibitory concentration, μM									
	Bactopeptone (1%)					Bactopeptone (0.3%)				
	kaliocin-1	Lfpep	hLf	hTf	Of	kaliocin-1	Lfpep	hLf	hTf	Of
Gram-negative bacteria										
E. coli ML-35	>200	23	>50	>150	>100	100	23	25	9	50
M. morganii	>200	>90	>50	>150	>100	200	>90	>50	19	ND
P. aeruginosa	>200	45	>50	>150	>100	75	45	25	19	ND
S. typhimurium	>200	23	>50	>150	>100	200	23	>50	75	100
Gram-positive bacteria										
K. rosea (*M. roseus*)	>200	ND	>50	>150	>100	75	6	1	9	25
S. aureus	>200	11	>50	>150	>100	75	11	23	19	>100
S. haemolyticus	>200	ND	>50	>150	>100	75	45	50	19	ND
S. mitis	>200	>90	>50	>150	>100	150	>90	50	ND	ND

Note: Minimal inhibitory concentration (MIC) of kaliocin-1 was determined in 1% and 0.3% Bactopeptone media. Control experiments were performed with human lactoferrin (hLf), human transferrin (hTf), ovotransferrin (Of), and lactoferricin-derived synthetic peptide (Lfpep). Results are the means of three independent experiments. ND, not determined.

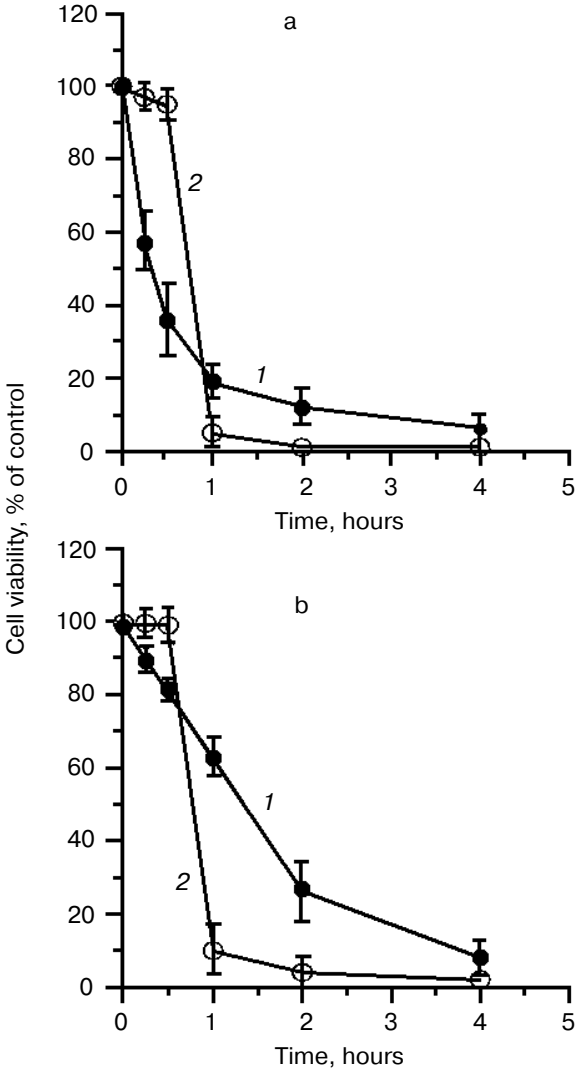

Fig. 2. Kinetics of killing of *E. coli* and *K. rosea* by kaliocin-1 and human lactoferrin. Cell suspensions of *K. rosea* (a) were incubated with 75 μM kaliocin-1 (*1*) or 1 μM hLf (*2*) in 5 mM PPB (pH 7.0). The *E. coli* cells (b) were incubated with 100 μM kaliocin-1 or 25 μM hLf. Untreated cells were used as control. Bacterial viability was determined by plating aliquots of the cell suspensions. Results are the mean ± standard deviations from duplicates from three separate experiments.

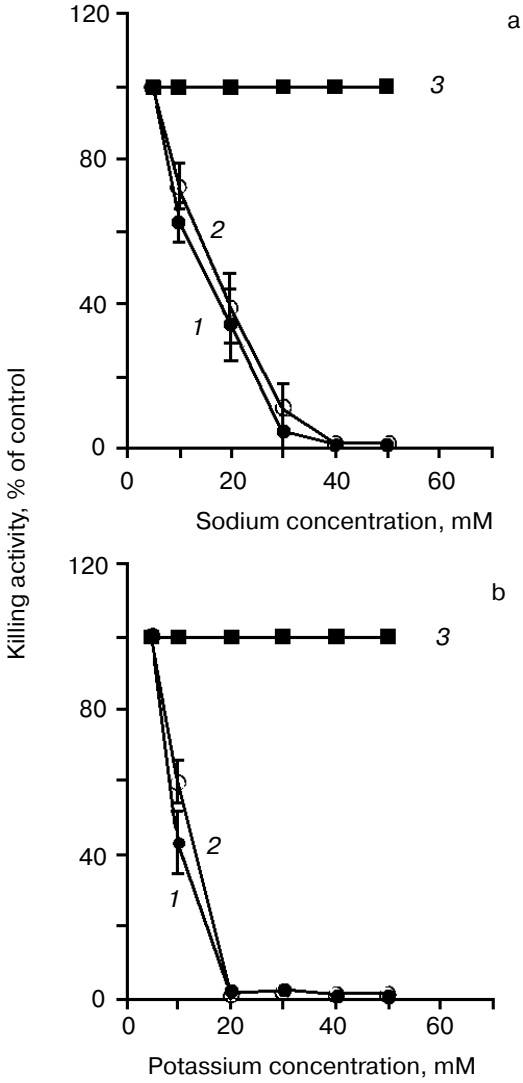

Fig. 3. Effect of cation concentration on the bactericidal effect of kaliocin-1 and human lactoferrin. *E. coli* cells were incubated with 100 μM kaliocin-1 (*1*), 25 μM hLf (*2*), or 25 μM Lfpep (*3*) at different concentration of sodium phosphate buffer (pH 7.0) (a) or potassium phosphate buffer (pH 7.0) (b). Bacterial viability was determined by plating aliquots of the cell suspensions. Results are the mean ± standard deviations from duplicates from three separate experiments.

using the NPN probe (Fig. 4a). The results showed the inability of kaliocin-1 to permeate the bacterial OM even at the highest concentration assayed (175 μM). In control assays, the OM was slightly permeabilized by hLf (≥6 μM) but the permeabilization degree was independent of the hLf-concentration tested. However, Lfpep increased the NPN fluorescence to significant rates even at a concentration as low as 3 μM, indicating an extensive disruption of the outer membrane.

The IM of the permease-negative mutant *E. coli* ML-35 was not permeabilized by kaliocin-1 (175 μM) or hLf (35 μM), whereas Lfpep (≥10 μM) caused a significant IM permeability as measured by the unmasking of cytoplasmic β-galactosidase (Fig. 4b).

K^+-efflux induced by kaliocin-1. The addition of growth inhibitory concentrations of kaliocin-1 and hLf to the cell suspensions of *E. coli* resulted in a slow and progressive cytoplasmic K^+-efflux, which showed also a high

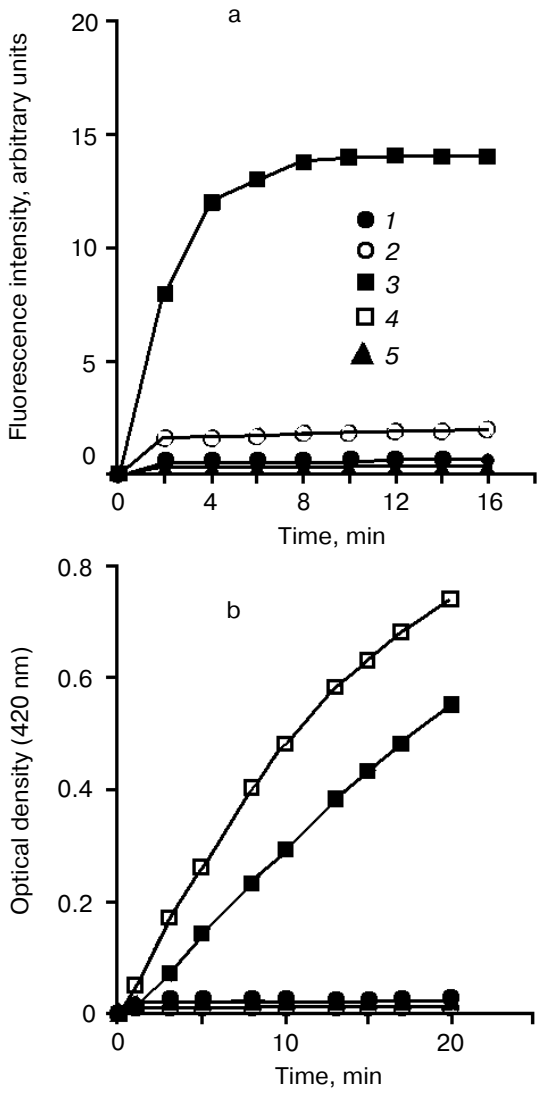

Fig. 4. Effect of kaliocin-1 on the outer and inner membranes of *E. coli*. *E. coli* ML-35 cells were treated with 175 μM kaliocin-1 (*1*), 35 μM hLf (*2*), or 50 μM Lfpep (*3*), and the OM-permeabilization (a) was monitored for the increase of the fluorescence caused by NPN-partitioning in the interior of the OM. The IM-permeabilization (b) was determined by monitoring the ONPG-hydrolysis. Sonicated bacteria (*4*) and untreated cells (*5*) were used as positive and negative control, respectively.

initial rate in Lfpep-treated cells (Fig. 5). Interestingly, kaliocin-1 and hLf did not increase the extracellular Na^+-concentration. However, the presence of Na^+ in the supernatant raised in the bacterial suspensions treated with the peptide Lfpep reaching at 60 min the maximum value (2.3 ± 0.6 μM).

Effect of kaliocin-1 on electrical potential and cytoplasmic pH in intact cells. To qualitatively assess the effect of kaliocin-1 on the Δψ of *E. coli* cells, the fluorescent probe DiS-C_3-(5) was used in spectrofluorometric assays. In the presence of kaliocin-1 (≥13 μM) or hLf (≥3 μM) a slow Δψ-dissipation was observed. In a representative assay using 100 μM kaliocin-1 and 6 μM hLf the maximal dissipation was reached 37 and 28 min after the addition of the peptide and protein, respectively (Fig. 6a). A similar dissipation degree was observed in control assays using the K^+-ionophore valinomycin. In contrast, the addition of the peptide Lfpep (≥18 μM) caused a faster loss of the Δψ of *E. coli* cells (Fig. 6a), similar to the Δψ-dissipation observed by adding gramicidin S (data not shown).

The monitoring of the internal pH of BCECF-loaded *E. coli* cells revealed that the addition of kaliocin-1 (150 μM) or hLf (25 μM) to the suspensions had no effect on the internal pH (Fig. 6b). However, Lfpep (≥18 μM) caused a rapid increase in the BCECF fluorescence and to a similar degree to that observed for gramicidin S used as control [42]. Thus, the ΔpH remained unchanged in kaliocin-1 and hLf-treated *E. coli* cells, indicating the absence of proton conductance, whereas Lfpep caused a total ΔpH dissipation.

Effect of kaliocin-1 on electrical potential and pH gradient in liposomes. The ability of kaliocin-1, compared to that of hLf and Lfpep, to interact with phospholipid membranes was assessed using K^+-loaded liposomes diluted in a K^+-free buffer. The liposomal preparation contained valinomycin to generate a Δψ and the fluores-

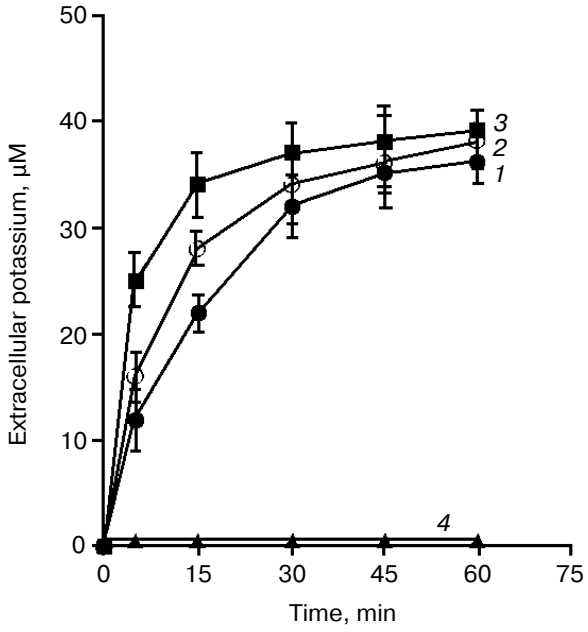

Fig. 5. Kinetics of potassium efflux from *E. coli* cells. Cells of *E. coli* were incubated with 175 μM kaliocin-1 (*1*), 25 μM hLf (*2*), or 50 μM Lfpep (*3*), and the extracellular K^+-concentration was quantified by atomic emission spectrometry at different times. Untreated cells were used as control (*4*). Results are the mean ± standard deviations from duplicates from at least four independent determinations.

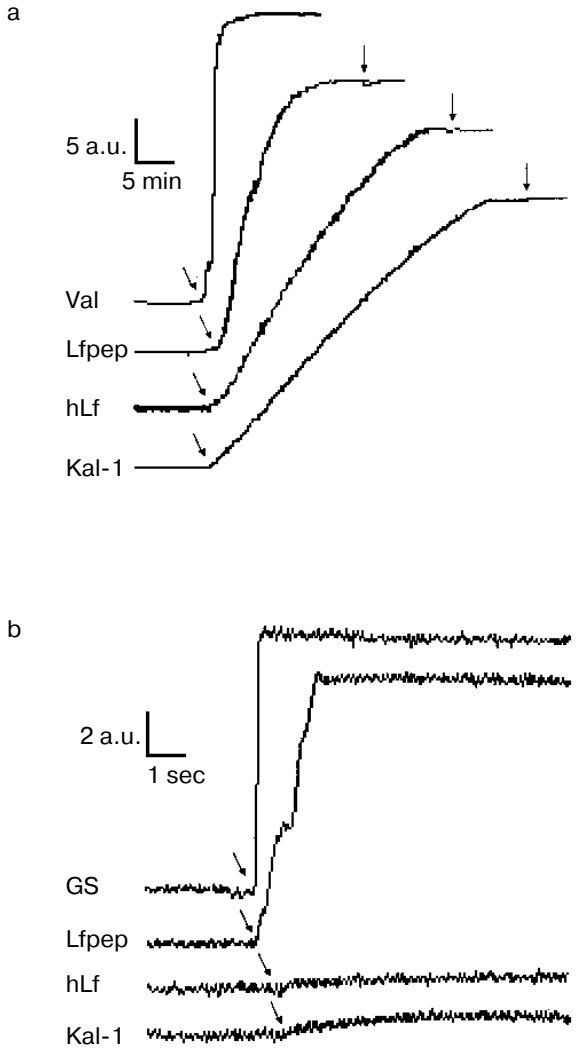

Fig. 6. Effect of kaliocin-1 on electrical and pH gradients of *E. coli* cells. a) Effect of kaliocin-1 on transmembrane electrical potential (Δψ) of *E. coli* cells. Changes in Δψ were monitored using the fluorescent indicator DiS-C_3-(5), after the addition (sloped arrows) of 100 μM kaliocin-1 (Kal-1), 6 μM human lactoferrin (hLf), or 18 μM Lfpep. Valinomycin (Val, 1 μM) was used as positive control and was also added (vertical arrows) to obtain the maximal Δψ dissipation. b) Effect of kaliocin-1 on pH gradient (ΔpH) of *E. coli* cells. The fluorescence of BCECF-loaded cells was monitored, and 150 μM Kal-1, 25 μM hLf, 50 μM Lfpep, or 40 μM gramicidin S (GS) were added at indicated times (arrows). Fluorescence intensity is expressed in arbitrary units (a.u.).

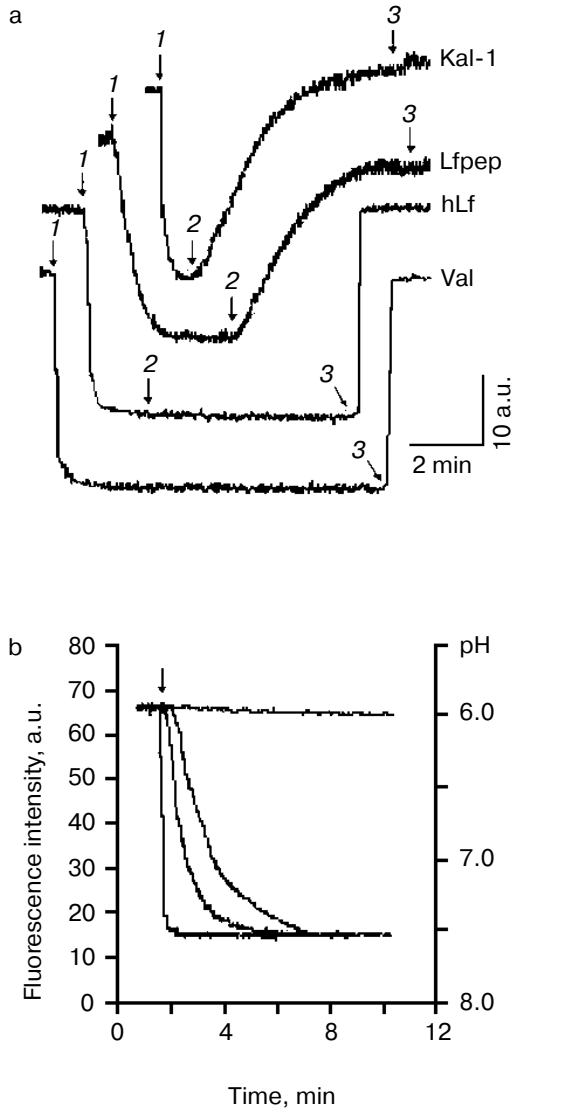

Fig. 7. Effect of kaliocin-1 on electrical and pH gradients generated in liposomes. a) Effect of kaliocin-1 on Δψ of liposomes. Liposomes containing 50 mM potassium phosphate (pH 6.0) were diluted 100-fold in 50 mM sodium phosphate (pH 6.0). Electrical potential (Δψ) was generated using 1 μM valinomycin (arrow *1*) and the fluorescence quenching of the potential-indicator DiS-C_3-(5) was monitored. The addition of 0.2 μM kaliocin-1, 25 μM hLf, 0.2 μM Lfpep, or 1 μM valinomycin (Val, control) was performed at indicated times (arrow *2*). Nigericin (1 μM) was added as a Δψ dissipation control (arrow *3*). b) Effect of kaliocin-1 on the ΔpH of liposomes. Liposomes containing 100 μM pyranine, 20 mM potassium phosphate, and 100 mM potassium acetate (pH 6.0) were 100-fold diluted in buffer (20 mM potassium phosphate, 100 mM K-PIPES, pH 7.5). Addition of kaliocin-1, hLf, and Lfpep was performed at the indicated time (arrow). The upper curve corresponds to hLf (25 μM). Progressively lower curves correspond to kaliocin-1 (0.2 μM), Lfpep (0.2 μM), and nigericin (1 μM, control). Change in the ΔpH was monitored by the fluorescence quenching of pyranine, used as pH indicator. a.u., arbitrary units.

cent indicator DiS-C_3-(5) to monitor changes in the artificial electrical potential. Concentrations ≥0.2 μM of kaliocin-1 or Lfpep caused the dissipation of the previously valinomycin-generated transmembrane electrical potential, whereas hLf (25 μM) was unable to dissipate the Δψ (Fig. 7a).

Changes in the ΔpH as a result of an increased proton permeability of the liposomal membrane were

assessed using pyranine-loaded liposomes (Fig. 7b). The addition of kaliocin-1 or Lfpep resulted in a total ΔpH dissipation at concentrations ≥0.2 μM, in a similar way to that caused by nigericin used as a positive control. In contrast, hLf (25 μM) was unable to modify the ΔpH of the liposomes.

Circular dichroism. The secondary structure of the kaliocin-1 in buffer and in aqueous mixtures of the structure-promoting solvent TFE was analyzed by far-UV circular dichroism (Fig. 8). TFE solutions are widely used to analyze conformational propensities of proteins and peptides and also as a sort of membrane-mimicking environment. In potassium phosphate buffer, a defined conformation was not observed in the CD spectrum of kaliocin-1, which had little ellipticity in the 215-230 nm range and a marked minimum at 199 nm, typical of random coil structures. In the presence of increasing amounts of TFE, the spectra of kaliocin-1 showed only slight changes, including a shift of the ellipticity minimum to 203 nm, indicating an intrinsic structural rigidity. The CD spectrum of kaliocin-1 in the presence of liposomes showed slight changes similar to those observed in the presence of TFE.

Hemolytic and cytotoxic activity assays. The synthetic peptide kaliocin-1 had no hemolytic activity in human, mouse, or rabbit erythrocytes when used at concentrations up to 150 μM. Permeabilization of PI to human lymphocytes and rat GH3 cells was not observed, indicating the absence of cytotoxicity of kaliocin-1 in these cells (data not shown).

To determine whether the permeabilization of ions could be exerted on eukaryotic cells, patch-clamp assays were performed using GH3 cells. The addition of several concentrations of kaliocin-1 (10-100 μM) and hLf (25 μM) to the cell suspension did not modify the initial cellular transmembrane electrical potential, estimated at -42 ± 7 mV ($n = 6$). Control of the cell function was tested by inducing depolarization of the cells by perfusion of 60 mM KCl and restoring the initial transmembrane electrical potential by perfusion of the bathing solution (data not shown).

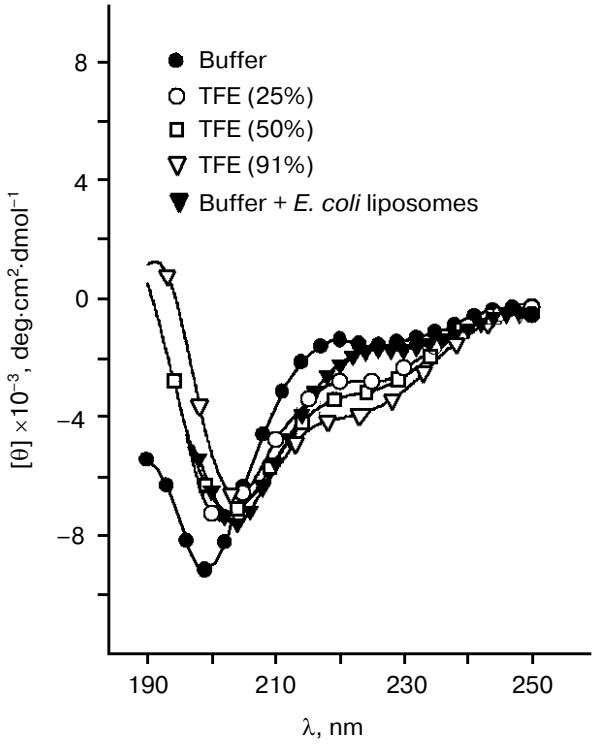

Fig. 8. Far-ultraviolet circular dichroism spectra of kaliocin-1. Measurements were made in 50 mM potassium phosphate buffer, pH 6.0, in presence or absence of *E. coli* liposomes, using a 1-mm pathlength cuvette, at 25°C. The CD spectra of kaliocin-1 were also recorded in water containing different percentages of trifluoroethanol (TFE). The concentrations of the kaliocin-1 and phospholipids were 50 μM and 4 mM, respectively. The spectra are averages of four scans.

DISCUSSION

Previous reports have demonstrated the antimicrobial effect of human lactoferrin, human transferrin, and ovotransferrin [8-10, 13]. Interestingly, our results show that these proteins exhibit a bactericidal effect only when the killing assays are performed in low-ionic-strength buffers or minimal basal media (i.e., 0.3% Bactopeptone). Although the killing effectiveness was different for each protein, the observation of a bactericidal effect only under similar conditions suggested a common antimicrobial mechanism of action for hLf, hTf, and Of. We assumed that this hypothetical mechanism could be different than that described for lactoferricin, a hLf-derived antimicrobial peptide that exerts a strong bactericidal effect independently of the assayed culture media. Moreover, the similarity of the amino acid sequence of lactoferricin with homologous sequences of other transferrin family proteins is weak. Based on these factors, we supposed that a new antimicrobial amino acid sequence of hLf could be obtained. The selected sequence was homologous to other present in all members of the transferrin family proteins, and the effects on bacterial cells and artificial membranes of a homologous synthetic peptide, termed kaliocin-1, were compared to that caused by the native hLf.

Interestingly, the synthetic peptide kaliocin-1 was able to inhibit the growth of most of the bacterial species tested but only in low-ionic-strength buffers (i.e., 5 mM PPB, pH 7.4) or minimal basal media. In a similar way, human lactoferrin, human transferrin, and ovotransferrin exhibited an antimicrobial activity dependent of the buffer or media assayed. However, the killing effect of the

peptide kaliocin-1 was less effective respect to that determined by hLf. Consequently with these observations, and a remarkable finding of the present work, was the striking influence of the cation concentration present in the testing buffer on the bactericidal effect of kaliocin-1 and hLf. The bactericidal effect was inversely correlated with the extracellular Na^+ or K^+ concentrations, being totally abolished when the assays were performed in buffer containing relatively low concentrations of Na^+ (≥40 mM) or K^+ (≥20 mM). Of note is also the remarkable similarity of the patterns of inhibition mediated by cations on the bactericidal effect of kaliocin-1 and hLf. The clear influence of the cation concentration on the bactericidal effect of both molecules could explain in part the absence of antimicrobial activity observed in growth media (i.e., 1% Bactopeptone).

Comparison of the permeabilization data indicated that kaliocin-1 and hLf were unable to grossly disturb the permeability of the membranes of *E. coli.* However, the K^+ efflux observed by kaliocin-1, similar to that described for hLf [21], suggested a direct interaction with the bacterial cytoplasmic membrane. In this case, like other natural antimicrobial peptides, kaliocin-1 probably could bind first to the highly anionic lipopolysaccharides of the OM of *E. coli*, gaining access to the cytoplasmic membrane via a process termed "self-promoted uptake" (reviewed in [43]). In contrast, the results obtained with Lfpep, an antimicrobial lactoferricin-like peptide [20], shown a significant increase in the permeabilization of *E. coli* membranes (OM and IM) probably due to its higher polycationic activity (pI = 12) in comparison with kaliocin-1 (pI = 7.9) and hLf (pI = 8.7). The peptide Lfpep caused also a simultaneous efflux of K^+ and Na^+ through the cytoplasmic membrane of *E. coli.* Recently, a bactericidal fragment of 92 residues (OTAP-92) obtained by acid proteolysis of ovotransferrin, that includes the sequence of kaliocin-1, has been reported [44, 45]. However, this Of-derived cationic peptide permeabilized the *E. coli* (OM and IM) and liposomal membranes, and exhibited a strong bactericidal effect on several Gram-negative bacteria [45]. These effects are more similar to those described for other cationic peptides (reviewed in [46]) but different to that observed for kaliocin-1. The differences in the mode of action of this larger Of-fragment and kaliocin-1 could be reflecting a strong interaction of OTAP-92, rich in positively charged residues, with the phospholipid membrane or it could be due to its inability to interact with a putative membrane component (i.e., a membrane protein).

The different flux of ions induced by kaliocin-1 and hLf (K^+ efflux) or by Lfpep (permeabilization of K^+, Na^+, and H^+) correlated well with the induced changes on the electrical potential ($\Delta\psi$) and pH gradient (ΔpH) of *E. coli* cells. Kaliocin-1 and hLf caused a selective loss of the $\Delta\psi$ but preserving the pH gradient of the cells meanwhile Lfpep collapsed the electrical and pH gradients as we described previously [20]. The kinetic bactericidal assays showed similar killing effects of kaliocin-1 and hLf that seem correlated to the K^+ efflux and $\Delta\psi$ dissipation. A similar K^+ efflux and selective loss of $\Delta\psi$ have been reported for other natural antimicrobial peptides such as lactococcin G and enterocin P [47, 48]. Thus, the futile cycle of ATP-driven potassium uptake and peptide-mediated potassium release proposed for lactococcin G [47] could be implicated in the cell death induced by kaliocin-1 and hLf.

To assess the influence of membrane proteins of *E. coli* on the activity of kaliocin-1, the peptide was tested on K^+-loaded liposomes prepared from *E. coli* total phospholipids. The peptides kaliocin-1 and Lfpep, but not hLf, were able to dissipate previously generated electrical and pH gradients. These results showed the inability of kaliocin-1 to permeate selectively K^+ in model membranes despite the fact that the secondary structure of the peptide, determined in the CD assays, remained unchanged by the interaction with the liposomes. These data differ from those obtained from cell assays, where kaliocin-1 and hLf showed the following common effects: 1) potassium-efflux from *E. coli* cells; 2) selective dissipation of the transmembrane electrical potential preserving the cellular pH gradient; 3) similar inability to disrupt the OM and IM of *E. coli*; 4) bactericidal activity showing dependence of similar extracellular cation concentration.

Based on the above arguments, it is tempting to hypothesize that the K^+ efflux induced by kaliocin-1 in cells may be due to its interaction with a membrane surface component (i.e., a membrane protein) as occurs with other antimicrobial peptides [49]. Another hypothesis based on the similarity of the mechanism of action of kaliocin-1 and hLf is the putative implication of this amino acid sequence in the antimicrobial activity of hLf, as well as hTf and Of, which exhibit homologous amino acid sequences on their molecular surfaces. However, further work will be necessary to assess the possible interaction of kaliocin-1 with a membrane component, and more studies must be performed to determine the structural similarities between kaliocin-1 and the homologous domains of the transferrin family proteins.

In summary, we report a novel hLf-derived synthetic peptide active on the bacterial cytoplasmic membrane, able to mimic the K^+ efflux and the selective $\Delta\psi$ dissipation induced by hLf on this cellular structure. Different reports have proposed a direct or adjunctive therapeutic application of hLf and hLf-derived peptides (reviewed in [50]). Since kaliocin-1 is not cytotoxic, it could be suggested that optimized analogs of this peptide with improved antimicrobial activity could be potential antimicrobial agents able to mimic the antimicrobial effect of human lactoferrin.

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REFERENCES

1. Metz-Boutigue, M. H., Jollés, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J., and Jollés, P. (1984) *Eur. J. Biochem.*, **145**, 659-676.
2. Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W., and Baker, E. N. (1989) *J. Mol. Biol.*, **209**, 711-734.
3. Spik, G., Strecker, G., Fournet, B., Bouquelet, S., Montreuil, J., Dorland, L., van Halbeek, H., and Vliegenthart, J. F. (1982) *Eur. J. Biochem.*, **121**, 413-419.
4. Masson, P. L., Heremans, J. F., and Dive, J. H. (1966) *Clin. Chim. Acta*, **14**, 735-739.
5. Masson, P. L., Heremans, J. F., and Schönne, E. (1969) *J. Exp. Med.*, **130**, 643-658.
6. Lönnerdal, B., and Iyer, S. (1995) *Annu. Rev. Nutr.*, **15**, 93-110.
7. Kanyshkova, T. G., Buneva, V. N., and Nevinsky, G. A. (2001) *Biochemistry (Moscow)*, **66**, 1-7.
8. Oram, J. D., and Reiter, B. (1968) *Biochim. Biophys. Acta*, **170**, 351-365.
9. Arnold, R. R., Cole, M. F., and McGhee, J. R. (1977) *Science*, **197**, 263-265.
10. Valenti, P., Visca, P., Antonini, G., and Orsi, N. (1986) *FEMS Microbiol. Lett.*, **33**, 271-275.
11. Levy, O. (1996) *Eur. J. Haematol.*, **56**, 263-277.
12. Kontoghiorghes, G. J., and Weinberg, E. D. (1995) *Blood Rev.*, **9**, 33-45.
13. Ellison III, R. T., Giehl, T. J., and LaForce, F. M. (1988) *Infect. Immun.*, **56**, 2774-2781.
14. Ellison III, R. T. (1994) in *Lactoferrin: Structure and Function* (Hutchens, T. W., Rumball, S. V., and Lönnerdal, B., eds.) Plenum Press, New York, pp. 71-90.
15. Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., and Tomita, M. (1992) *Biochim. Biophys. Acta*, **1121**, 130-136.
16. Odell, E. W., Sarra, R., Foxworthy, M., Chapple, D. S., and Evans, R. W. (1996) *FEBS Lett.*, **382**, 175-178.
17. Strom, M. B., Rekdal, O., and Svendsen, J. S. (2000) *J. Pept. Res.*, **56**, 265-274.
18. Bellamy, W. R., Wakabayashi, H., Takase, M., Kawase, K., Shimamura, S., and Tomita, M. J. (1993) *J. Appl. Bacteriol.*, **75**, 478-484.
19. Chapple, D. S., Mason, D. J., Joannou, C. L., Odell, E. W., Gant, V., and Evans, R. W. (1998) *Infect. Immun.*, **66**, 2434-2440.
20. Aguilera, O., Quirós, L. M., and Fierro, J. F. (1999) *FEBS Lett.*, **462**, 273-277.
21. Aguilera, O. (2000) *Specific Permeabilization of Ions Mediated by Transferrins on Biological and Artificial Membranes*: M. Sc. Thesis, University of Oviedo, Oviedo, Spain.
22. Mitchell, P. (1966) *Biol. Rev. Camb. Philos. Soc.*, **41**, 445-502.
23. Rottenberg, H. (1979) *Meth. Enzymol.*, **55**, 547-569.
24. De Lillo, A., Quirós, L. M., and Fierro, J. F. (1997) *FEMS Microbiol. Lett.*, **150**, 89-94.
25. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.*, **25**, 3389-3402.
26. Corpet, F. (1988) *Nucleic Acids Res.*, **16**, 10881-10890.
27. Turchany, J. M., Aley, S. B., and Gillin, F. D. (1995) *Infect. Immun.*, **63**, 4550-4552.
28. Wu, M., and Hancock, R. E. W. (1999) *J. Biol. Chem.*, **274**, 29-35.
29. Ehret-Sabatier, L., Loew, D., Goyffon, M., Fehlbaum, P., Hoffmann, J. A., van Dorsselaer, A., and Bulet, P. (1996). *J. Biol. Chem.*, **271**, 29537-29544.
30. Loh, B., Grant, C., and Hancock, R. E. W. (1984) *Antimicrob. Agents Chemother.*, **26**, 546-551.
31. Lehrer, R. I., Barton, A., Daher, K. A., Harwig, S. S. L., Ganz, T., and Selsted, M. E. (1989) *J. Clin. Invest.*, **84**, 553-561.
32. Kung, F. C., Raymond, J., and Glaser, D. A. (1976) *J. Bacteriol.*, **126**, 1089-1095.
33. Sims, P. J., Waggoner, A. S., Wang, C. H., and Hoffmann, J. F. (1974) *Biochemistry*, **13**, 3315-3330.
34. Schuldiner, S., and Kaback, H. R. (1975) *Biochemistry*, **14**, 5451-5461.
35. Molenaar, D., Abee, T., and Konings, W. N. (1991) *Biochim. Biophys. Acta*, **1115**, 75-83.
36. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta*, **812**, 55-65.
37. Clement, N. R., and Gould, J. M. (1981) *Biochemistry*, **20**, 1534-1538.
38. Bartlett, G. R. (1959) *J. Biol. Chem.*, **234**, 466-468.
39. Gao, F. H., Abee, T., and Konings, W. N. (1991) *Appl. Environ. Microbiol.*, **57**, 2164-2170.
40. Ruano, M. L. F., García-Verdugo, I., Miguel, E., Pérez-Gil, J., and Casals, C. (2000) *Biochemistry*, **39**, 6529-6537.
41. Barros, F., Delgado, L. M., Maciá, C., and de la Peña, P. (1991) *FEBS Lett.*, **279**, 33-37.
42. Kondejewski, L. H., Farmer, S. W., Wishart, D. S., Kay, C. M., Hancock, R. E. W., and Hodges, R. S. (1996) *J. Biol. Chem.*, **271**, 25261-25268.
43. Hancock, R. E. W., and Chapple, D. S. (1999) *Antimicrob. Agents Chemother.*, **43**, 1317-1323.
44. Ibrahim, H. R., Iwamori, E., Sugimoto, Y., and Aoki, T. (1998) *Biochim. Biophys. Acta*, **1401**, 289-303.
45. Ibrahim, H. R., Sugimoto, Y., and Aoki, T. (2000) *Biochim. Biophys. Acta*, **1523**, 196-205.
46. Epand, R. M., and Vogel, H. J. (1999) *Biochim. Biophys. Acta*, **1462**, 11-28.
47. Moll, G., Ubbink-Kok, T., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I. F., Konings, W. N., and Driessen, A. J. M. (1996) *J. Bacteriol.*, **178**, 600-605.
48. Herranz, C., Chen, Y., Chung, H. J., Cintas, L. M., Hernández, P. E., Montville, T. J., and Chikindas, M. L. (2001) *Antimicrob. Agents Chemother.*, **67**, 1689-1692.
49. Jack, R. W., Tagg, J. R., and Ray, B. (1995) *Microbiol. Rev.*, **59**, 171-200.
50. Weinberg, E. D. (2001) *J. Pharm. Pharmacol.*, **53**, 1303-1310.