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Mechanisms of Antibacterial Action of Tachyplesins and Polyphemusins, a Group of Antimicrobial Peptides Isolated from Horseshoe Crab Hemocytes

MICHIO OHTA,[1]* HIDEO ITO,[1] KOJI MASUDA,[2] SHIGENORI TANAKA,[3] YOSHICHIKA ARAKAWA,[1] ROCHAPORN WACHAROTAYANKUN,[1] AND NOBUO KATO[1]

Department of Bacteriology[1] and Department of Oral Surgery,[2] Nagoya University School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466, and Tokyo Institute, Seikagaku Kogyo Co., Tateno 3-1253, Higashiyamato 189,[3] Japan

Received 25 October 1991/Accepted 1 May 1992

Tachyplesins I and II and polyphemusins I and II, cationic peptides isolated from the hemocytes of horseshoe crabs, show bactericidal activities with similar efficiencies for both gram-negative and gram-positive bacteria. Tachyplesin I inhibited bacterial growth irreversibly within 40 min. A subinhibitory concentration of tachyplesin I sensitized gram-negative bacteria to the bactericidal actions of novobiocin and nalidixic acid, although polymyxin B-resistant strains which have altered lipopolysaccharides were susceptible to tachyplesin I. This implies that tachyplesin permeabilizes the outer membrane and that the likely target of its action is outer membrane constituents other than lipopolysaccharides. On the other hand, a defensin-susceptible *phoP* strain of *Salmonella typhimurium* was also susceptible to tachyplesin I. Tachyplesin I rapidly depolarized the inverted inner-membrane vesicles of *Escherichia coli*. These results suggest that depolarization of the cytoplasmic membrane, preceded by the permeabilization of the outer membrane for gram-negative bacteria, is associated with tachyplesin-mediated bactericidal activity. The similarity between the actions of tachyplesin and those of defensin was discussed.

There are a number of cationic peptides which exert antimicrobial activities. These include antibiotic polymyxins (27), nisin (22), Pep 5 (23), AS-48 (6), a small bacteriocin (C3603) (9), cationic polyamino acids (3, 11, 27), protamines (28), platelet-derived basic peptides (4), seminal plasmin (21), cecropins from the silk moth (2), magainins from *Xenopus* skin (33), and defensins (8, 26). In 1988, a cationic peptide, tachyplesin I, was found in the hemocytes of Japanese horseshoe crabs (*Tachypleus tridentatus*) as an inhibitor of the bacterial lipopolysaccharide (LPS)-mediated activation of factor C, the initiation factor in the *Limulus* clotting cascade (19). Tachyplesin I formed a complex with LPS and inhibited the growth of both gram-negative and gram-positive bacteria at low concentrations. Later, similar cationic peptides, tachyplesin II, polyphemusin I, and polyphemusin II, were isolated from hemolysate from American horseshoe crabs (*Limulus polyphemus*) (17). Tachyplesin III was isolated from hemolysate from southeast Asian horseshoe crabs (*Carcinoscorpius rotundicauda* and *Tachypleus gigas*) (18). These peptides inhibited the growth of not only gram-negative and gram-positive bacteria, but also that of *Candida albicans*.

Tachyplesins I, II, and III consist of a total of 17 amino acid residues with a COOH-terminal arginine α-amide, and polyphemusins consist of 18 residues with an additional Arg at the NH_2 terminus (17, 18). These peptides contain 6 basic amino acid residues which make peptides cationic. Two intramolecular disulfide linkages and three tandem repeats of a tetrapeptide, hydrophobic amino acid-Cys-aromatic amino acid-Arg, are characteristic structures of these peptides. Since these cationic peptides are highly abundant (approximately 10 mg in the total hemolymph of an individual horseshoe crab), they may play a role in the self-defense mechanisms of horseshoe crabs. In this report, we describe the mechanisms of bactericidal activity of these endogenous antibiotic peptides.

MATERIALS AND METHODS

Organisms. *Escherichia coli* SC9251 and its polymyxin B-resistant mutants SC9252 and SC9253 and *Salmonella typhimurium* SH9178 and SH6482 and their polymyxin B-resistant mutants SH7426 and SH6497, respectively, were provided by E. J. McGroarty (20). *E. coli* K003 was provided by T. Mizuno, Faculty of Agriculture, Nagoya University. *S. typhimurium* LT2 was from M. Nakano, Jichi Medical College. *S. typhimurium* 14028s and its *phoP* mutant, MS7953, were provided by F. Heffron (5). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were recent clinical isolates obtained from Nagoya University Hospital. Unless otherwise noted, bacteria were cultivated in Luria-Bertani (LB) broth (1% tryptone [Difco], 0.5% yeast extract [Difco], and NaCl) and on LB agar plates. A portion of an overnight culture of each strain was transferred into new LB broth and cultivated with constant shaking. A late-log-phase culture was diluted to an appropriate concentration with T broth (0.5% tryptone, 0.5% NaCl) and used for the antibacterial assay.

Compounds. Native tachyplesin I was purified from the acid extract of horseshoe crab hemocytes as described by Nakamura et al. (19). Synthetic tachyplesins I and II and polyphemusins I and II were kindly provided by N. Fujii, Faculty of Pharmaceutical Sciences, Kyoto University (1) and M. Ohno, Faculty of Science, Kyushu University. Synthetic tachyplesins and polyphemusins were used unless indicated otherwise. Polymyxin B and novobiocin were

* Corresponding author.

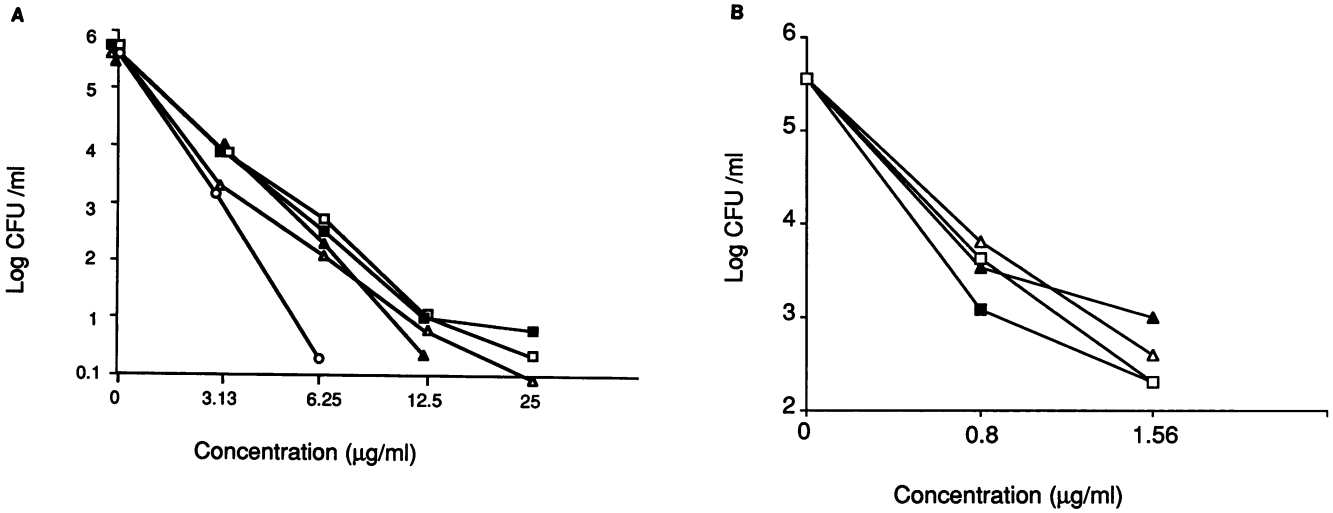

FIG. 1. Bactericidal activities of tachyplesins and polyphemusins against *S. typhimurium* LT2 (A) and *S. aureus* 198 (MRSA) (B). CFUs are per milliliter of culture. Symbols: ▲, tachyplesin I; △, tachyplesin II; ■, polyphemusin I; □, polyphemusin II; ○, native tachyplesin I.

purchased from Sigma Chemical Co. Nalidixic acid was kindly provided by Daiichi Pharmaceutical Co. NADH was purchased from Boehringer Mannheim, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was from Sigma Chemical Co., and oxonol V was from Molecular Probes, Inc.

Susceptibility testing. Because of poor diffusion in agar and/or the presence of inhibitory contaminants, the standard twofold-dilution method in agar and the disk diffusion method could not be used. Therefore, MICs were determined by using the broth microdilution method in 0.2-ml volumes of T broth with approximately 10^5 CFU/ml as the inoculum. Microdilution plates were incubated at 37°C for 18 h, and the MICs were determined by finding the lowest concentration with no visible growth. In some tests, 50-μl aliquots of cultures were removed 60 min after exposure to drugs at 37°C and plated onto LB agar plates to measure the bacteria-killing effects of tachyplesins. The plates were incubated for 18 h at 37°C before colonies were counted.

Time-kill study. The bactericidal kinetics of tachyplesin I against *E. coli* and *S. typhimurium* were determined in T broth. The late-log-phase culture was diluted with T broth to give final cell densities of approximately 10^5 to 10^6 CFU/ml. Tachyplesin I was added to T broth before inoculation to achieve the final concentration of 25 μg/ml for testing. A separate culture of T broth without tachyplesin I served as a control for growth. For testing tachyplesin-induced susceptibility to novobiocin and nalidixic acid, antibiotics were diluted with the T broth which contained tachyplesin I. After inoculation of the bacterial suspension, the final concentration of tachyplesin I was one-half its MIC for bacteria. At designated time intervals after exposure to drugs, 20-μl aliquots of cultures were removed, diluted in saline, and plated onto LB agar plates. The plates were incubated for 18 h at 37°C for colony counting.

Preparation of inverted membrane vesicles. Inverted membrane vesicles were prepared according to the method described by Yamada et al. (31). *E. coli* K003 was used as the source of inverted membrane vesicles. Bacteria at late log phase were collected and converted to spheroplasts by treatment with EDTA-lysozyme in Tris-acetate buffer (pH 7.8). After DNase was added (50 μg/ml), the spheroplasts were treated with an Aminco French pressure cell and a Teflon homogenizer. The cell lysate was centrifuged at 150,000 × *g* for 2 h, and the membrane vesicles were precipitated. The membrane vesicles were then layered over a linear gradient of 30 to 44% (wt/wt) sucrose dissolved in Tris-dithiothreitol-glycerol buffer and centrifuged at 60,000 × *g* for 15 h for the fractionation of the inner membrane. Much of the outer membrane was pelleted on the bottom under these conditions. The inner-membrane vesicles were dispersed in a minimal volume of 50 mM Tris buffer (pH 7.8) and stored at −80°C. The amount of membrane vesicles was expressed as that of proteins.

Determination of membrane potential (Δψ). The generation of Δψ (inside positive) in membrane vesicles was monitored by following the fluorescence quenching of oxonol V (25). The reaction mixtures at 37°C contained 50 mM potassium phosphate (pH 7.5), 5 mM $MgSO_4$, membrane vesicles (50 μg/ml), and 1 μM oxonol V. The fluorescence emission of oxonol V was measured at 635 nm, with excitation at 580 nm.

RESULTS

Antibacterial activities of tachyplesins. The MICs of synthetic tachyplesins I and II, polyphemusins I and II, and native tachyplesin I for *S. typhimurium* LT2, determined by using the broth microdilution method, were 3.13, 3.13, 3.13, 6.25, and 3.13 μg/ml, respectively. Figure 1A shows the bactericidal effects of these peptides at various concentrations after 60 min of exposure at 37°C. Tachyplesins I and II and polyphemusin I were similarly effective against *S. typhimurium* LT2, and polyphemusin II was less effective than the others. The preparation of native tachyplesin I was slightly more effective than that of the synthetic one. The dose responses of the antibacterial activities of these four peptides for a clinical isolate of *S. aureus* were also tested. As shown in Fig. 1B, all four peptides have the same bactericidal activity against *S. aureus*. The effect of tachyplesin I on 65 clinical isolates of MRSA was measured. All MRSA isolates tested were susceptible to tachyplesin I; the MIC for 12 isolates was 3.13 μg/ml; for 48 isolates, it was 6.25 μg/ml; and for 5 isolates, it was 12.5 μg/ml. All of these isolates were highly resistant to polymyxin B (data not

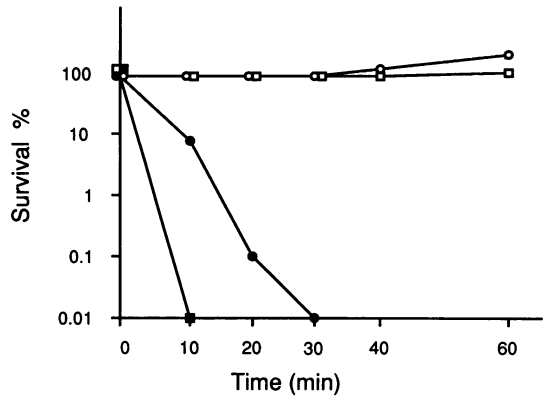

FIG. 2. Killing kinetics of tachyplesin I at 25 μg/ml with *E. coli* SC9251 and *S. typhimurium* LT2. Details are given in Materials and Methods. Data are presented as percent survival relative to initial inocula. Bacteria are *S. typhimurium* LT2 in the absence (○) or presence (●) of tachyplesin I and *E. coli* SC9251 in the absence (□) or presence (■) of tachyplesin I.

shown). The time-kill study shows that tachyplesin I inhibited the growth of bacteria rapidly and irreversibly within 40 min at a concentration of 25 μg/ml (Fig. 2). When bacteria were exposed to tachyplesin I in 0.01 M phosphate-buffered saline (pH 7.2) instead of T broth, the bactericidal effect of tachyplesin I was only weak (data not shown).

Mechanisms of the action of tachyplesins. Testing of tachyplesin-induced susceptibility to novobiocin and nalidixic acid was performed by using the method described by Viljanen and Vaara (30) when they studied the effect of polymyxin B nonapeptide on sensitization of bacteria to hydrophobic antibiotics. Table 1 shows the MICs of novobiocin and nalidixic acid for *E. coli* SC9251 and *S. typhimurium* SH9178 under the influence of a subinhibitory concentration of tachyplesin I. Tachyplesin I at one-half its MIC sensitized these bacteria to novobiocin and nalidixic acid by a factor of 4 to 16. Time-kill curves confirmed this effect (Fig. 3A and B). Some degree of synergistic effect on the killing of bacteria was observed after 120 min of incubation with the combination of tachyplesin I and novobiocin or nalidixic acid. These hydrophobic antibiotics are believed to penetrate the outer membrane through the hydrophobic pathway.

Because of the cationic character and high binding affinity to LPS of gram-negative bacteria (19), a functional similarity of tachyplesins to a cationic peptide antibiotic, polymyxin B, was expected. We accordingly compared the antibacterial activity of tachyplesin I with that of polymyxin B. The resistance of *S. typhimurium* SH7426 and SH6497 and *E. coli* SC9252 and SC9253 to polymyxin B resulted from increased esterification of phosphate residues of their LPSs and therefore from a decrease in the binding of polycationic antibiotics to LPSs (20). As shown in Table 2, however, these polymyxin B-resistant strains were as susceptible as or somewhat more susceptible to tachyplesin I than their parent strains. Moreover, all MRSA isolates tested were susceptible to tachyplesin I rather than polymyxin B. These results indicate that the mechanism of antibacterial action of tachyplesin I is likely different from that of polymyxin B.

TABLE 1. MICs of novobiocin and nalidixic acid against *E. coli* SC9251 and *S. typhimurium* LT2 in the presence of tachyplesin I

Organism and antibiotic	MIC (μg/ml) at a tachyplesin I concn (μg/ml) of:	
	0	1/2 MIC[a]
E. coli SC9251		
Novobiocin	100	12.5
Nalidixic acid	12.5	1.56
S. typhimurium LT2		
Novobiocin	100	6.25
Nalidixic acid	6.25	1.56

[a] MICs of tachyplesin I against *E. coli* SC9251 and *S. typhimurium* LT2 in this experiment are 3.13 and 1.56 μg/ml, respectively. 1/2 MIC, one-half the MIC required.

Another hypothesis is that the bactericidal action of tachyplesins would resemble that of the defensin family. Defensins are small cationic peptides isolated from the granules of macrophages and neutrophils (8). It was demonstrated previously that *S. typhimurium phoPQ* mutants showed increased susceptibility to defensins (5, 16). We therefore tested the susceptibility of a *phoP Salmonella* mutant to tachyplesin. As shown in Fig. 4, tachyplesin I had a strong bactericidal effect on a *phoP* mutant, MS7953s. The survival of the mutant after 60 min of incubation with tachyplesin I was 100-fold lower than that of the wild strain 14028s.

Effect of tachyplesin I on inverted membrane vesicles. It was reported previously that defensin-mediated bactericidal activity against *E. coli* is associated with sequential permeabilization of the outer and inner membranes and that inner-membrane permeabilization appears to be the lethal event (15) and, moreover, that defensins form voltage-dependent, weakly anion-selective channels in planar lipid bilayer membranes (10). Accordingly, the effect of tachyplesin I on inverted inner-membrane vesicles was tested. Inverted membrane vesicles were prepared from K002 spheroplasts by disruption with a French pressure cell and subsequent fractionation by sucrose gradient centrifugation as described by Yamada et al. (31). The generation of membrane potential $\Delta\psi$ (inside positive) in membrane vesicles was monitored by observing the fluorescence quenching of oxonol V (25). As shown in Fig. 5, the membrane potential generated by NADH was disrupted by the addition of carbonyl cyanide *m*-chlorophenylhydrazone, a membrane-depolarizing agent. Tachyplesin I also inhibited the generation of $\Delta\psi$ in a dose-dependent manner. Once membrane vesicles were depolarized completely by tachyplesin I, no additional effect of carbonyl cyanide *m*-chlorophenylhydrazone was observed. The preexposure of membrane vesicles with tachyplesin I abolished the generation of membrane potential by NADH. These observations indicate that the inner membrane of bacteria is the primary target of tachyplesin I.

DISCUSSION

Although tachyplesins have in vitro high affinities to LPS as well as cationic character, the mechanism of their antibacterial activity seems different from that of polymyxin B. The present data show that polymyxin B-resistant strains which have altered LPS structures are susceptible to tachyplesin I and that gram-positive cocci such as *S. aureus* strains (MRSA) are also susceptible to it. Vaara and Vaara have reported that polymyxin B nonapeptide sensitizes gram-negative bacteria to the hydrophobic antibiotics novobiocin, fusidic acid, and erythromycin (28). The cationic peptides protamine and synthetic lysine polymers had a

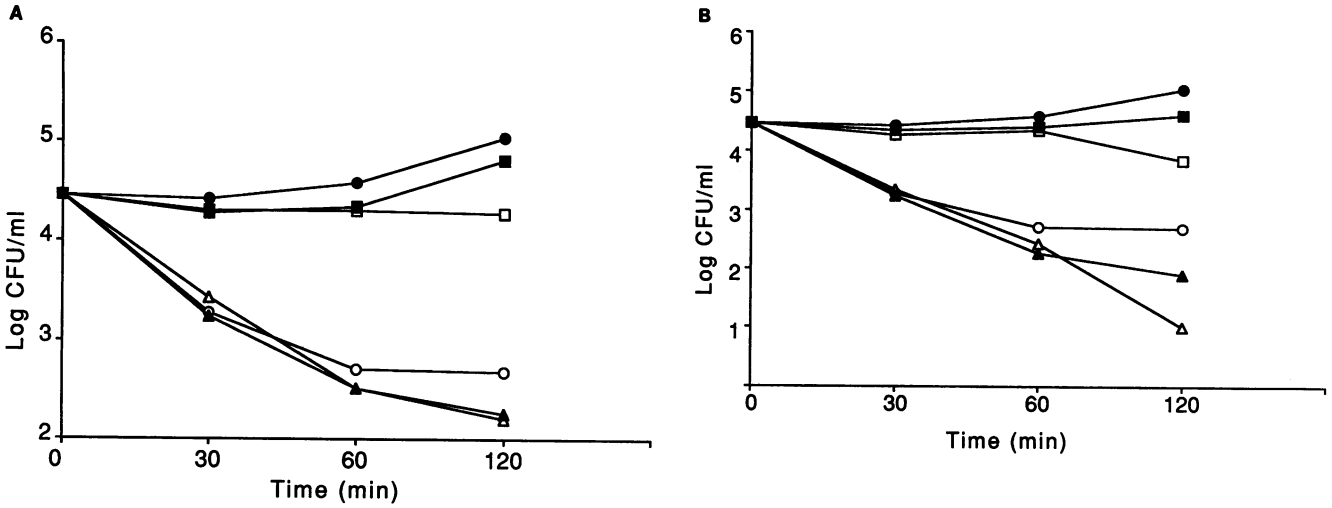

FIG. 3. Kinetic studies of the combined effect of tachyplesin I and novobiocin (A) and nalidixic acid (B) against *S. typhimurium* LT2. CFUs are the viable cells per milliliter of culture at the indicated times. (A) Symbols: ●, T broth; ○, tachyplesin I (0.78 μg/ml, one-half the MIC); □, novobiocin (50 μg/ml, one-half the MIC); ■, novobiocin (12.5 μg/ml, one-eighth the MIC); △, tachyplesin I (0.78 μg/ml) plus novobiocin (50 μg/ml); ▲, tachyplesin I (0.78 μg/ml) plus novobiocin (12.5 μg/ml). (B) Symbols: ●, T broth; ○, tachyplesin I (0.78 μg/ml); □, nalidixic acid (3.13 μg/ml, one-half the MIC); ■, nalidixic acid (0.78 μg/ml, one-eighth the MIC); △, tachyplesin I (0.78 μg/ml) plus nalidixic acid (3.13 μg/ml); ▲, tachyplesin I (0.78 μg/ml) plus nalidixic acid (0.78 μg/ml).

similar effect. This was explained as follows. Tight binding of these cationic peptides to LPS disrupted the outer membrane as a permeability barrier to allow increased penetration of hydrophobic antibiotics (28). Tachyplesin I also showed some degree of synergistic effect on the hydrophobic antibiotics novobiocin and nalidixic acid. Taken together, these observations show that tachyplesin permeabilizes the outer membrane of gram-negative bacteria, but unlike polymyxin B, the target of its action is likely different from LPS.

Defensins are a family of 29- to 34-amino-acid peptides found in the granules of mammalian phagocytes. The members of this family are arginine rich, and all share 6 conserved cysteine residues that participate in three intramolecular disulfide bonds (7). These intramolecular disulfide bonds between cysteine residues make the molecules cyclic. Because of the structural similarity between defensins and tachyplesins, common mechanisms in the antimicrobial activities were anticipated for them. Tachyplesins have microbicidal activities against a variety of gram-negative and gram-positive bacteria (17; also this study) and fungi (17), as do defensins (7). Recently, it has been reported that the *phoPQ* genetic locus, a two-component regulon, has a role in

TABLE 2. Activities of tachyplesin I against polymyxin B-resistant strains and their parents

Organism and strain	MIC (μg/ml)	
	Polymyxin B	Tachyplesin I
E. coli		
SC9251	3.13	6.25
SC9252	>100	3.13
SC9253	>100	3.13
S. typhimurium		
SH9178	3.13	6.25
SH7426	50	3.13
SH6482	3.13	3.13
SH6497	50	3.13

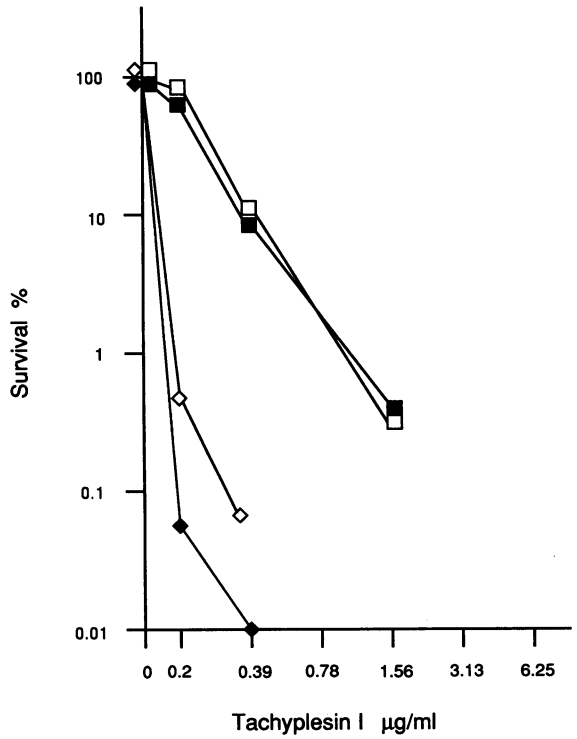

FIG. 4. Sensitivity of a *phoP* mutant to tachyplesin I. Data are presented as percent survival relative to cells incubated without tachyplesin I. Symbols: ■, *S. typhimurium* 10428, a wild-type strain (1.5×10^5 CFU/ml as the inoculum); □, 10428 (3.0×10^4 CFU/ml); ◆, *S. typhimurium* MS7953, a *phoP* mutant strain (1.7×10^5 CFU/ml); ◇, MS7953 (3.4×10^4 CFU/ml).

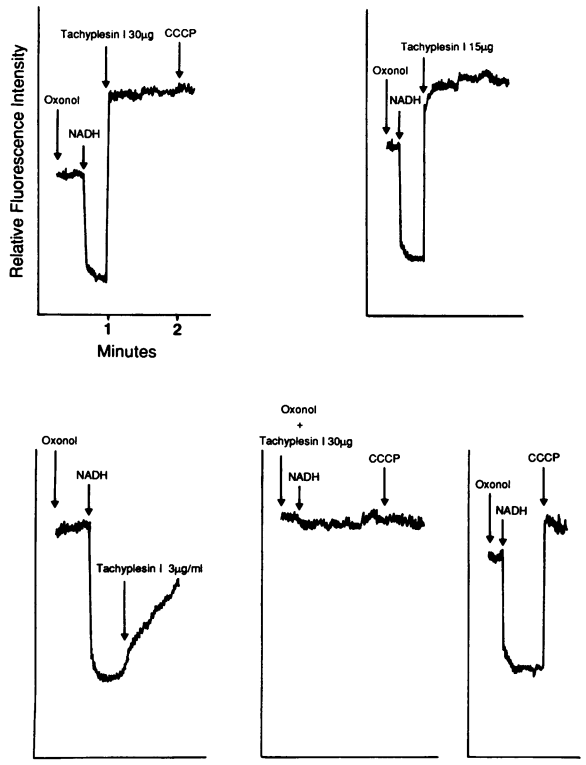

FIG. 5. Effect of tachyplesin I on the generation of $\Delta\psi$ by inverted membrane vesicles. The generation of $\Delta\psi$ (inside positive) by inverted membrane vesicles was monitored by means of fluorescence quenching as described in Materials and Methods. The membrane vesicles were energized by the addition of 1 mM NADH. A membrane-depolarizing agent, carbonyl cyanide *m*-chlorophenylhydrazone, was used at 10 μM.

resistance to defensins and is required for virulence of *Salmonella* species (5, 16). Mutants with defects in either the *phoP* or *phoQ* gene had increased sensitivities to defensins. In this study, we have shown that an *S. typhimurium phoP* mutant is highly susceptible to tachyplesin I compared with the parent strain.

In a previous study, it was suggested that defensins, like other polycations, interacted with the outer membrane of gram-negative bacteria and that one of their major functions might be the permeabilization of the outer membrane (24). Later, Lehrer et al. observed that defensin sequentially permeabilized the outer membrane and inner membrane of *E. coli* and that the rate and extent of bacterial death more closely paralleled a loss of inner-membrane integrity than outer-membrane permeabilization (10, 15). Tachyplesin I inhibited the generation of membrane potential in the inverted inner membrane vesicles rapidly. We therefore suggest that this ability, preceded by the permeabilization of the outer membrane for gram-negative bacteria, contributes to the bactericidal activity of tachyplesin I. Indeed, the results obtained with tachyplesin I are strikingly similar to those obtained with Pep 5 (14, 23), nisin (22), melittin (12), AS-48 (6), and colicins (E1, Ia, Ib, A, K, and V) (13, 29, 32), as well as defensins. The cytoplasmic membrane is the primary target for these antimicrobial peptides disrupting the membrane potential by forming ion-permeable channels. Except for AS-48, these peptides require membrane potential for disruption of the cytoplasmic membrane. This voltage dependency of channel formation may explain the observation that energized cells growing aerobically in rich medium are more susceptible to tachyplesins than are cells in saline (data not shown). Although we do not have direct evidence for the channel-formation ability of tachyplesins, permeabilization of the cytoplasmic membrane must be the lethal event in the action of tachyplesins.

Tachyplesins consist of a total of 17 amino acid residues (tachyplesin) or 18 amino acid residues (polyphemusin) and are 11 to 17 amino acid residues shorter than defensins, though these peptides resemble each other in their chemical structure. The potential of bactericidal activities of tachyplesins seems much higher than that of defensins in our preliminary experiments. These advantages of tachyplesins may help us to explore new antimicrobial drugs based on these natural peptides.

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