Mode of Action of the Gramicidin S Analogs Lacking Hydrophilic Amino Acid Residues on Biomembranes

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The gramicidin S analog lacking basic ornithine residues, *cyclo*(–Val–Ala–Leu–ΔPhe–Pro–)$_2$ (where ΔPhe represents α,β-dehydrophenylalanine), increased the K^+ permeability of human erythrocytes and *Staphylococcus aureus* similarly to the parent gramicidin S. This analog altered the normal discoid shape of human erythrocytes to an invaginated form. The direction of the shape change was opposite to the case of gramicidin S causing crenated cells. We suppose that the analog accumulated predominantly into the inner half monolayer of membrane and destabilized the membrane structure, resulting in a break in the membrane.

Keywords gramicidin S; structure–activity relationship; membrane–peptide interaction; membrane permeability; hemolysis; cell shape

An antibiotic gramicidin S, *cyclo*(–Val–Orn–Leu–D-Phe–Pro–)$_2$, takes a β-sheet structure whose cationic ornithine residues are located on one side of the molecular plane and hydrophobic amino acid residues on the other side, forming a strong amphiphilic structure.[1] It has long been believed that this amphiphilicity of gramicidin S is important for the antimicrobial activity. In support of this, the gramicidin S analogs of high bioactivity so far prepared had contained ornithine or other basic amino acid residues in the structure.[1] However, it was recently found that one gramicidin S analog, *cyclo*(–Val–Ala–Leu–ΔPhe–Pro–)$_2$ (where ΔPhe represents α,β-dehydrophenylalanine), lacking cationic amino acid residues, had a potent antimicrobial activity against gram-positive bacteria.[2] It is interesting that the analog in which only Orn was replaced by Ala did not induce any antimicrobial activity and further substitution of D-Phe for ΔPhe was necessary. These bioactive and bioinactive analogs are hereafter abbreviated to [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-gramicidin S and [Ala$^{2,2'}$]-gramicidin S, respectively. It was found that [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-gramicidin S increased the K^+ permeability of human erythrocytes and *Staphylococcus aureus*, but it did not increase that of *Escherichia coli*. [Ala$^{2,2'}$]-gramicidin S did not cause any K^+ permeability enhancement of these cells. The action mechanism was discussed in connection with the shape change of human erythrocytes induced by the peptides.

Materials and Methods

S. aureus FDA 209P and *E. coli* K12 strain W3110 were grown as reported previously.[3] Washed *S. aureus* and *E. coli* cells were suspended in 100 mM choline chloride and 50 mM 4-morpholinepropanesulfonic acid (Mops)–2-amino-2-hydroxymethylpropane-1,3-diol (Tris) (pH 7.2) at 5×10^9 cells/ml. Human erythrocytes were suspended in 0.15 M NaCl and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)–NaOH (pH 7.4) at 1% hematocrit. The number of cells was 1×10^8 cells/ml. Cells were incubated with a peptide at 37 °C for 30 min. The amount of K^+ efflux was measured with a K^+ ion-selective electrode.[3] The total amount of K^+ was determined by disrupting cells with a surfactant Triton X-100 or cetyltrimethylammonium bromide.[4] Hemolysis was estimated by measuring the absorbance at 540 nm.[3] The viability of cells was determined by counting colonies.[3] Cells were fixed with 2% glutaraldehyde dissolved in 1/30 M NaH_2PO_4/Na_2HPO_4 (pH 7.4) and observed under an optical microscope connected to a video camera and a display television.

Results and Discussion

Figure 1 shows changes in the K^+ permeability of human erythrocytes, *S. aureus* and *E. coli* cells induced by gramicidin S and [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-gramicidin S. [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-gramicidin S enhanced the efflux of K^+ from erythrocytes and *S. aureus* cells similarly to the parent gramicidin S, but it did not act on *E. coli* cells. This bioactive gramicidin S analog also lowered the viability of *S. aureus* cells, but it did not decrease that of *E. coli* cells at all (data not shown), indicating that the antimicrobial activity resulted from an increase in the membrane permeability as in the case of gramicidin S.[3] Under the same conditions,

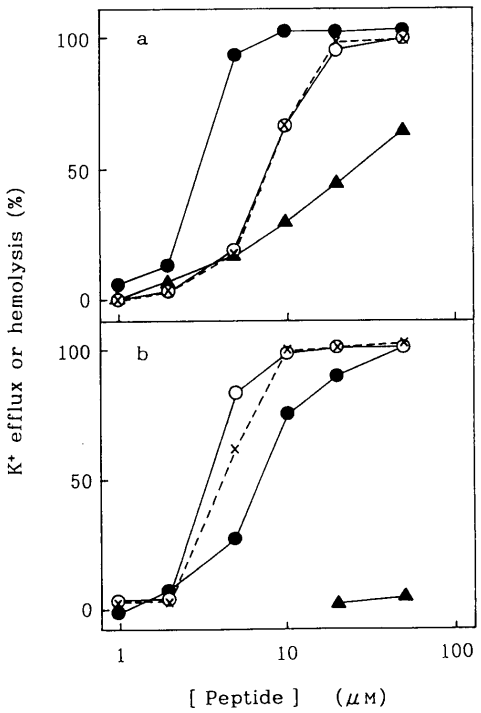

Fig. 1. The Dose–Response Curves of K^+ Efflux from Human Erythrocytes (○), *S. aureus* (●) and *E. coli* (▲) Cells and Hemolysis (×) Induced by (a) Gramicidin S and (b) [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-Gramicidin S

Erythrocytes were suspended in 0.15 M NaCl and 5 mM Hepes–NaOH (pH 7.4) at 1×10^8 cells/ml. *S. aureus* and *E. coli* cells were suspended in 100 mM choline chloride and 50 mM Mops–Tris (pH 7.2) at 5×10^9 cells/ml. Cells were incubated with the peptide at 37 °C for 30 min.

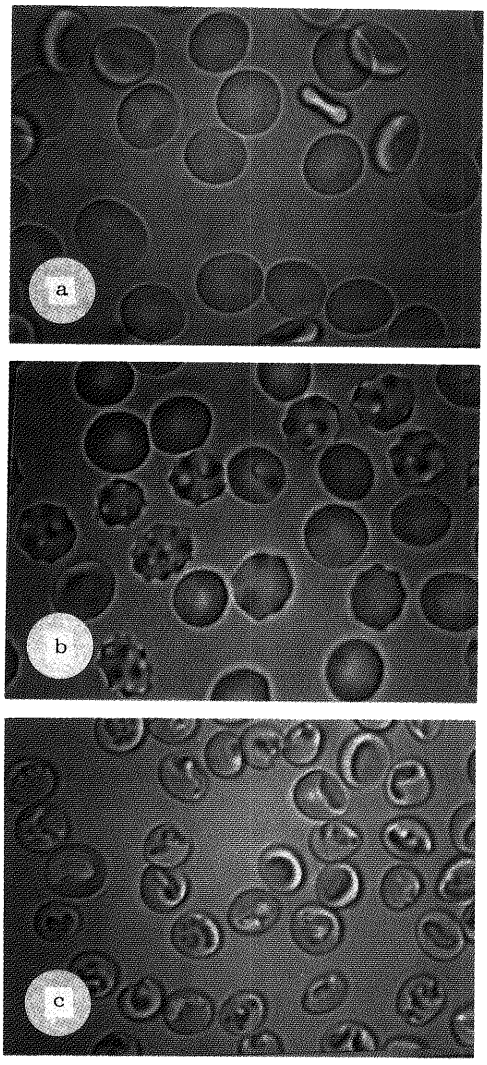

Fig. 2. Changes in the Morphology of Human Erythrocytes

(a) Intact cells. Cells were incubated with (b) gramicidin S (5 μM) or (c) [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-gramicidin S (2 μM) at 37 °C for 30 min.

[Ala$^{2,2'}$]-gramicidin S did not induce any K^+ permeability enhancement in accordance with its low antimicrobial activity. When the outer membrane structure of *E. coli* cells was disrupted by using Tris–ethylenediaminetetraacetic acid (EDTA),[3] the cells were markedly sensitive to [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-gramicidin S, indicating that the weakness of the action against *E. coli* cells was due to the lack of ability to disrupt the outer membrane structure. This result is compatible with the previous observations[5] that the outer membrane, differently from a cytoplasmic membrane, forms a permeability barrier against hydrophobic antibiotics.

Then, we estimated the size of peptide-induced membrane lesion in erythrocytes by means of an osmotic-protection experiment.[6] It was found that [Ala$^{2,2'}$,ΔPhe$^{4,4'}$]-gramicidin S formed a lesion of extremely large size (>3 nm) even at 10 μM addition of this peptide. This fact indicates that the peptide did not act as a carrier type ionophore as has been observed with many hydrophobic antibiotics.[7]

Figure 2 shows the morphological change of human erythrocytes induced by peptides. In a previous study,[3] we observed that gramicidin S disrupted the membrane structure of erythrocytes immediately after the appearance

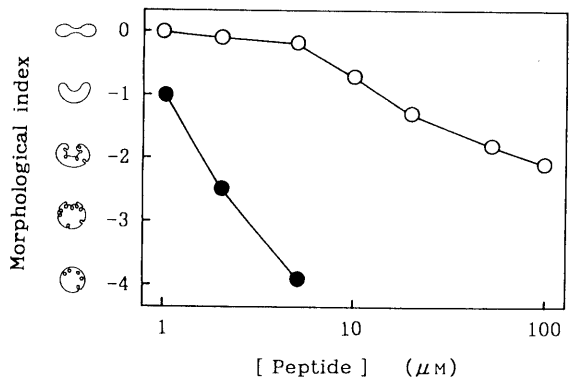

Fig. 3. Changes in a Morphological Index as a Function of the Concentration of Peptide

Erythrocytes were incubated with [Ala$^{2,2'}$,ΔPhe$^{4,4'}$]-gramicidin S (●) or [Ala$^{2,2'}$]-gramicidin S (○) at 37 °C for 30 min.

of the crenated cells (Fig. 2b). In contrast, [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-gramicidin S caused a change to an invaginated form (Fig. 2c); the direction of this change was opposite to the case of gramicidin S. We expressed the dose-dependence of the shape change using the morphological indices defined by Fujii and co-workers.[8] As shown in Fig. 3, [Ala$^{2,2'}$, ΔPhe$^{4,4'}$]-gramicidin S induced a rapid change to the higher stages of the invaginated form at lower concentrations, while [Ala$^{2,2'}$]-gramicidin S did not do so remarkably. It is now reasonable to speculate that the invaginated form arises from the predominant accumulation of substances into the inner half monolayer of erythrocyte membrane.[9-11] The large accumulation of [Ala$^{2,2'}$,ΔPhe$^{4,4'}$]-gramicidin S molecules into the inner leaflet of the membrane will destabilize the membrane structure, resulting in a break in the membrane. At present, however, we are unable to explain the mechanism by which the peptide is transported into the inner half of the membrane.

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