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Lactoferricin B causes depolarization of the cytoplasmic membrane of *Escherichia coli* ATCC 25922 and fusion of negatively charged liposomes

H. Ulvatne*, H.H. Haukland, Ø. Olsvik, L.H. Vorland

Department of Medical Microbiology, P.O. Box 56, University Hospital of Tromsø, N-9038 Tromsø, Norway

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Abstract Antimicrobial peptides have been extensively studied in order to elucidate their mode of action. Most of these peptides have been shown to exert a bactericidal effect on the cytoplasmic membrane of bacteria. Lactoferricin is an antimicrobial peptide with a net positive charge and an amphipatic structure. In this study we examine the effect of bovine lactoferricin (lactoferricin B; Lfcin B) on bacterial membranes. We show that Lfcin B neither lyses bacteria, nor causes a major leakage from liposomes. Lfcin B depolarizes the membrane of susceptible bacteria, and induces fusion of negatively charged liposomes. Hence, Lfcin B may have additional targets responsible for the antibacterial effect.

Key words: Antimicrobial peptide; Lactoferricin; Liposome; Mode of action

1. Introduction

Cationic antimicrobial peptides have been isolated from a vast number of organisms [1]. They are a heterogeneous group of peptides, varying both in primary and secondary structure. These peptides have been extensively studied for the past decade, in order to elucidate their mode of action. The bactericidal effect of many of these peptides is thought to be due to the action on the cytoplasmic membrane of susceptible bacteria. The interaction may be through the formation of pores [2], thinning of the membrane [3], or destabilization of the bilayer [4]. Common for these models is a subsequent lysis of the bacterial cell.

Some antimicrobial peptides have been shown to have additional effects on the bacterial cell. Examples are indolicidin and PR-39, which act by inhibiting protein and DNA synthesis [5,6], and buforin, which inhibits cellular functions by binding to DNA and RNA [7]. Most of these peptides also exhibit effects on the outer and inner membranes of bacteria, and controversy has arisen about the bactericidal mode of action of antimicrobial peptides. Whether or not the killing event is due to the effect on the membrane or disturbance of the cellular metabolism is debated [8].

Bovine lactoferricin (lactoferricin B; Lfcin B) is a 25 amino acid residue peptide with a net charge of +8 [9], derived from the N-terminal part of bovine lactoferrin [10]. It has been proven to be effective against Gram-positive and Gram-negative bacteria [11], the latter in the same concentration ranges as magainin 2 [12]. Magainin 2 acts on the cytoplasmic membrane, forming transient pores causing the death of the bacterial cells.

The mode of action of Lfcin B is not yet elucidated, but due to its amphipatic structure and positive charge, it has been postulated to act on the cytoplasmic membrane [10]. The selectivity of Lfcin B may be due to the presence of negatively charged fatty acids in the bacterial membrane. To test these hypotheses, we have studied the effect of Lfcin B on membranes.

2. Materials and methods

2.1. Reagents

Lfcin B was prepared by pepsin digestion of bovine lactoferrin by Centre for Food Technology (Qld., Australia). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide) was obtained from Molecular Probes (OR, USA), while valinomycin, mellitin, propidium iodide (PI), phospholipids, sodium cholate, Triton X-100 and calcein were purchased from Sigma, St. Louis, MO, USA. PD10 columns containing Sephadex G-25 were obtained from Amersham Pharmacia Biotech AB, Sweden.

2.2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination

Determination of MIC and MBC of Lfcin B was performed using a standard microdilution technique in 1% bacto peptone water (BPW) pH 6.8, as previously described by Vorland et al. [13]. For determination of the MBC, aliquots of 10 μl were transferred onto agar plates. The plates were incubated overnight at 37°C and the colony forming units (CFU) determined. The MBC was set as the concentration reducing the CFU by 99%.

2.3. Preparation of liposomes

To study leakage of vesicle content, the self-quenching dye calcein was entrapped in liposomes, using a buffer of pH 7.5 containing 5 mM calcein, 100 mM NaCl and 5 mM EDTA [14]. Intermediate unilamellar vesicles (IUV) were prepared by dissolving lipids and sodium cholate at a ratio of 1:1 (mol:mol) in calcein buffer to a final concentration of 5 mg lipid per ml buffer. The lipids were incubated at 60°C overnight to allow formation of micelles. The micelle solutions were dialyzed using a Liposomat (Dianorm, Munich, Germany) at 60°C against buffer until conversion to liposomes. The liposomes were filtered through a 0.22 μm filter, and analyzed by dynamic light scattering (Coulter N4S, Beckman) to ensure size distribution and

*Corresponding author. Fax: (47)-77-62 70 15.
E-mail: hilde.ulvatne@rito.no

Abbreviations: Lfcin B, bovine lactoferricin; DPPC, diphosphatidyldipalmitoylcholine; DPPG, diphosphatidyldipalmitoylglycerol; DPPS, diphosphatidyldipalmitoylserine; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide; PI, propidium iodide; CFU, colony forming units; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; IUV, intermediate unilamellar vesicles; SUV, small unilamellar vesicles; BPW, bacto peptone water

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lamellarity. Neutral liposomes were made of diphosphatidyldipalmitoylcholine (DPPC), and negatively charged liposomes of 80% DPPC and 20% diphosphatidyldipalmitoylglycerol (DPPG) or 20% diphosphatidyldipalmitoylserine (DPPS). Palmitoyl (C16:0) was chosen to mimic the natural character of bacterial membranes.

Empty liposomes were prepared for the fusion study, and contained 33% DPPG or DPPS in DPPC vesicles. Small unilamellar vesicles (SUV) were prepared using high-pressure extrusion technique [15]. A solution of 20% phospholipids in buffer (100 mM NaCl and 5 mM EDTA) was extruded 10 times through a Micron Lab 40 APV Gaulin-Homogenizer (Lubeck, Germany) at a pressure of 700 bar. Size distribution was measured using dynamic light scattering.

2.4. Leakage from liposomes

The liposomes were separated from unentrapped calcein by double filtration through Sephadex G-25. The liposomes were then diluted in calcein free buffer to a final concentration of 10 μM lipid. Lfcin B was added to the liposomes to yield concentrations of 10, 50 and 100 μg/ml liposomes, and incubated at 37°C for 15, 30, 45, 60 and 120 min. Release of the entrapped dye will cause a dilution of calcein and an increase in fluorescent intensity. Relative fluorescence intensity was measured on a Perkin Elmer Luminescens Fluorimeter LS-50B (λ_{ex} 490 nm, λ_{em} 520 nm). The background fluorescent intensity from liposomes without peptide or detergent was measured as negative control at each time point. 10% Triton X-100 was added to the liposomes to induce maximum release of the entrapped dye. Relative leakage was calculated using Graph PadPrism 3.0 software for Windows (Graph Pad Software Inc.). The following formula was used: relative leakage = $((F_x-F_0)\times 100)/(F_t-F_0)$ where F_x is the intensity measured at a given concentration of Lfcin B, F_0 is the intensity of the liposomes (background) and F_t is the intensity after collapse by Triton X-100.

2.5. Fusion of liposomes

Fusion of SUV was determined using changes in absorbance at 400 nm as previously described by Fujii et al. [16]. Measuring the absorbance at 400 nm gives an indication of changes in liposome size, due to fusion of vesicles. The absorbance was measured after exposing the SUV to 0, 10, 50 and 100 μg/ml Lfcin B. Further, changes in particle size were measured using dynamic light scattering after exposure to the same concentrations of Lfcin B as performed in the leakage assay.

2.6. Effect on membranes in whole cells

Escherichia coli ATCC 25922 and *Proteus mirabilis* ATCC 35659 were grown to mid-logarithmic phase in 2% BPW and adjusted to a density of approximately 1×10^6 CFU/ml. Six aliquots of the bacterial suspension were drawn; one served as negative control, one as positive control. To the last four, Lfcin B was added to a final concentration of 15, 30, 70 and 100 μg/ml. The peptide–bacteria solutions were incubated at 37°C for 1 and 2 h before staining. PI was dissolved in double distilled sterile water to a stock concentration of 10 mg/ml, and added to the bacteria to give a final concentration of 10 μg/ml, then allowing 5 min to stain before analyzing [17]. Ethanol-treated cells were used as a positive control, indicating maximal uptake of PI. JC-1 was dissolved in DMSO to a stock concentration of 10 mg/ml, and stored in the dark at room temperature until used. JC-1 was added to the bacteria to give a final concentration of 1 μg/ml. The cells were incubated in the dark at room temperature for 15 min to allow uptake of the dye [18]. The cells were then washed twice and resuspended in 2% BPW before analyzing. Valinomycin-treated cells (200 nM) were used as positive control.

2.7. Flow cytometric analysis

The analysis was performed on a FACScan (Becton Dickinson, New Jersey, USA) equipped with an argon laser with a single wavelength at 488 nm. Uptake of JC-1 was detected using FL2 (λ_{em} 585 nm ± 25 nm) and depolarization detected as a shift in fluorescence from FL2 to FL1 (λ_{em} 530 nm ± 30 nm). PI (λ_{em} 617 nm) uptake was detected as an increase in FL2 emission.

2.8. Electron microscopy

E. coli ATCC 25922 was grown to mid-logarithmic phase in 2% BPW, and further diluted in 2% BPW to a final concentration of 2×10^6 CFU/ml. Equal amounts of bacteria and Lfcin B (dissolved in water) were mixed to give a total volume of 100 ml, yielding a final concentration of 24 μg/ml Lfcin B ($0.8\times$MIC). The solutions were placed in a water shaker (37°C) for 60 min, and then centrifuged for 10 min at $1700\times g$, and the pellet kept for electron microscopy.

The specimens were cut and washed in phosphate buffer prior to postfixation in 1% aqueous OsO_4 for 2 h. The specimens were then block-stained in 2% aqueous uranyl acetate, dehydrated in graded ethanol concentrations, and subsequently embedded in Epon/Araldite. Ultrathin sections were cut on a RMC MT-7 ultramicrotome, contrasted for 12 min in 5% aqueous uranyl acetate and 10 min in Reynolds lead citrate. The sections were examined with a JEOL JEM 1010 electron microscope, and micrographs taken on Kodak Electron Microscope film No. 4489.

2.9. Lysis assay

E. coli ATCC 25922 was grown in 2% BPW to 2×10^8 CFU/ml, harvested, washed twice in phosphate-buffered saline (pH 7.4) and then resuspended in 2% BPW to yield $\sim10^7$ CFU/ml. Aliquots of 100 μl bacteria and 100 μl Lfcin B (yielding concentrations ranging from zero to 100 μg/ml) were added to a microtiter tray. The $OD_{630\ nm}$ was read in a Spectramax®PLUS (Molecular Devices, CA, USA) for 1 h, reading interval 1 min at 37°C. A reduction in $OD_{630\ nm}$ was interpreted as lysis of the bacteria [7].

2.10. Effect of osmotic pressure

Swelling of bacterial cells was monitored at 420 nm using Spectramax®PLUS [19]. In a microtiter tray, aliquots of 75 μl *E. coli* at mid-logarithmic growth, 75 μl Lfcin B (dissolved in water) and 75 μl water or 0.3 poly ethylene glycol were added to yield final concentrations of Lfcin B between zero and 100 μg/ml. The increase in absorbance at 420 nm was monitored in a Spectramax®PLUS at intervals of 10 min for 2 h, and the data analyzed using Graph PadPrism 3.0.

3. Results

The MICs and MBCs of Lfcin B against *E. coli* and *P. mirabilis* were determined. *E. coli* had a MIC of 30 μg/ml and MBC of 70 μg/ml, while *P. mirabilis* was less susceptible with a MIC above 200 μg/ml (MBC not detected).

Liposomes of different composition were exposed to 10, 50 and 100 μg/ml Lfcin B. Release of vesicle content was measured as an increase in relative fluorescent intensity caused by dilution of the self-quenching dye calcein. Fig. 1 shows the relative leakage from vesicles after exposure to Lfcin B. An initial increase in fluorescent intensity was observed (15 min), before the intensity decreased (1 h), and then maximum release of vesicle content from DPPS vesicles was detected at 2 h. The leakage from all liposomes was minimal, and at no point of time did the leakage exceed 25%. A minor leakage was detected from the neutral liposomes (DPPC), but this was not significant compared to the leakage from the charged liposomes.

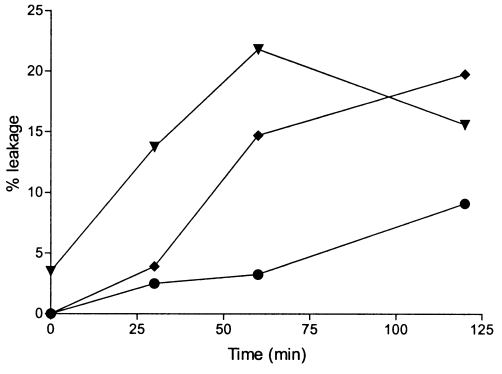

Fig. 1. Relative leakage from IUV induced by 100 μg/ml Lfcin B plotted as a function of time (▼ DPPG, ♦ DPPS, ● DPPC).

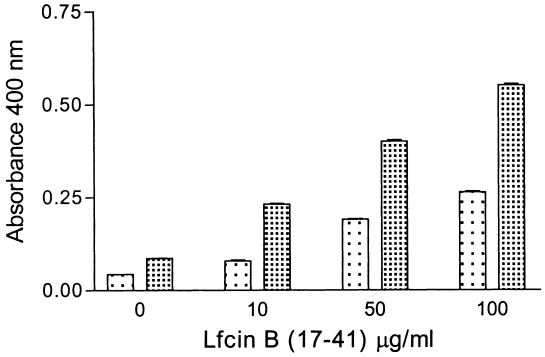

Fig. 2. Fusion of negatively charged vesicles represented by change in absorbance at 400 nm. The absorbance increases with increasing size of the vesicles. SUV were exposed to 0, 10, 50 and 100 μg/ml Lfcin B for 60 min at room temperature. Bars indicate S.E.M. ▭, DPPG; ▦, DPPS.

Fig. 2 shows that the absorbance of the liposome:peptide solution increased with increasing peptide concentration. The increase was greatest when measured on the DPPS containing liposomes. To verify the increase in vesicle size, the mean particle size and size distribution were measured using dynamic light scattering. These results were consistent, and showed that the vesicle size increased 200-fold with increasing concentrations of Lfcin B (data not shown). The DPPS population displayed the largest liposomes.

Fig. 3 illustrates the effect on the cytoplasmic membrane of whole bacteria. Lfcin B caused a depolarization of the cytoplasmic membrane in *E. coli*, whilst the membrane of *P. mirabilis* was not depolarized. The depolarization was concentration dependent, indicated by a greater JC-1 uptake with increasing concentrations of Lfcin B. A greater proportion of the *E. coli* population was depolarized and damaged than in the population of *P. mirabilis*. There was only a minor uptake of PI by the bacteria exposed to Lfcin B.

Morphological changes as visualized by electron microscopy were evident at concentrations below the MIC. Fig. 4 shows a micrograph of *E. coli* exposed to 30 μg/ml Lfcin B. The bacterial cells were intact, and the content of the cell was still inside. The bacterial cells did not appear swollen, but there appeared to be a separation of the cell envelope. Further

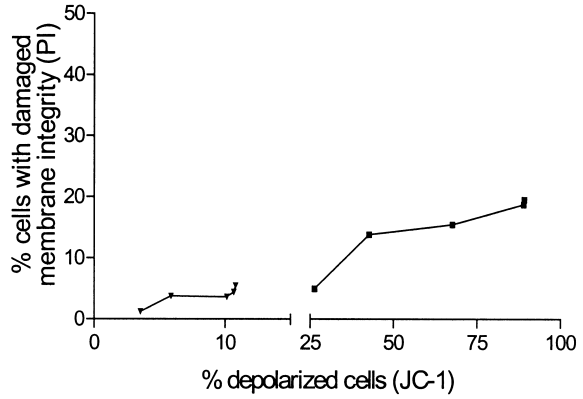

Fig. 3. The effect on bacterial membranes induced by Lfcin B after 2 h as % cells with depolarized membranes (measured as uptake of JC-1) plotted against % cells with damaged membrane integrity (measured by PI uptake). ■ *E. coli*. ▼ *P. mirabilis*.

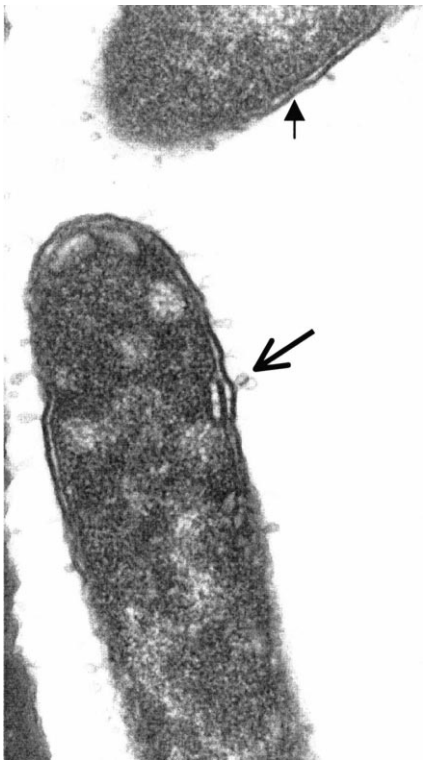

Fig. 4. Electron micrograph of *E. coli* exposed to 30 μg/ml Lfcin B. Morphological changes include the formation of blebs on the bacterial surface. The open arrow points out the formation of blebs on the bacterial surface, and the closed arrow the separation of the cell envelope.

examination reveals blebs containing bilayer structures on the bacterial surface, extruding from the cytoplasmic membrane, and released to the surroundings of the bacteria.

Lfcin B did not lyse *E. coli*, as measured by $OD_{630\,nm}$. No reduction in OD was observed. An increase in OD was observed even at concentrations above the MIC. Adding poly ethylene glycol to the external medium had no effect on the measured absorbance at 420 nm. Equalizing the osmotic pressure did not counteract the effect Lfcin B exerts on the bacteria.

4. Discussion

Several studies report a correlation between increased permeability and antimicrobial activity of antimicrobial peptides. Hence, the paradigm of today is that the membrane destabilizing effect is responsible for the antimicrobial activity.

Our results show that Lfcin B destabilizes liposomes, causing leakage and fusion. The greatest leakage of liposome content was observed from negatively charged liposomes. This is in accordance with the results presented by Matsuzaki et al. in a study performed with magainin 2 and liposomes [20]. However, the leakage induced by Lfcin B is minor when compared to other antimicrobial peptides with a known effect on the cytoplasmic membrane [21,22]. Subsequent to the initial leakage, further exposure to Lfcin B caused the acidic vesicles to rearrange (i.e. fuse) rather than to leak, and Lfcin B induced a 10-fold increase in size of the negatively charged liposomes. Defensin-induced fusion of liposomes has been reported, but the biological significance of the fusion process is poorly

understood [16]. However, the event does enable a translocation of the peptide molecules across the membrane bilayer.

The findings reported above are supported by the results obtained from the experiments performed on whole bacteria. Lfcin B does not lyse the bacterial strain tested here, nor is the osmotic pressure important for the effect of the peptide. As indicated by a low uptake of PI, we reveal no significant damage to the integrity of the cytoplasmic membrane. PI is a small cationic dye excluded from cells with intact membrane [23]. Lfcin B caused a depolarization of the cytoplasmic membrane, indicated by a shift in fluorescent properties of JC-1. JC-1 is a lipophilic cationic probe, originally used to analyze the membrane potential of mitochondria [18]. All cells take up JC-1, but JC-1 changes fluorescent properties as the cells become depolarized. This effect is only exerted on *E. coli*, the most susceptible strain. Whether the depolarization is a result of a direct action on the membrane, or a secondary result from an attack on metabolic pathways, is not possible to determine based on these experiments.

We show that Lfcin B favors interactions with negatively charged phospholipids, inducing a small leakage of vesicle content. The small leakage observed could be an effect of a transient channel formation process, as proposed for magainin 2 and mellitin [20]. This is however not likely, since the effect of Lfcin B is not affected by osmotic pressure. The major effect on acidic liposomes is fusion of the vesicles. The initial increase in fluorescent intensity observed after exposure to Lfcin B may not be a result of a true leakage, but mainly a result of re-encapsulation of dye during the fusion process itself as proposed for other peptides [16]. The fusion process may represent a mean of transport across the lipid barrier, enabling translocation and opening for the possibility for the peptide to reach cytoplasmic targets.

We show that Lfcin B selectively induces fusion of negatively charged liposomes, an event representing the probable selectivity basis of the peptide. Lfcin B does not cause increased permeability of membranes in intact bacteria, but causes depolarization and formation of blebs on the bacterial surface. The peptide may cause the cytoplasmic membrane to curl up on itself, fuse due to the hydrophobic character of the bilayer, and thus forming blebs. The peptide may be anchored to the blebs, or may be translocated into the cytoplasm during this event. The effect of Lfcin B is not dependent upon osmotic pressure, since Lfcin B exerts its effect on the bacteria even after the osmotic pressure was equalized across the envelope. Neither do the cells undergo lysis, which is an essential result of pore formation. A more detailed model for the mechanisms responsible for the bactericidal effect of Lfcin B is not elucidated at this point.

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