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Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria

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Abstract

Magainin peptides, isolated from *Xenopus* skin, have broad spectra of antimicrobial activity and low toxicities to normal eukaryotic cells, thus being good candidates for therapeutic agents. The mechanism of action is considered to be the permeabilization of bacterial membranes. A number of studies using lipid vesicles have elucidated its molecular detail. However, their interactions with bacteria are not yet well understood. In this paper, we synthesized several magainin analogs with different charges (0 to +6) and hydrophobicities, and systematically studied their interactions with the outer and inner membranes of three species of Gram-negative bacteria (*Escherichia coli*, *Acinetobacter calcoaceticus*, *Proteus vulgaris*). The treatment of the *E. coli* cells with native magainin 2 (+4) immediately induced the efflux of the intracellular K^+ ions and the cell death. A number of blebs were formed on the bacterial surface and the outer membrane became leaky. An increase in the peptide's positive charge enhanced the outer membrane permeabilization and the bactericidal activity. The cationic peptides also effectively permeabilized the inner membranes rich in acidic phospholipids, indicating the importance of electrostatic interactions. Substitution of Trp for Phe simultaneously increased the bactericidal activity and the hemolytic activity. A strategy to develop potent antimicrobial peptides was discussed on the basis of these results.

Keywords: Magainin; Outer membrane; Inner membrane; Gram-negative bacterium; Membrane permeabilization; Electrostatic interaction

1. Introduction

The widespread use of exogenous antibiotics increases the resistance of pathogenic microorganisms to them, posing a threat to their medical use in the future. For the last decade, new classes of endogenous peptidic antibiotics of animal origin have been extensively investigated as alternatives (for review see [1,2]), e.g., cecropins from insects [3], tachyplesins from horseshoe crab hemolymph [4], magainins from frog skin [5], and defensins from mam-

Abbreviations: LPS, lipopolysaccharide; Fmoc, fluoren-9-ylmethoxycarbonyl; RP-HPLC, reverse phase high-performance liquid chromatography; TLC, thin-layer chromatography; FID, flame-ionization detector; ATCC, American Type Culture Collection; CFU, colony-forming units; MIC, minimal inhibitory concentration; HED, heptaethylene glycol dodecyl ether; MG2, magainin 2

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malian neutrophils [6]. These peptides possess the common features of net positive charges due to the presence of multiple Arg and Lys residues and of forming amphipathic structures. Despite very diverse peptide sequences and structures, the action mechanisms of most host defense peptides are considered to enhance the permeability of bacterial membranes [1,7–9]. A number of studies by use of lipid bilayers as a model membrane system have been accumulated to elucidate the molecular mechanisms of the membrane permeabilization. However, the interactions of these peptides with bacterial membranes have been less extensively investigated.

Magainin peptides are isolated from the skin of the African frog, *Xenopus laevis*, and possess antibiotic activities against various microorganisms, including Gram-positive and Gram-negative bacteria, fungi, and protozoa and even cancer cells [5,10,11]. It is generally accepted that membrane lipids constitute the main target of the peptides, because (1) magainins permeabilize lipid bilayers [12–14] and form ion channels [15,16], and (2) all-D enantiomer peptides are equipotent relative to the parent all-L peptides; the action mechanism does not involve interactions with a chiral receptor nor enzyme [17,18]. We have elucidated the molecular mechanisms of the magainin-induced membrane permeabilization as follows [12–14,19–21]. Positively charged magainins preferentially interact with acidic phospholipids to form amphiphilic helices, which lie parallel to the membrane surface. Five helices spontaneously form membrane-spanning pores of short duration. Upon the disintegration of the pores, a fraction of the peptides translocates into the inner leaflets, causing pore deactivation. The pore lining is composed of not only the hydrophilic residues of the peptides but also the lipid head groups, allowing the rapid flip-flop of the lipids by lateral diffusion through the pores. Thus, the peptides can simultaneously dissipate the membrane potential and the lipid asymmetry.

Magainins are expected to be good candidates for therapeutic agents because they are preferentially active toward prokaryotic membranes. Three factors regulate this membrane selectivity [20]. (1) The outer leaflets of normal eukaryotic cell membranes are predominantly composed of zwitterionic lipids such as phosphatidylcholine and sphingomyelin [22], with which the interactions of magainins are rather weak. On the other hand, bacterial membranes abundantly contain negatively charged lipids such as phosphatidylglycerol, cardiolipin and lipopolysaccharides (LPS), which significantly enhance the membrane binding of the peptides. (2) Eukaryotic cell membranes are rich in cholesterol, while bacterial cell membranes are devoid of it. Cholesterol inhibits magainin-induced lysis of both erythrocytes and liposomes. (3) Bacterial cells have large, inside-negative transmembrane potentials promoting magainins' attack.

The development of potential antibiotic peptides requires the detailed knowledge of peptide–bacteria interactions, which is extremely lacking. Magainins disorder the outer membrane of *Salmonella typhimurium* [23–25] and dissipate the membrane potential of the inner membrane of outer membrane-permeabilized *Escherichia coli* [26]. Do they lyse this membrane? Is there a correlation between the acidic lipid contents of the membrane and the susceptibility of the bacteria to magainins? How do electrostatic interactions play a role in the magainin–bacteria interactions? To shed light on these problems, we carried out a systematic study using a series of magainin 2 analogs of different electric charges. The peptides with higher positive charges were found to effectively permeabilize both the outer and inner membranes. The lysis of the latter membrane was correlated with the acidic phospholipid content.

2. Materials and methods

2.1. Materials

Magainin 2, its analogs, and tachyplesin I were synthesized by use of a Fmoc-based solid phase method and purified by RP-HPLC and gel filtration, as reported previously [13,27,28]. Highly purified melittin was obtained from Boehringer Mannheim (Mannheim, Germany). The absence of phospholipid hydrolysis due to possible contamination of phospholipase A_2 was confirmed by a TLC/FID analyzer Iatroscan MK-5 (Iatron Laboratories, Tokyo, Japan). The purity and the concentration of the peptides were determined by quantitative amino acid analysis. The concentration of the Trp-containing peptides was routinely determined by the optical density at 280 nm.

Lysozyme from chicken egg white was purchased from Wako (Tokyo, Japan). All other chemicals from Wako were of special grade.

2.2. Bacteria

The bacterial strains used were *Escherichia coli* (ATCC 8739), *Acinetobacter calcoaceticus* (ATCC 14987), and *Proteus vulgaris* (ATCC 13315) from the Institute for Fermentation (Osaka, Japan). The bacteria were grown at 37°C in a medium (g/l) (10 casein peptone/5 yeast extract/10 NaCl (pH 7.0)). The cells were harvested in the mid-logarithmic growth phase and washed three times with a Hepes buffer (10 mM Hepes/150 mM NaCl (pH 7.0)).

2.3. Antimicrobial activities

The peptides were sterilized through a 0.22 μm filter and stepwise-diluted. The bacterial cells were mixed with each peptide and the medium in a 96-well tissue culture plate [28]. The final composition of the bacterial suspension was 10 mM Hepes/150 mM NaCl/1% (w/v) casein peptone/0.5% (w/v) yeast extract/peptide/10^6 CFU/ml bacterial cells (pH 7.0). Microbial growth was determined by the increase in optical density at 405 nm, after an 8 h incubation at 37°C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the peptides at which there was no change in optical density between time 0 and 8 h. The ζ-potential of the bacteria was calculated by use of Smoluchowski's equation [29] from their electrophoretic mobilities in the Hepes buffer at 25°C measured on an electrophoretic light scattering spectrophotometer (Zetasizer 4; Malvern Instruments).

2.4. K^+ efflux from E. coli cells

E. coli cells were grown to the mid-logarithmic growth phase, washed three times with the Hepes buffer and then resuspended in the same buffer at $5 \cdot 10^8$ CFU/ml. The K^+ efflux from the cells after addition of magainin 2 was monitored by a K^+ ion-selective electrode connected to a microprocessor ion analyzer model 901 (Orion Research, Boston, USA) [30].

2.5. Measurement of cell viability

The cell viability was measured under the same conditions as the K^+ permeability measurements. Samples (10 μl) collected at intervals were immediately diluted 100-fold to stop the antimicrobial activity of the peptide, and 100 μl of the diluted sample was dispersed onto five petri dishes with an agar medium (g/l) (15 agar/10 casein peptone/5 yeast extract/10 NaCl). The cell viability was determined by counting colonies after a 20 h incubation at 37°C.

2.6. Outer membrane permeability

Bacterial cells in the mid-logarithmic phase were suspended at a density of $1 \cdot 10^7$ CFU/ml in the Hepes buffer and preincubated with the peptides at 37°C for 10 min. A nonionic surfactant, HED, was then added to a final concentration of 0.005% (w/v), and the incubation was continued. The change in turbidity was monitored by measuring the optical density at 400 nm.

2.7. Scanning electron microscopy

E. coli cells were incubated with magainin 2 at 37°C for 30 min in the Hepes buffer. The cells were then chemically fixed with 2.5% glutaraldehyde for 2 h, collected on a Nuclepore filter (pore size, 0.2 μm) by filtration, and washed three times with the buffer. The cells on the filter were fixed with 1% osmic acid for 1 h and subsequently dehydrated with a graded ethanol series. The samples were lyophilized, coated with gold in an ion coater, and examined by scanning electron microscopy on an Hitachi S450 instrument.

2.8. Spheroplast lysis

Spheroplasts of Gram-negative bacteria were prepared by treating stationary phase cells with lysozyme-EDTA [31] and washed three times with a Hepes buffer (10 mM Hepes/150 mM NaCl/250 mM sucrose (pH 7.0)). The spheroplasts were added to the peptide, and the optical density at 500 nm was monitored at 37°C for 10 min. The lysis of the spheroplasts was measured as a decrease in optical density. For the maximal lysis, the suspension was

sonicated twice for 5 min in an ice bath. Lipids were extracted from each spheroplast suspension by the Bligh–Dyer method [32]. The individual phospholipids were quantitated by the TLC/FID analyzer.

2.9. Hemolytic assays

The hemolytic activities of the peptides were determined by use of fresh human erythrocytes (blood type A). The blood was centrifuged and washed three times with a Hepes buffer (10 mM Hepes/150 mM NaCl/1 mM EDTA (pH 7.4)) to remove the plasma and buffy coat. Peptide solutions were incubated with an erythrocyte suspension (final erythrocyte concentration, 1% v/v) at 37°C for 1 h. The percent hemolysis was determined from the optical density at 540 nm of the supernatant after centrifugation ($800 \times g$, 10 min), as described elsewhere [33]. Hypotonically lysed erythrocytes were used as a standard for 100% hemolysis.

3. Results

3.1. Magainin analogs

In the present study, we have assessed four analog peptides of magainin 2 (MG2) to investigate the effect of charge on the bactericidal activity (Fig. 1). The strategy of the chemical modification was described in detail elsewhere [34]. The parent MG2 possesses a net charge of +4 under physiological conditions. (We assume that the α-NH_2 and His groups are protonated, because magainins preferentially interact with negatively charged membranes, the local pH of whose surface is slightly acidic.) To reduce the positive charge, we synthesized two analogs in which negatively charged Glu residues were substituted for Lys residues at the 10th and/or 11th positions (10E,12W-MG2 and 10E,11E,12W-MG2). In these analogs, a Trp residue was introduced at the 12th position [28]. A 19Q-MG2a analog with an increased net charge had a Gln residue for Glu at the 19th position and the amidated C-terminus.

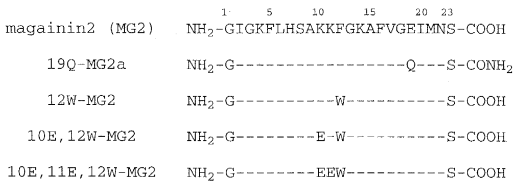

Fig. 1. Amino acid sequences of magainin 2 (MG2) and its analogs.

3.2. Antimicrobial assay

The antimicrobial activities of MG2, its four analogs, tachyplesin I, and melittin were assayed against Gram-negative bacteria (Table 1). All peptides, except inactive 10E,11E,12W-MG2, exhibited similar antibacterial spectra: *A. calcoaceticus* and *E. coli* were rather susceptible to these agents, whereas *P. vulgaris* was relatively resistant. No correlation was found between the susceptibility and the ζ-potential of the bacterial surface.

The rank order of antimicrobial activity of the peptide was tachyplesin I (+7) > melittin (+6) > 19Q-MG2a (+6) ≥ 12W-MG2 (+4) > MG2 (+4) > 10E,12W-MG2 (+2) > 10E,11E,12W-MG2 (±0). The antimicrobial activity tends to increase with the net positive charge.

3.3. Hemolytic activities

As a measure of toxicity, the hemolysis of human erythrocytes induced by the magainin analogs was

Table 1
Antimicrobial activity of membrane-active peptides and ζ-potential of bacteria

Bacteria	ζ-potential	MIC (μM)						
	(mV)	tachyplesin I (+7) [a]	melittin (+6)	19Q-MG2a (+6)	MG2 (+4)	12W-MG2 (+4)	10E,12W-MG2 (+2)	10E,11E,12W-MG2 (±0)
A. calcoaceticus	−8.9	0.781	3.13	6.25	25	6.25	50	> 100
E. coli	−12.9	0.781	> 12.5	6.25	50	12.5	100	> 100
P. vulgaris	−14.9	3.13	> 12.5	> 100	> 200	> 200	> 200	> 100

[a] The net charge of each peptide at physiological conditions is shown in parentheses.

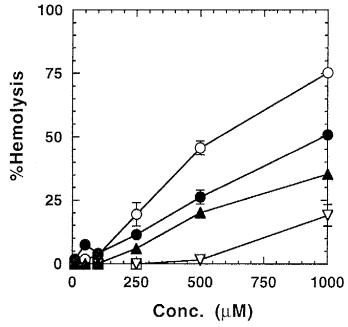

Fig. 2. Hemolytic activity of MG2 and its analogs. Each peptide solution at various concentrations was incubated with human erythrocyte suspension (final erythrocyte concentration, 1% v/v) at 37°C for 1 h. The percent hemolysis value was colorimetrically determined. Peptides: ▲, 19Q-MG2a; ●, MG2; ○, 12W-MG2; ▽, 10E,12W-MG2; □, 10E,11E,12W-MG2. The error bars represent the standard deviation of triplicate samples. The 10E,11E,12W-MG2 peptide was insoluble at concentrations greater than 250 μM.

measured (Fig. 2). The hemolytic activity of 19Q-MG2a (closed triangles) was approximately equivalent to the potency of MG2 (closed circles). The Glu-substituted magainin 2 analogs (open triangles and open squares) showed weaker hemolytic activities. The introduction of Trp enhanced the toxicity (cf. open and closed circles).

3.4. K^+ efflux from *E. coli* cells

To gain an insight into the relationship between the inner membrane permeabilization and cell death, we simultaneously measured the efflux of intracellular potassium ions and the cell viability by a K^+-selective electrode and an agar plate method, respectively. Fig. 3 shows that the addition of magainin 2 to *E. coli* cells induced a K^+ efflux from the cells (solid lines) and a reduced cell viability (closed circles). The percent cell viability value rapidly decreased to reach a plateau value of ca. 50% within 5 min at a peptide-to-cell ratio of 0.1 nmol/10^6 CFU (Fig. 3A). The K^+ efflux also started immediately after the addition of the peptide. The ion leakage leveled off at about 20 min. Even at a lower peptide-to-cell ratio of 0.01 nmol/10^6 CFU, a short contact of the peptide with the bacteria caused the K^+ ion leakage and the cell death (Fig. 3B). The presence of 10 mM Mg^{2+}, however, prevented both the K^+ efflux and the cell viability loss (open circles and dotted line in Fig. 3A).

3.5. Outer membrane permeability

The peptide-induced permeabilization of *E. coli* outer membranes was examined on the basis of resistance of the bacteria to a low concentration of a surfactant. We used a nonionic surfactant HED instead of SDS [35] to avoid a complicated interpretation of data due to the involvement of electrostatic interactions. Fig. 4 depicts that the preincubation of the *E. coli* cells with the peptide for 10 min rendered the cells susceptible to 0.005% w/v HED. They were rapidly lysed upon the addition of the surfactant (dashed line), whereas in the absence of the peptide, the detergent addition hardly reduced the turbidity (solid line). This result suggests that the peptide disrupts the barrier function of the outer membrane of the Gram-negative bacteria to allow the surfactant to permeate, leading to bacterial lysis. The ΔO.D. value 10 min after the HED treatment shown in the figure

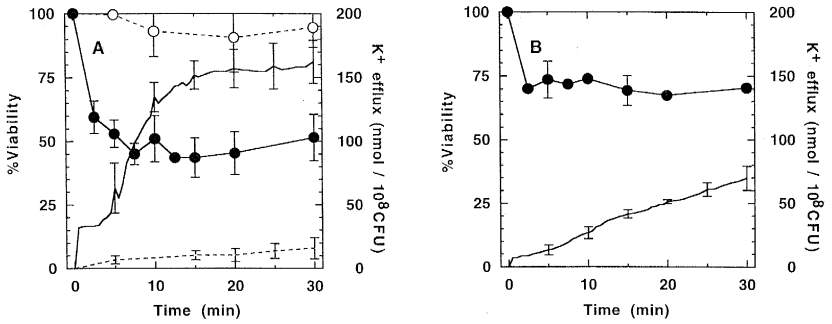

Fig. 3. Time course of the amount of K^+ efflux (solid lines) from *E. coli* cells and the cell viability (solid circles) after the addition of MG2. The data in the presence of 10 mM $MgCl_2$ are shown by dotted lines and open circles. MG2 was added to an *E. coli* cell suspension ($5 \cdot 10^8$ CFU/ml) at time 0. Peptide-to-cell ratio (nmol/10^6 CFU): panel A, 0.1; panel B, 0.01.

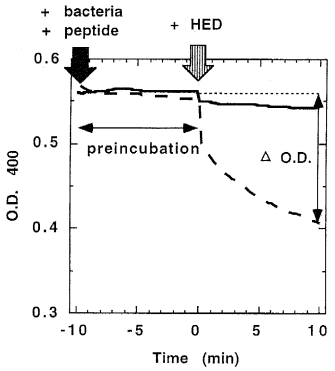

Fig. 4. Time course of outer membrane permeabilization. An *E. coli* cell suspension ($1 \cdot 10^7$ CFU/ml) preincubated with (dashed line) or without (solid line) 12W-MG2 (0.5 μM, 0.05 nmol/10^6 CFU) at 37°C for 10 min. HED (final concentration, 0.005% w/v) was then added at time 0, and the incubation was continued. The change in turbidity (ΔO.D.) was monitored by the optical density at 400 nm.

can be used as a measure of the outer membrane lesion caused by the peptide.

We examined the effect of the peptide charge on the peptide-induced permeabilization of the outer membranes of the *E. coli* cells (Fig. 5A). The percent change in the O.D. value during 10-min incubation was plotted as a function of the amount of the added peptide per cell. The rank order of the damage to the outer membrane was 19Q-MG2a $(+6)$ > 12W-MG2 $(+4)$ ≈ MG2 $(+4)$ > 10E,12W-MG2 $(+2)$ > 10E,11E,12W-MG2 (± 0), again in accordance with the decreasing order of the positive charges. The potency was roughly reduced by an order of magnitude for every loss of 2 positive charges.

Fig. 5B compares the outer membrane disruption activities of 12W-MG2 against the three Gram-negative bacteria. The outer membranes of *P. vulgaris* (circles) appeared to be significantly less susceptible to the peptide than those of *E. coli* (triangles) and *A. calcoaceticus* (squares). This observation can at least partially explain the difference in the antimicrobial activities (Table 1).

3.6. Scanning electron microscopy

In order to gain a more direct insight into the interaction of MG2 with *E. coli* , we examined the bacteria treated with the peptide by scanning electron microscopy. The untreated *E. coli* cells (Fig. 6A) had a smooth surface. In contrast, as shown in Fig. 6B, the *E. coli* cells showed clear morphological changes under the conditions where the bacterial viability had decreased by about 30% at the peptide-to-cell ratio of 0.01 nmol/10^6 CFU (Fig. 3B). The changes caused by MG2 appeared to involve the formation of blebs on the cell surface. Furthermore, string-like substances, which are considered to be cellular debris arising from cell lysis, were seen around the cells when the concentration of MG2 was increased to 0.1 nmol/10^6 CFU (Fig. 6C).

3.7. Inner membrane permeability

The peptide-induced permeabilization of bacterial cytoplasmic membranes was measured on the basis of the lysis (a decrease in O.D. 500) of the spheroplasts of the Gram-negative bacteria. The kinetics of lysis was very rapid without a measurable time lag

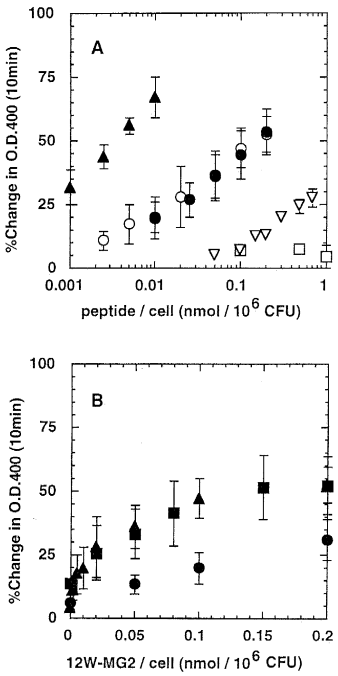

Fig. 5. Dependence of outer membrane permeability on peptide charge and bacterial species at 37°C. The percent charge in O.D. 400 10 min after the addition of HED was determined as shown in Fig. 4. (A) *E. coli* cells were incubated with various peptides. ▲, 19Q-MG2a; ●, MG2; ○, 12W-MG2; ▽, 10E,12W-MG2; □, 10E,11E,12W-MG2. (B) 12W-MG2 was applied to various bacterial species. ▲, *E. coli*; ■, *A. calcoaceticus*; ●, *P. vulgaris*. The error bars represent the standard deviation of three experiments.

(Fig. 7). Fig. 8A shows the dose response curves of the lysis of the *E. coli* spheroplasts induced by the magainin 2 analogs. The concentration of the cells was expressed as that of the extracted membrane lipids. At a peptide-to-lipid molar ratio of 0.25, 12W-MG2 (+4) lysed 50% of the cells during 10 min (open circles). The lysis triggered by 10E,12W-MG2 (+2, open triangles) was one order of magnitude weaker than that by 12W-MG2 (+4). Neutral 10E,11E,12W-MG2 was additionally less potent (open squares). In contrast to the outer membrane permeabilization, the lytic activity of 19Q-MG2a (+6, closed triangles) was similar to those of MG2 (+4, closed circles) and 12W-MG2 (+4).

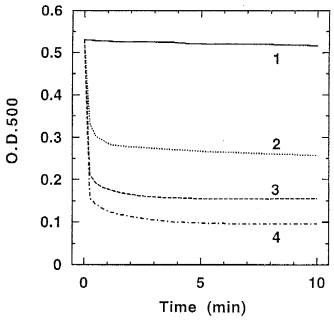

Fig. 7. Time course of *E. coli* spheroplast lysis. *E. coli* spheroplasts were incubated with 12W-MG2 at 37°C for 10 min. The change in turbidity was monitored by the optical density at 500 nm. Peptide concentration (μM): trace 1, 0; trace 2, 2; trace 3, 4; trace 4, 6.

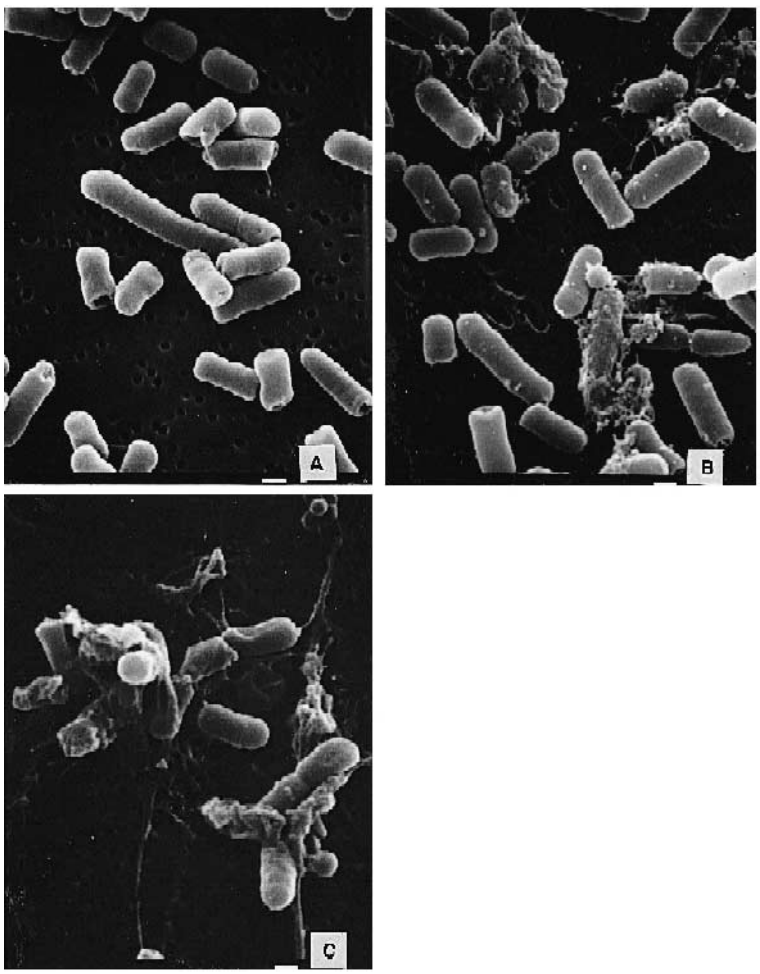

Fig. 6. Scanning electron microscopy of *E. coli* treated with MG2. *E. coli* cells were incubated with MG2 at 37°C for 30 min and the specimens were then prepared for scanning electron microscopy. (A) Intact bacteria; (B) bacteria treated with MG2 (0.01 nmol/10^6 CFU); (C) bacteria treated with MG2 (0.1 nmol/10^6 CFU). The bars represent 0.5 μm.

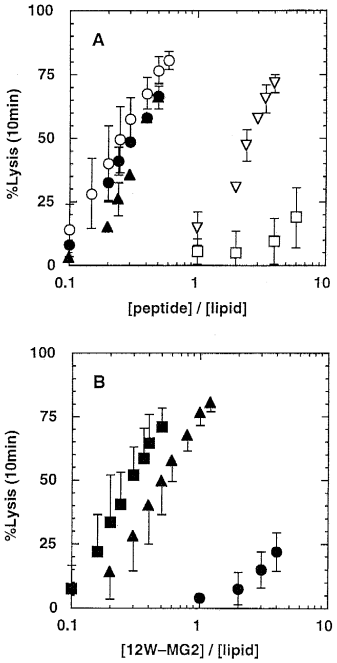

Fig. 8. Dependence of inner membrane permeability on peptide charge and bacterial species at 37°C. The percent change in O.D. 500 10 min after the addition of each peptide was determined as depicted in Fig. 7. (A) *E. coli* spheroplast were incubated with various peptides. ▲, 19Q-MG2a; ●, MG2; ○, 12W-MG2; ▽, 10E,12W-MG2; □, 10E,11E,12W-MG2. (B) 12W-MG2 was added to various spheroplasts. ▲, *E. coli*; ■, *A. calcoaceticus*; ●, *P. vulgaris*. The error bars represent the standard deviation of three experiments.

Fig. 8B illustrates the sensitivity of the spheroplasts of the three bacteria to 12W-MG2-induced lysis. The spheroplasts of *P. vulgaris* (circles) were much less susceptible to the peptide than those of *E. coli* (triangles) and *A. calcoaceticus* (squares). Table 2 summarizes the phospholipid compositions of the three spheroplast membranes, which were found to be composed of zwitterionic phosphatidylethanolamine, anionic phosphatidylglycerol and cardiolipin. A good correlation was found between the acidic phospholipid content (Table 2) and the peptide susceptibility (Fig. 8B).

Table 2
Phospholipid composition of the spheroplasts of Gram-negative bacteria [a]

Bacteria	% of lipid		
	cardiolipin	phosphatidyl-glycerol	phosphatidyl-ethanolamine
A. calcoaceticus	12.6 ± 0.7	34.7 ± 2.5	52.7 ± 2.1
E. coli	8.2 ± 0.7	23.9 ± 0.9	67.9 ± 1.1
P. vulgaris	5.8 ± 0.5	16.8 ± 1.0	77.4 ± 1.3

[a] Phospholipids were extracted from each spheroplast suspension by the Bligh–Dyer method [32]. The individual phospholipids were quantitated by the TLC/FID analyzer. Mean ± S.D. ($n = 5$).

4. Discussion

There is a consensus that the action mechanism of this class of peptides is to disrupt the barrier properties of bacterial membranes. However, it is still controversial because of the lack of basic knowledge if their ultimate target is the outer membrane or the inner membrane. Westerhoff and his coworkers [26,36] proposed that the cytoplasmic membrane, containing the electron transport chain and the enzymes for oxidative phosphorylation, is the site of action. The group of Blazyk [23–25] pointed out the importance of magainin-outer membrane interactions in addition to the involvement of the inner membrane. It was recently reported by use of magainin 2 covalently fixed to a resin that magainin's actions on the bacterial surface are sufficient for its lethal activity [37]. We have systematically examined the interactions of the magainin peptides with both outer and inner membranes to map out a strategy for the development of potent peptidic antibiotics.

4.1. Inner membrane

Magainin 2 and its analogs rapidly lysed the spheroplasts of the Gram-negative bacteria without any discernible lag (Fig. 7), indicating that the peptides can directly permeabilize the cytoplasmic membranes. Electrostatic interactions between the positively charged peptides and the negatively charged lipids play important roles in this process: The lysis of the inner membranes was correlated with the acidic phospholipid content (Fig. 8B, Table 2). Furthermore, the peptides with higher positive charges (19Q-MG2a (+6), MG2 (+4), and 12W-MG2 (+4)) were more active than the less basic peptides (10E,12W-MG2 (+2) and 10E,11E,12W-MG2 (±0)). The observation that the most basic 19Q-

MG2a is not more potent compared with the +4 peptides can be attributed to the fact that the 19Q-MG2a pore formed in the membrane is rather unstable because of the closely spaced positive charges [34].

4.2. Outer membrane

Magainins should cross the outer membranes to reach the cytoplasmic membrane. To demonstrate the alteration of the outer membrane permeability, we used the amphiphilic agent, HED (Fig. 4). It penetrates the intact outer membrane only poorly, because the membrane serves as a barrier to amphiphilic and hydrophobic molecules [38]. The peptides disrupt the barrier function of the outer membrane. Electrostatic interactions also appear to be one of the factors controlling the process. More positive peptides caused stronger membrane disturbances (Fig. 5A). In contrast to the inner membrane permeabilization, 19Q-MG2a more effectively disrupts the outer membrane, suggesting that the mode of action could be different between the two membrane systems. The presence of Mg^{2+} ions protected *E. coli* cells from the magainin-induced loss of the viability and the K^+ efflux (Fig. 3A; [39]). Because divalent cations normally function as cationic bridges between adjacent phosphates of LPS, cationic peptides were suggested to compete with the cations, thus destroying the LPS cross-bridging and disturbing the outer membrane ('self-promoted pathway'; [40]), which accompanies morphological changes in the *E. coli* surface (Fig. 6).

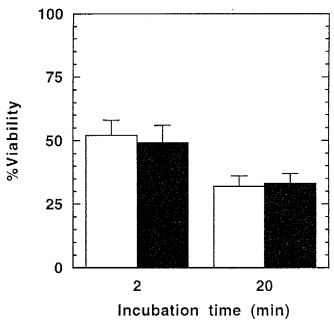

Fig. 9. Viability of MG2-treated *E. coli* cells. The *E. coli* cells were incubated with MG2, as in Fig. 3A. After 2 and 20 min, aliquots from the specimen were diluted $5 \cdot 10^5$-fold. The diluted cells were plated onto petri dishes with the agar medium immediately (empty bars) or after another 1 h incubation (filled bars). The percent viability was determined by counting colonies after a 20 h incubation at 37°C.

On the other hand, factors other than electrostatic interactions should be taken into account to explain the susceptibility differences among various bacterial species. The outer membrane of *P. vulgaris* is less susceptible to 12W-MG2 than those of *E. coli* and *A. calcoaceticus* in spite of its most negatively charged surface (Fig. 5B and Table 1). *Proteus* species are inherently very resistant to polymyxin [41]. The mechanism of polymyxin resistance in *Proteus mirabilis* is related to the high content of phosphate-linked 4-aminoarabinose in its LPS [42].

4.3. Bactericidal activity

Fig. 3 clearly shows that instantaneously after the peptide treatment, both the cell viability decrease and the K^+ efflux were observed, suggesting that the rapidly formed lesion in the inner membrane is closely related to cell death. It should be noted here that the cell viability was determined by the agar plate method immediately after the dilution of the specimen. For a microbial cell, death is defined as the *irreversible* loss of the ability to reproduce (grow and divide). The conditions of incubation in the first hour are critical in the determination of killing [43]. Therefore, we confirmed that the death of the cells induced by MG2 is irreversible. The *E. coli* cells were incubated with MG2, as in Fig. 3. After 2 and 20 min, aliquots from the specimen were diluted 5×10^5 fold, and the diluted cells were further incubated for 1 h. The colony-forming potency was then examined. Fig. 9 shows that the post-incubation failed to repair the bacterial reproducing ability. Therefore, we conclude that the initial contact of the cells with the peptide triggers the membrane disruption, causing the bacterial death. The leakage of a cytoplasmic enzyme, β-galactosidase, was undetectable (data not shown) implying that the size of the peptide-induced lesion is not very large. Similar phenomena have been reported for other peptides. Polymyxin did not release a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, from *Salmonella typhimurium* when a majority of the bacteria are killed [44].

4.4. Development of potent peptidic antibiotics

Antimicrobial peptides of therapeutic value should simultaneously fulfill the two requirements, i.e., a low toxicity and a high bactericidal activity. Both factors are closely related to the peptide–membrane interactions, which are largely affected by the affinity of the peptide for the target membrane. The binding energy originates from both the intrinsic chemical affinity, mainly due to hydrophobic interactions, and electrostatic contributions. The former dominates in the peptide-induced hemolysis, because the erythrocyte surface is exclusively composed of zwitterionic phospholipids, such as phosphatidylcholine and sphingomyelin [22]. Actually, we demonstrated that the lytic activities of several peptides against phosphatidylcholine liposomes correlate with those against human erythrocytes [20]. Furthermore, Blondelle and Houghten [45] found a good correlation between the hemolytic activities of the position-specific omission analogs of melittin and their retention times on RP-HPLC. The observation that the introduction of Trp enhanced the sensitivity to the erythrocytes (Fig. 2) may also be attributed to the enhanced hydrophobicity, although Trp shows variability in assigned hydrophobicity among different scales. The hydrophobicity difference of ca. 0.6 kcal/mol [46] would increase the binding affinity 3-fold. The presence of the NH group in Trp capable of hydrogen-bonding may also be related to the higher activity of the Trp containing peptide. Another possibility would be the bulkiness of the side chain compared to that of Phe [47]. The most basic magainin derivative, 19Q-MG2a, possesses a hemolytic activity even slightly weaker than that of MG2 (Fig. 2), probably because of the shorter life span of the former peptide [34], as in the case of the inner membrane permeabilization. The reason for the weak hemolytic potency of 10E,12W-MG2 is not clear at present. One possibility would be that the peptide with the less positive charge is easier to self-aggregate in aqueous solution at high concentrations.

In the peptide–bacteria interactions, electrostatic interactions become dominant because of the presence of LPS and acidic phospholipids. An increase in peptide positive charge enhanced the outer membrane disruption (Fig. 5A), leading to the augmentation in the antibacterial activity (Table 1). This strategy is often used to improve the bactericidal activity of peptides [39,48]. The observations that a free amino terminus is required to elicit the maximal antimicrobial activity [49] and that a decrease in pH from 7.2 to 6 enhances the bactericidal activity of magainin analogs [48] can be understood on the basis of the enhanced basicity of the peptide. However, an increase in the peptide positive charge does not always augment the bactericidal activity, suggesting a complex nature of the peptide–bacteria interactions [48]. Hydrophobicity enhancement is also a method of intensifying the antibiotic potency, as can be seen from the comparison between MG2 and 12W-MG2 (Table 1). However, this type of modification simultaneously raises the toxicity (vide supra). Chen et al. reported [49] that Gly → Ala and Ser → Ala substitutions greatly enhance the antimicrobial activity of magainin, whereas in most cases the hemolytic activity is simultaneously raised. One of the derivatives, magainin A, was suggested to have a higher membrane affinity than the parent peptide [50], probably because of its enhanced hydrophobicity.

The antibacterial spectra are regulated by the outer membrane permeability and the acidic lipid content of the cytoplasmic membrane: the insusceptibility of *P. vulgaris* to magainin can be understood on the basis of the resistance of its outer membrane (Fig. 5B) and the low acidic lipid content of the inner membrane (Fig. 8B and Table 2). It seems to be difficult to alter the antimicrobial spectrum (Table 1), as found for a set of magainin analogs [49].

In summary, we systematically investigated the interactions of a series of magainin analogs with three Gram-negative bacteria. An amphiphilic peptide of a high basicity and a low hydrophobicity would be a promising candidate for a therapeutic agent, in keeping with the results from a number of structure–activity relationship studies [51]. Other factors, such as helicity and chain length, are of course should be taken into consideration.

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