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Rapid Membrane Permeabilization and Inhibition of Vital Functions of Gram-Negative Bacteria by Bactenecins

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Bactenecins are a class of arginine-rich antibacterial peptides of bovine neutrophil granules. Two bactenecins with approximate molecular weights of 5,000 and 7,000 designated Bac5 and Bac7, respectively, exert in vitro a potent bactericidal activity toward several gram-negative bacteria (R. Gennaro, B. Skerlavaj, and D. Romeo, Infect. Immun. 57:3142–3146, 1989). We have now found that this activity shows an inverse relationship to the ionic strength of the medium and is inhibited by divalent cations and greatly potentiated by lactoferrin. Under conditions supporting marked bactericidal activity, the two peptides cause a rapid increase in the permeability of both the outer and inner membranes of *Escherichia coli*, as shown by unmasking of periplasmic β-lactamase and of cytoplasmic β-galactosidase. In addition, the two bactenecins inhibit the respiration of *E. coli* and *Klebsiella pneumoniae* but not of Bac5- and Bac7-resistant *Staphylococcus aureus*. Furthermore, they induce a drop in ATP content in *E. coli*, *K. pneumoniae*, and *Salmonella typhimurium* and a marked decrease in the rates of transport and incorporation of [^{3}H]leucine and [^{3}H]uridine into *E. coli* protein and RNA, respectively. In general, all these effects become evident within 1 to 2 min and reach their maximal expression within about 5 min. Overall, these data strongly suggest that the decrease in bacterial viability is causally related to the increase in membrane permeability and the subsequent fall in respiration-linked proton motive force, with the attendant loss of cellular metabolites and macromolecular biosynthesis ability.

Neutrophils are vehicles of rapid and effective animal defense against invading microorganisms, toward which they are guided by chemotaxins. Once the microorganisms are internalized by phagocytosis, a variety of antimicrobial factors are discharged into the phagosomes. These factors may either be generated from molecular oxygen concomitantly with phagocytosis (16) or preexist as proforms or mature protein molecules in different populations of cytoplasmic granules (7–9, 26, 27, 29, 30, 33, 35, 36; M. Zanetti, L. Litteri, R. Gennaro, H. Horstmann, and D. Romeo, J. Cell Biol., in press; R. Gennaro, D. Romeo, B. Skerlavaj, and M. Zanetti, *in* J. R. Harris, ed., *Blood Cell Biochemistry*, vol. 3, in press).

In bovine neutrophils the oxygen-independent antibacterial activity is exclusively stored in the so-called large granules (8). We have purified some of the cationic polypeptides responsible for this activity and shown that their cationic nature can be ascribed to a content of arginyl residues of at least 20% (9, 27). Among these peptides, which have been named bactenecins, there are two proline-rich components with molecular weights of about 5,000 and 7,000, designated Bac5 and Bac7, respectively (9). At concentrations of 2 to 50 μg/ml, Bac5 and Bac7 kill *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumoniae*. However, even at a concentration of 200 μg/ml they are unable to kill or suppress the growth of gram-positive organisms such as *Staphylococcus aureus* and *Streptococcus agalactiae* (9).

In an attempt to understand the mechanism by which Bac5 and Bac7 impair the capacity of at least some gram-negative bacteria to divide, we first carried out bactericidal assays under different conditions of pH, ionic strength, and content of divalent cations. We then investigated the effects of the bactenecins on some vital functions of the target microorganisms, such as respiration and permeability/transport of the outer membranes (OM) and inner membranes (IM). The data obtained suggest that Bac5 and Bac7 bind electrostatically to the OM, where they dramatically increase the permeability to small molecules. Within a few minutes they also cause a marked increase in the permeability of IM and inhibit the reduction of oxygen by the electron transport chain. This impairs the generation of the transmembrane potential and the production of ATP, required for several processes, such as transport of protein and nucleic acid precursors. In addition, we found that lactoferrin, an iron-binding glycoprotein widely distributed in mucosal secretions (20) and also present in neutrophil granules (3, 8), markedly potentiates the bactericidal activity of bactenecins.

MATERIALS AND METHODS

Antibacterial peptides. Bac7 and Bac5 were acid extracted from bovine neutrophil granules and purified by ion-exchange and high-performance reversed-phase chromatographies as previously described (9). Purification to homogeneity was confirmed by both analytical reversed-phase chromatography and acid-polyacrylamide gel electrophoresis in the presence of 4 M urea (9). Peptides were dissolved in 0.1% trifluoroacetic acid (TFA), and their concentrations were determined by the method of Waddell (9).

Lactoferrin. Bovine milk lactoferrin, a gift of B. Senft, University of Giessen, Federal Republic of Germany, was iron desaturated (apolactoferrin) by dialysis against 0.2 M sodium acetate buffer containing 40 mM EDTA and 0.2 M sodium phosphate (pH 4.0) (21). The iron-free protein was subsequently dialyzed extensively against deionized, distilled water and concentrated by evaporation. Concentration

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and iron desaturation were determined by A_{280} and A_{465}, respectively (10).

Bacteria. *E. coli* ATCC 25922, *S. typhimurium* ATCC 14028, and *K. pneumoniae* ATCC 13883 were grown aerobically at 37°C in nutrient broth (Difco Laboratories, Detroit, Mich.), whereas *S. aureus* ATCC 25923 was grown in brain heart infusion broth (Difco). Overnight cultures were transferred into fresh medium (1:25) and allowed to grow aerobically for 3 to 4 h to obtain mid- to late-logarithmic-phase cultures, (A_{600} of approximately 0.45 for *E. coli*). Bacteria were then harvested by centrifugation and resuspended in the media selected for the various assays. Bacterial density was assessed by turbidity at 600 nm, with reference to previously determined standards.

E. coli K-12 AB1157, transformed with plasmid pNO1523 (encoding a periplasmic β-lactamase) by the standard high-efficiency transformation procedure of Hanahan (12), was a gift of L. Dolzani, Institute of Microbiology, University of Trieste. The transformed *E. coli*, which was used for OM permeability assays, was grown aerobically in nutrient broth in the presence of 50 μg of ampicillin per ml. Logarithmic-phase cultures were washed twice with sterile physiological saline to remove ampicillin and harvested as described above.

E. coli ML-35, a lactose, permease-deficient strain with constitutive cytoplasmic β-galactosidase activity (*lacI lacZ+ lacY*), utilized for IM permeability assays, was obtained from D. Petit, Institute J. Monod, Paris, France, and from E. Ruby, University of Southern California, Los Angeles, Calif.

Bactericidal assays. The effects of different ionic strengths and pHs and the presence of Ca^{2+}, Mg^{2+}, and lactoferrin on the bactericidal capacity of Bac7 and Bac5 were tested on logarithmic-phase cells of *E. coli* ATCC 25922. Bacteria (1×10^6 to 1.5×10^6 CFU/ml or 8×10^6 to 15×10^6 CFU/ml in the assays with lactoferrin) were incubated in a final volume of 200 μl for 1 h at 37°C in a shaking water bath with different amounts of Bac7 or Bac5. The buffers used for pH dependency experiments were 10 mM sodium citrate at pHs 5, 5.5, and 6 or 10 mM sodium phosphate at pHs 6, 6.5, 7, 7.5, and 8, both supplemented with 100 mM NaCl. Controls lacked the bactenecins but contained TFA at a maximal concentration of 0.005%. At the end of the incubation, samples were serially diluted with sterile saline solution, plated in duplicate on nutrient agar, and incubated for 16 to 18 h to allow colony counts.

OM permeability. Periplasmic β-lactamase activity was measured in transformed *E. coli* K-12 with 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethyl-aminophenylazo)-pyridinium methyl]-3-cephem-4-carboxylic acid (PADAC) as a substrate (17, 38). Logarithmic-phase bacteria (about 6×10^6 CFU) were incubated at 37°C in 0.6 ml of 10 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl and 50 μM PADAC. After addition of either Bac5 or Bac7, the decrease of A_{570} was recorded. An equivalent volume of 0.1% TFA replaced the bactenecin solutions in control assays. To determine the maximal rate of PADAC hydrolysis by fully permeabilized cells, samples placed in an ice bath were submitted to four 30-s cycles of sonication at 4 A with a Branson sonicator equipped with a small probe. Total β-lactamase activity was measured in the presence or absence of bactenecins to exclude any potential interference by the cationic peptides on the enzyme activity.

IM permeability. The β-galactosidase activity of *E. coli* ML-35 was measured with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate (17). Logarithmic-phase bacteria (about 6×10^6 CFU) were incubated in 0.6 ml of 10 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl and 1.5 mM ONPG at 37°C. At zero time, different amounts of Bac5 or Bac7 were added and the rate of *o*-nitrophenol production was recorded at 405 nm. In controls, an equivalent volume of 0.1% TFA replaced the bactenecin solution. Total β-galactosidase activity was measured with bacteria lysed by ultrasonication (eight 30-s cycles at 4 A) in the presence or absence of bactenecins to exclude any potential interference by the cationic peptides with the enzyme activity.

Oxygen consumption. Respiration of logarithmic-phase cultures of aerobically grown *E. coli* ATCC 25922, *K. pneumoniae*, and *S. aureus* was measured with a Clark electrode. The electrode chamber contained 1.2 ml of 10 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl, 10 mM glucose, 0.4 ml of nutrient broth (*E. coli* and *K. pneumoniae*) or of BHI broth (*S. aureus*), and 0.1 ml of bacterial suspensions (1.5×10^7 to 3×10^7 CFU).

ATP determination. *E. coli* ATCC 25922, *K. pneumoniae*, or *S. typhimurium* (6×10^6 to 10×10^6 CFU/ml) in 10 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl and 5 mM glucose were incubated at 37°C with Bac7 or Bac5 for 0, 5, 10, 20, and 40 min. At the end of the incubation, 180-μl portions of the bacterial suspensions were extracted with 0.5% (wt/vol) trichloroacetic acid (TCA) in the presence of 2 mM EDTA. After 30 min in ice and centrifugation at 11,000 × *g* for 5 min at 4°C, the supernatants were collected and stored at −20°C. The ATP content in the TCA extracts was measured with a luminometer as bioluminescence generated in the presence of D-luciferin and luciferase (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden).

Uptake and incorporation of [^{3}H]uridine and [^{3}H]leucine. The effects of bactenecins on the active transport of [5,6-^{3}H]uridine and L-[4,5-^{3}H]leucine and their incorporation into bacterial RNA and proteins, respectively, were studied in *E. coli* ATCC 25922. Logarithmic-phase bacteria were resuspended in 10 mM sodium phosphate buffer (pH 7.5) with 100 mM NaCl and 5 mM glucose at a density of 1×10^7 to 1.5×10^7 CFU/ml. After preincubation at 37°C for 10 min, Bac7 or Bac5 was added. At 0, 10, and 30 min, 2.5-ml portions of bacterial suspensions were transferred to tubes containing 3 μCi of [5,6-^{3}H]uridine or L-[4,5-^{3}H]leucine, and the incubation at 37°C was continued for 3 min.

To assess the efficiency of transport, 500-μl samples were immediately diluted with 4.5 ml of physiological saline, filtered on 0.45-μm-pore-size Millipore membrane filters, and washed once with the same volume of saline. To evaluate the amount of radioactivity incorporated into macromolecules, the remaining 2-ml portions of the suspensions were treated with 2 ml of 10% TCA. After vigorous mixing, samples were placed in an ice bath, divided into two identical aliquots, and filtered as described above, except that 5% TCA was used to wash the filters. In both cases, filters were air dried and immersed in 10 ml of scintillation fluid and the radioactivity was counted in a liquid scintillation counter.

Chemicals. [5,6-^{3}H]uridine (50 Ci/mmol) and L-[4,5-^{3}H]leucine (120 to 190 Ci/mmol) were from Amersham International (Amersham, Buckinghamshire, United Kingdom). PADAC was from Calbiochem (La Jolla, Calif.), and ONPG was from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

We measured the bactericidal potential of Bac5 and Bac7 under different experimental conditions. In the pH range 5 to

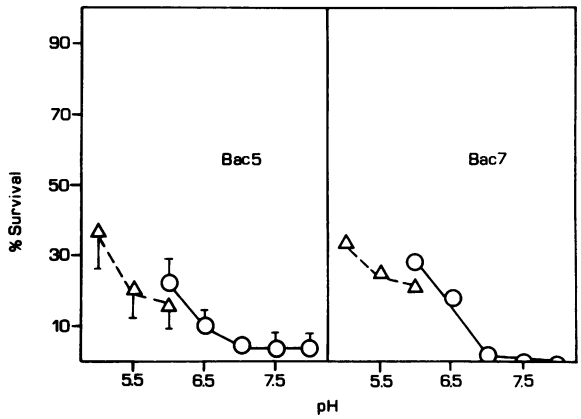

FIG. 1. Effect of pH on the bactericidal activity of Bac5 and Bac7. *E. coli* cells (1×10^6 to 1.5×10^6 CFU/ml) were treated with 25 μg of Bac5 (left panel, means ± standard errors of the means of three experiments) or Bac7 (right panel, means of two virtually identical, independent experiments) per ml in either 10 mM sodium citrate buffer (pHs 5.0, 5.5, and 6.0 [△]) or sodium phosphate pHs 6.0, 6.5, 7.0, 7.5, and 8.0 [○]) with 100 mM NaCl. The buffers used had no effect on bacterial viability in control (no treatment) assays.

8, optimal efficiency of the two bactenecins against *E. coli* was found at pH 7 to 8, while at pH 5 there was about 60% of maximal bactericidal activity (Fig. 1). Bac5 and Bac7 killed *E. coli* more effectively with decreasing ionic strength in the bactericidal assays (Table 1). Furthermore, the presence of Ca^{2+} and Mg^{2+} caused a significant reduction in the bactericidal activity of both bactenecins (Table 2), whereas 5 mM glucose had no effect (data not shown).

Assuming the OM of the susceptible organisms to be the most immediate target of Bac5 and Bac7, we then evaluated the effects of the bactenecins on OM permeability by utilizing *E. coli* K-12 transformed with plasmid pNO1523. In these cells, which displayed the same susceptibility to both Bac5 and Bac7 as the strain used in the bactericidal assays (data not shown), the periplasmic β-lactamase is virtually inaccessible to the substrate PADAC. The cleavage of PADAC by β-lactamase is thus an indication of increased leakiness of the permeability barrier of OM (38). Both Bac5 and Bac7 caused a dramatic increase in permeability to PADAC, which under the assay conditions used was already evident by 20 to 40 s and reached a maximum within 4 min (30 to 35% accessibility to β-lactamase compared with 1 to 5% in intact *E. coli* and 100% in bacterial sonicates; range of three independent experiments carried out with 50 μg of Bac5 or

TABLE 1. Effect of ionic strength on the bactericidal activity of Bac5 and Bac7[a]

NaCl concn (mM)	CFU/ml (log) with treatment:			
	Bac5		Bac7	
	Expt 1	Expt 2	Expt 1	Expt 2
0	<2.0	<3.0	<2.0	<2.0
50	<2.0	4.20	3.40	<2.0
100	4.79	5.15	4.56	4.20
150	5.31	5.44	5.01	5.37

[a] *E. coli* cells were treated for 60 min at 37°C with bactenecins (20 μg/ml) in 10 mM sodium phosphate (pH 7.5) containing different concentrations of NaCl. Data for untreated bacteria (60 min): 6.18 (experiment 1) and 5.97 (experiment 2) CFU/ml (log).

TABLE 2. Effect of Ca^{2+} and Mg^{2+} on the bactericidal activity of Bac5 and Bac7[a]

Treatment (concn [μg/ml])	CFU/ml (log)	
	$-(Ca^{2+}, Mg^{2+})$	$+(Ca^{2+}, Mg^{2+})$
Untreated	6.15	6.15
Bac5, 20	3.04	5.14
Bac5, 50	<2.0	4.95
Bac5, 100	<2.0	4.60
Bac7, 20	<2.0	5.44
Bac7, 50	<2.0	4.82
Bac7, 100	<2.0	4.13

[a] *E. coli* cells were treated with bactenecins for 60 min at 37°C in 10 mM sodium phosphate with 50 mM NaCl (pH 7.5) in the absence or presence of Ca^{2+} and Mg^{2+} (0.5 mM each).

Bac7 per ml) (Fig. 2). In keeping with the observed inhibition of bactericidal potency, the presence of Ca^{2+} and Mg^{2+} in the medium prevented the increase of OM permeability to PADAC. However, when the divalent cations were added a few minutes after Bac5 or Bac7 no change in the increased hydrolysis of PADAC was observed (data not shown).

The inhibitory effects shown in the presence of high ionic strength or of the divalent cations Ca^{2+} and Mg^{2+}, as well as the increased accessibility of the periplasmic β-lactamase to its substrate, are all manifestations of early events occurring when Bac5 or Bac7 binds to the OM of *E. coli*. To determine whether the two bactericidal peptides also exert some effects at the IM level, we evaluated their effects on bacterial respiration, as an indirect measure of the integrity of the electron transport chain, as well as the rate of the energy-dependent transport of macromolecular precursors.

Both Bac5 and Bac7 caused a marked inhibition of the respiration of *E. coli* and *K. pneumoniae* while not affecting the oxygen consumption by bactenecin-resistant *S. aureus*

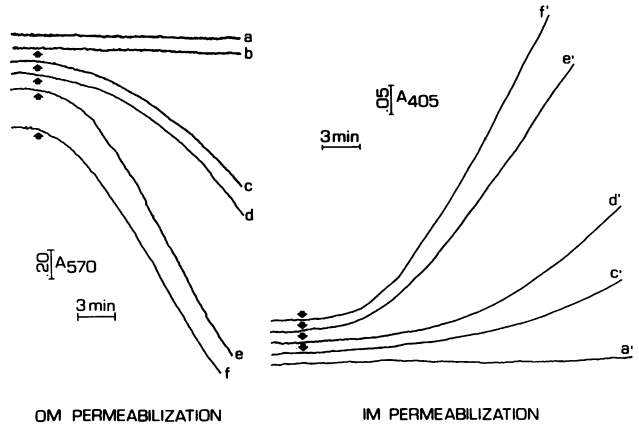

FIG. 2. Effect of bactenecins on the permeability of the OM and IM of *E. coli*. Shown is the unmasking of periplasmic β-lactamase activity in transformed *E. coli* K-12 (left panel) and of cytoplasmic β-galactosidase activity in *E. coli* ML-35 (right panel), measured as described in Materials and Methods. Bacteria (about 10^7 CFU/ml) were suspended in 10 mM sodium phosphate buffer (pH 7.5)—100 mM NaCl in the absence (all traces except trace b) or presence (trace b) of Ca^{2+} and Mg^{2+} (0.5 mM each). Traces a and a', Untreated bacteria; c and c', Bac5 (10 μg/ml); d and d', Bac7 (10 μg/ml); e and e', Bac5 (50 μg/ml); b, f, and f', Bac7 (50 μg/ml). Arrows indicate addition of bactenecins. The results shown are representative of three virtually identical, independent experiments.

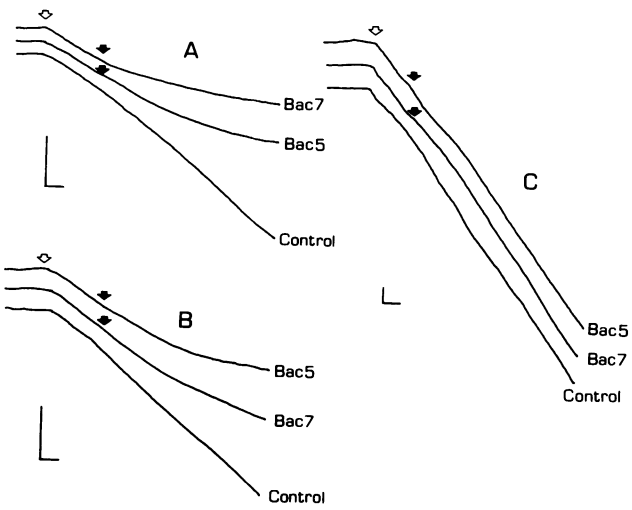

FIG. 3. Effect of bactenecins on oxygen consumption of *E. coli* (A), *K. pneumoniae* (B), and *S. aureus* (C). Bacteria (1.5×10^7 to 3×10^7 in 0.1 ml) were added (first arrows) to a medium made of 1.2 ml of 10 mM sodium phosphate (pH 7.5) with 100 mM NaCl, 10 mM glucose, and 0.4 ml of nutrient broth (*E. coli* and *K. pneumoniae*) or BHI broth (*S. aureus*). The second arrows indicate the additions of bactenecins at 150 μg/ml. Vertical bars, 10 nmol of O_2; horizontal bars, 1 min. The results shown are representative of several very similar, independent experiments.

(Fig. 3). The inhibition of respiration reached its maximal value (60 to 80%, range of at least three independent determinations) 1.5 to 3 min after the addition of the bactenecins to the electrode vessel, suggesting that in addition to the OM permeabilization there is also a rather rapid effect on the IM.

The inhibition of respiration was very likely caused by a direct effect on one of the components of the electron transport chain. This was indirectly checked by measuring the ability of an uncoupler to release from respiratory control a bactenecin-inhibited system. Because bacteria tend to have poor respiratory control, the system used in this assay was rat liver mitochondria. We found that the oxygen consumption by these organelles was also inhibited by bactenecins (data not shown), consistent with previous observations made with a cationic antibacterial fraction of neutrophil granules (24). When the inhibition reached a steady state and an uncoupling concentration of Ca^{2+} was added to the mitochondria, no recovery of the respiration rate was observed, thereby suggesting that the antibacterial peptides may directly affect the function of a component (or components) of the electron transport system.

Concurrent with the respiratory inhibition in *E. coli*, as well as in *K. pneumoniae* and *S. typhimurium*, Bac5 and Bac7 also induced a drop in the ATP content (Fig. 4). This effect was time and dose dependent, without apparent differences in susceptibility among the three organisms tested. In parallel, there was also a decrease in the rate of transport of [^{3}H]leucine (Fig. 5) and [^{3}H]uridine (Fig. 6) which was accompanied by an even greater reduction of incorporation of these precursors into protein and RNA, respectively. The adverse effects on both the ATP turnover and the transport/incorporation of solutes reached a significant value within 3 to 5 min.

The effects of Bac5 and Bac7 on IM functions suggest that there may also be a perturbation of the integrity of this membrane. This was checked with *E. coli* ML-35, whose susceptibility to the bactericidal action of Bac5 and Bac7 was similar to that of *E. coli* ATCC 25922 (data not shown). In the ML-35 strain, which is deficient in lactose permease and thus virtually impermeable to lactose and its analogs, both bactenecins caused a significant increase in IM permeability, as measured by the unmasking of cytoplasmic β-galactosidase. With Bac5 or Bac7 at 50 μg/ml, the IM permeabilization occurred after a lag of 1.5 min and reached a steady state by about 7 min (Fig. 2). Compared with the enzyme activity of sonicates of an equivalent number of bacteria, this steady state corresponds to about 40% (37 to 44%, range of three independent experiments) of total β-galactosidase accessibility to ONPG.

Finally, we found that apolactoferrin, which in bovine neutrophils is associated with the specific and the large granules (8), markedly potentiated the bactericidal effect of both bactenecins. Although per se ineffective at the concentrations tested (50 to 300 μg/ml), apolactoferrin greatly increased the antibacterial capacity against *E. coli* of suboptimal and optimal concentrations of Bac7 (Table 3) and Bac5 (data not shown) both in the absence and in the presence of Ca^{2+} and Mg^{2+}.

DISCUSSION

Normal neutrophils of different animal species kill ingested *S. typhimurium* and *E. coli* with considerable efficacy

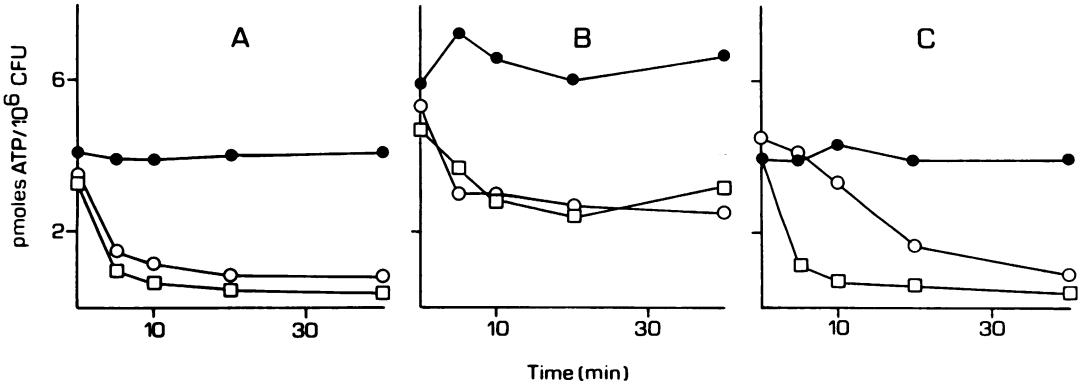

FIG. 4. Effect of bactenecins on the ATP content of *E. coli* (A), *K. pneumoniae* (B), and *S. typhimurium* (C). Bacteria (6×10^6 to 10×10^6/ml) were incubated for various times at 37°C with 50 μg (final concentration) of Bac5 (○) or Bac7 (□) per ml in 10 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl and 5 mM glucose. ●, Untreated bacteria. Experiments were carried out in duplicate.

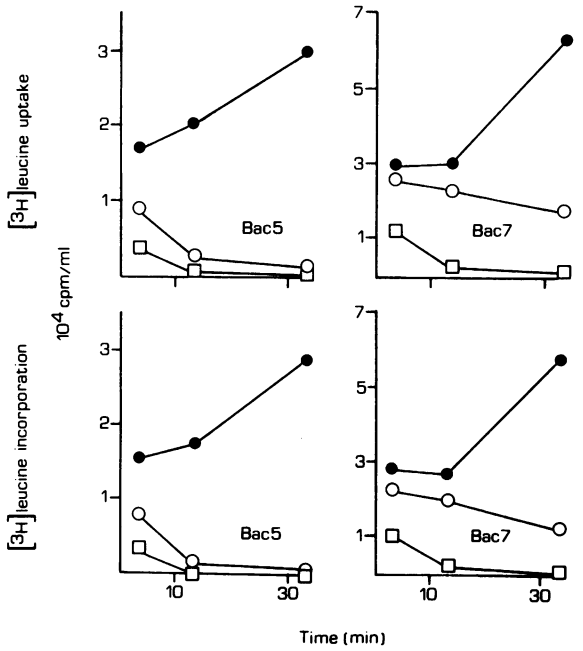

FIG. 5. Inhibition by bactenecins of [^{3}H]leucine uptake and incorporation into proteins of *E. coli*. Bacteria (1×10^7 to 1.2×10^7/ml) were treated at 37°C with Bac5 or Bac7 in 10 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl and 5 mM glucose. ●, Untreated bacteria; ○, 10 μg of each bactenecin per ml; □, 50 μg of each bactenecin per ml. Assays of L-[4,5-^{3}H]leucine uptake and incorporation into bacterial proteins were performed as described in Materials and Methods. Data from two independent but very similar experiments, both carried out in duplicate, were combined.

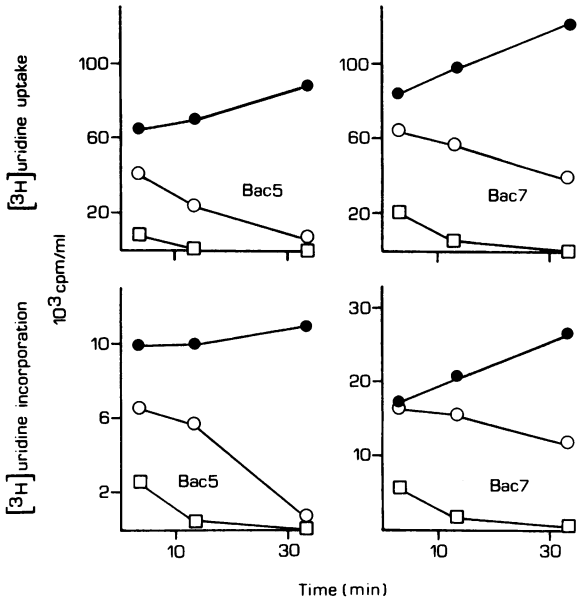

FIG. 6. Inhibition by bactenecins of [^{3}H]uridine uptake and incorporation into RNA of *E. coli*. Bacteria (1×10^7 to 1.5×10^7/ml) were treated with Bac5 or Bac7 under the conditions described in the legend to Fig. 5. Assays of [5,6-^{3}H]uridine uptake and incorporation into RNA were performed as described in Materials and Methods. Symbols are as in Fig. 5. Data from two independent but very similar experiments, both carried out in duplicate, were combined.

under anaerobic conditions (23, 36) or in the presence of myeloperoxidase inhibitors (25), thereby suggesting that these cells possess potent oxygen- or hydrogen peroxide-independent bactericidal mechanisms against at least some gram-negative bacteria.

In bovine neutrophils this mechanism may involve two proline- and arginine-rich peptides, Bac5 and Bac7, that we have purified from granule extracts. In fact, in vitro both peptides markedly reduce the ability of a variety of gram-negative organisms to form colonies (9). Here we have shown that Bac5 and Bac7 permeabilize both the OM and IM of *E. coli* and inhibit its respiration, ATP synthesis, and transport functions. Some of these effects were also investigated with other gram-negative microorganisms, such as *S. typhimurium* and *K. pneumoniae*.

Inhibition of bactericidal activity by increasing concentrations of NaCl suggests that the early interaction of cationic Bac5 and Bac7 with the target cells involves electrostatic binding to negatively charged surface molecules. These molecules are very likely the lipopolysaccharide (LPS) molecules of the outer envelope, which contain clusters of carboxylic and phosphate groups in their core region (18). This is further supported by the observed inhibitory action of Ca^{2+} and Mg^{2+}, which are known to stabilize the LPS molecules into an ordered and cohesive layer by binding to their negatively charged groups (18). An immediate binding to LPS has also been suggested for other cationic bactericidal polypeptides of neutrophils (6, 34). In particular, the bactericidal/permeability-increasing protein of human neutrophils is homologous to a LPS-binding protein found in acute-phase sera (11, 32). A prompt enzymatic trimming of the carbohydrate chains of the LPS of ingested bacteria, with a concomitant relaxation of the molecular complexity masking the negative charges in the LPS core region, would thus facilitate bactenecin binding and the subsequent bactericidal processes.

In this frame one should consider the potentiating effect of apolactoferrin on the bactericidal activity of Bac5 and Bac7. In fact, this protein has been shown to promote a LPS release from the OM of *E. coli* similar to that promoted by the metal chelator EDTA (5). Concurrently with the LPS release, the OM becomes more permeable to some agents that are normally excluded. One such agent is the antibiotic rifampin, whose antibacterial effects at subinhibitory concentrations are increased by the presence of lactoferrin (5).

Under some circumstances, apolactoferrin is bacteriostatic or bactericidal for a variety of gram-negative and

TABLE 3. Effect of lactoferrin on the bactericidal activity of Bac7[a]

Treatment	CFU/ml (log) with lactoferrin concn (μg/ml)					
	0	50	300	0	50	300
Untreated	6.95	7.22	7.23	6.96	7.11	6.93
Bac7 (2 μg/ml)	6.58	6.35	4.11	6.94	6.65	4.07
Bac7 (10 μg/ml)	5.88	3.94	<2.70	6.38	4.50	2.66
Untreated	6.98		7.03	7.23		7.12
Bac7 (25 μg/ml)	5.04		<2.70	6.10		1.90
Bac7 (25 μg/ml) + Ca^{2+} and Mg^{2+}	6.51		5.58	6.90		4.96

[a] *E. coli* cells were treated for 60 min at 37°C with Bac7 in 10 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl with or without Ca^{2+} and Mg^{2+} (0.5 mM each) in the presence or absence of apolactoferrin. Results of two independent experiments are shown.

gram-positive microorganisms (2, 4, 5, 20, 22). We have employed apolactoferrin concentrations which are not toxic to *E. coli* and observed that under these conditions there is a remarkable potentiation of the bactericidal activity of Bac5 and Bac7. These observations suggest that in phagocytosing neutrophils, bactenecins and lactoferrin may act synergistically when both granular components are discharged in the phagosomes (8) or extracellularly at inflammatory sites.

The drop in viability of *E. coli* treated with either Bac5 or Bac7 was more marked in the pH range between 7 and 8 than at a more acidic pH. However, the observed reduction in bactericidal efficiency below pH 7 should not be considered too significant in considering potential bactenecin functions in vivo. In fact, the effects of Bac5 and Bac7 on gram-negative bacteria reach a maximum within only a few minutes, when the pH of the phagocytic vacuoles is still slightly alkaline (28, 30).

Computer modeling of Bac5 and Bac7 reveals that several conformations are possible with an asymmetrical distribution of polar and apolar amino acid residues on the surfaces of both bactenecins (S. Pongor, R. Gennaro, and D. Romeo, unpublished data), implying that both peptides may have an amphiphilic character. The adoption of an amphiphilic helical conformation has been predicted for and subsequently found in a variety of antimicrobial polypeptides, such as melittin (14), magainin 2 (19), and cecropin A (13), whose functions are related to perturbation of cell membranes. Actually, both Bac5 and Bac7 appear to perturb the integrity of the OM of *E. coli* K-12, as assessed by an increased accessibility of periplasmic β-lactamase to PADAC. Furthermore, within the time interval in which they increase OM permeability, but with a slightly delayed onset, they also make the IM permeable to ONPG, a hydrophilic substrate of β-galactosidase.

Bac5 and Bac7 also affect respiration, which in aerobically grown gram-negative bacteria is carried out by two oxidase systems localized in the IM and oxidizing ubiquinol generated by dehydrogenases (1). This inhibition is very likely due to a direct interaction with one of the components of these electron transport chains, as suggested by indirect evidence provided by experiments carried out with rat liver mitochondria. The transport of electrons to oxygen is coupled to the formation of a transmembrane electrochemical proton gradient, utilized to drive bacterial ATP synthesis, solute uptake, and other energy-requiring, membrane-associated processes. As expected, bactenecin-treated *E. coli* cells show both a reduced content of ATP and a reduced ability to take up [^{3}H]uridine and [^{3}H]leucine, coupled to a parallel inhibition of incorporation of these precursors into RNA and protein, respectively.

The effects of Bac5 and Bac7 on IM functions suggest that these polypeptides cross the OM and reach the cytoplasmic membrane. It is feasible that in the phagosome, concomitantly with Bac5 and Bac7, other polypeptides may pass through the OM, possibly aggravating the lethal effects of bactenecins (31). Thus, the biochemical lesion responsible for the ultimate death of Bac5- and Bac7-susceptible microorganisms may be a combination of an increased permeability of both the OM and IM and the disruption of the proton motive force, with the related sequelae of loss of cellular metabolites and alteration of energy-requiring, membrane-associated processes.

Other antibacterial cationic polypeptides of human neutrophils, such as bactericidal/permeability-increasing protein and defensins, have also been reported to induce increased membrane permeability and inhibition of respiration of some gram-negative bacteria (15, 17, 33). Unlike bactenecins, defensins require that the susceptible microorganisms are in a state of active metabolism (17). This might be linked to the necessity of actively transporting defensins to their molecular targets, whereas bactenecins, as discussed above, after binding to the OM might assume an amphiphilic conformation suitable for passive diffusion to their target(s).

Staphylococci and streptococci are resistant to Bac5 and Bac7 (9). Furthermore, even at 300 μg/ml neither polypeptide lyses erythrocytes (unpublished results) and Bac7, corresponding to the anti-herpesvirus polypeptide IIIa$_2$β (9, 37), is devoid of cytotoxicity to the mammalian cells used to support viral growth. Resistance to Bac5 and Bac7 by some gram-positive bacteria as well as by mammalian cells might simply be explained by failure of effective binding of the antimicrobial peptides, which thereby could not reach an apolar environment necessary for the adoption of an amphiphilic, membrane-perturbing conformation.

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LITERATURE CITED

1. **Anraku, Y.** 1988. Bacterial electron transport chain. Annu. Rev. Biochem. **57**:101–132.
2. **Arnold, R. R., M. Brewer, and J. J. Gauthier.** 1980. Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. Infect. Immun. **28**:893–898.
3. **Baggiolini, M., D. deDuve, P. L. Masson, and J. F. Heremans.** 1970. Association of lactoferrin with specific granules in rabbit heterophil leukocytes. J. Exp. Med. **13**:559–570.
4. **Bortner, C. A., R. D. Miller, and R. R. Arnold.** 1986. Bactericidal effect of lactoferrin on *Legionella pneumophila*. Infect. Immun. **51**:373–377.
5. **Ellison, R. T., III, T. J. Giehl, and F. M. LaForce.** 1988. Damage to the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect. Immun. **56**:2774–2781.
6. **Farley, M. M., W. M. Shafer, and J. K. Spitznagel.** 1988. Lipopolysaccharide structure determines ionic and hydrophobic binding of a cationic antimicrobial neutrophil granule protein. Infect. Immun. **56**:1589–1592.
7. **Ganz, T., M. E. Selsted, D. Szklarek, S. S. L. Harwig, K. Daher, and R. I. Lehrer.** 1985. Defensins: natural peptide antibiotics of human neutrophils. J. Clin. Invest. **76**:1427–1435.
8. **Gennaro, R., B. Dewald, U. Horisberger, H. U. Gubler, and M. Baggiolini.** 1983. A novel type of cytoplasmic granule in bovine neutrophils. J. Cell Biol. **96**:1651–1661.
9. **Gennaro, R., B. Skerlavaj, and D. Romeo.** 1989. Purification, composition, and activity of two bactenecins, antibacterial peptides of bovine neutrophils. Infect. Immun. **57**:3142–3146.
10. **Gordon, W. G., J. Ziegler, and J. J. Basch.** 1962. Isolation of an iron-binding protein from cow's milk. Biochim. Biophys. Acta **60**:410–411.
11. **Gray, P. W., G. Flaggs, S. R. Leong, R. J. Gumina, J. Weiss, C. E. Ooi, and P. Elsbach.** 1989. Cloning of the cDNA of a human neutrophil bactericidal protein. J. Biol. Chem. **264**:9505–9509.
12. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning. A practical approach, vol. 1. IRL Press, Oxford.
13. **Holak, T. A., A. Engstrom, P. J. Kraulis, G. Lindeberg, H. Bennich, T. A. Jones, A. M. Gronenborg, and G. M. Clore.** 1988. The solution conformation of the antibacterial peptide cecropin A: a nuclear magnetic resonance and dynamical simulated annealing study. Biochemistry **27**:7620–7629.
14. **Inagaki, F., I. Shimada, K. Kawaguchi, M. Hirano, I. Terasawa, T. Ikura, and N. Go.** 1989. Structure of melittin bound to perdeuterated dodecylphosphocholine micelles as studied by

two-dimensional NMR and distance geometry calculations. Biochemistry **28**:5985–5991.
15. **In't Veld, G., B. Mannion, J. Weiss, and P. Elsbach.** 1988. Effects of the bactericidal/permeability-increasing protein of polymorphonuclear leukocytes on isolated bacterial cytoplasmic membrane vesicles. Infect. Immun. **56**:1203–1208.
16. **Klebanoff, S. J.** 1988. Phagocytic cells: products of oxygen metabolism, p. 391–444. *In* J. I. Gallin, I. M. Goldstein, and R. Snyderman (ed.), Inflammation: basic principles and clinical correlates. Raven Press, New York.
17. **Lehrer, R. I., A. Barton, K. A. Daher, S. S. L. Harwig, T. Ganz, and M. E. Selsted.** 1989. Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. J. Clin. Invest. **84**:553–561.
18. **Lugtenberg, B., and L. van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737**:51–115.
19. **Marion, D., M. Zasloff, and A. Bax.** 1988. A two-dimensional NMR study of the antimicrobial peptide magainin 2. FEBS Lett. **227**:21–26.
20. **Masson, P. L., and J. F. Heremans.** 1966. Studies on lactoferrin, the iron-binding protein of secretions, p. 115–124. *In* H. Peeters (ed.), Protides of the biological fluids, vol. 14. Elsevier/North Holland Publishing Co., Amsterdam.
21. **Mazurier J., and G. Spik.** 1980. Comparative study of the iron-binding properties of human transferrins. I. Complete and sequential iron saturation and desaturation of the lactotransferrin. Biochim. Biophys. Acta **629**:399–408.
22. **Nonnecke, B. J., and L. K. Smith.** 1984. Inhibition of mastitic bacteria by bovine milk apo-lactoferrin evaluated by in vitro microassay of bacterial growth. J. Dairy Sci. **67**:606–613.
23. **Okamura, N., and J. K. Spitznagel.** 1982. Outer membrane mutants of *Salmonella thyphimurium* LT2 have lipopolysaccharide-dependent resistance to the bactericidal activity of anaerobic human neutrophils. Infect. Immun. **36**:1086–1095.
24. **Pennial, R., and H. I. Zeya.** 1971. The effects of cationic proteins of rabbit polymorphonuclear leukocyte lysosomes on the respiratory activity of liver mitochondria. Biochem. Biophys. Res. Commun. **45**:6–13.
25. **Rainard, P.** 1985. Assessment of attachment, ingestion, and killing of *Escherichia coli* by bovine polymorphonuclear cells with combined micromethods. Vet. Immunol. Immunopathol. **10**:155–166.
26. **Rice, W. G., T. Ganz, J. M. Kinkade, M. E. Selsted, R. I. Lehrer, and R. T. Parmley.** 1987. Newly identified defensin-rich dense granules of human neutrophils. Blood **70**:757–765.
27. **Romeo, D., B. Skerlavaj, M. Bolognesi, and R. Gennaro.** 1988. Structure and bactericidal activity of an antibiotic dodecapeptide purified from bovine neutrophils. J. Biol. Chem. **263**:9573–9575.
28. **Segal, A. W., M. Geisow, R. Garcia, A. Harper, and R. Miller.** 1981. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. Nature (London) **290**:406–409.
29. **Selsted, M. E., and S. S. L. Harwig.** 1987. Purification, primary structure, and antimicrobial activities of a guinea pig neutrophil defensin. Infect. Immun. **55**:2281–2286.
30. **Shafer, W. M., L. E. Martin, and J. K. Spitznagel.** 1986. Late intraphagosomal hydrogen ion concentration favors the in vitro antimicrobial capacity of a 37-kilodalton cationic granule protein of human neutrophil granulocytes. Infect. Immun. **53**:651–655.
31. **Thorne, K. J. I., R. Oliver, and A. J. Barrett.** 1976. Lysis and killing of bacteria by lysosomal proteinases. Infect. Immun. **14**:555–563.
32. **Tobias, P. S., J. C. Mathison, and R. U. Ulevitch.** 1988. A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. J. Biol. Chem. **263**:13479–13481.
33. **Viljanen, P., P. Koski, and M. Vaara.** 1988. Effect of small cationic leukocyte peptides (defensins) on the permeability barrier of the outer membrane. Infect. Immun. **56**:2324–2329.
34. **Weiss, J., M. Hutzler, and L. Kao.** 1986. Environmental modulation of lipopolysaccharide chain length alters the sensitivity of *Escherichia coli* to the neutrophil bactericidal/permeability-increasing protein. Infect. Immun. **51**:594–599.
35. **Weiss, J., and I. Olsson.** 1987. Cellular and subcellular localization of the bactericidal/permeability increasing protein of neutrophils. Blood **69**:652–659.
36. **Weiss, J., M. Victor, O. Stendahl, and P. Elsbach.** 1982. Killing of gram-negative bacteria by polymorphonuclear leukocytes. Role of an O_2-independent bactericidal system. J. Clin. Invest. **69**:959–970.
37. **Zerial, A., B. Skerlavaj, R. Gennaro, and D. Romeo.** 1987. Inactivation of herpes simplex virus by protein components of bovine neutrophil granules. Antiviral Res. **7**:341–352.
38. **Zimmerman, W., and A. Rosselet.** 1977. Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. Antimicrob. Agents Chemother. **12**: 368–372.