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Mechanism of membrane damage induced by the amphipathic peptides gramicidin S and melittin

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The action of gramicidin S and melittin on human erythrocytes, *Staphylococcus aureus* and *Escherichia coli* was studied as an extension of the previous study (Katsu, T., Ninomiya, C., Kuroko, M., Kobayashi, H., Hirota, T. and Fujita, Y. (1988) Biochim. Biophys. Acta 939, 57–63). These amphipathic peptides stimulated the release of membrane phospholipids outside cells in a concentration range causing permeability change. The shape change of erythrocytes from normal discoid to spiculate form was observed just prior to the release of membrane components. We have proposed the following action mechanism of gramicidin S and melittin. The peptide molecules were predominantly accumulated in the outer half of the bilayer, deforming the erythrocyte cell into crenature. A large accumulation made the membrane structure unstable, resulting in the release of membrane fragments and the simultaneous enhancement of permeability. The action mechanism of these peptides was compared with that of simple surfactants.

Introduction

Many amphipathic peptides increase the permeability of membrane, and the action mechanism is discussed in connection with the conformation of peptides. These peptides consist of, for example, melittin [1], mastoparan [1,2], cecropin [3] and some analogues of mitochondrial protein precursors [4] taking an α-helical conformation in which hydrophobic and hydrophilic amino acid residues are located in opposite directions in the molecule. Another example is gramicidin S, *cyclo*(-Val-Orn-Leu-D-Phe-Pro-)$_2$, which forms a β-sheet structure with two cationic ornithine residues on one side of the molecular plane and hydrophobic amino acid residues on the other side [5]. Increases in permeability have been discussed in view of a channel formation [1,6] or their ability to make a disordered region in lipid bilayer [7,8].

However, we have recently found that one mode of action of gramicidin S and melittin on erythrocyte membrane differs from those discussed above [9]. Both peptides had an ability to stimulate the release of membrane phospholipids outside cells, resulting in an enhancement in permeability. We were particularly interested in whether a common mechanism exists in the action of gramicidin S and melittin on various biological membranes. In the present study, using three different types of cells (i.e., human erythrocytes, *Staphylococcus aureus* as Gram-positive bacteria and *Escherichia coli* as Gram-negative bacteria), we have examined in detail the correlation between the K^+ permeability enhancement of the cells and the release of lipid components. The release of phospholipids was directly analysed by the phosphorus assay instead of the previous fluorescent probe method [9]. We also examined morphological changes of erythrocytes induced by peptides. The action of peptides was compared with that of simple surfactant molecules. Based on these results, we discussed a molecular mechanism of peptide action on biomembranes.

Part of this work has been presented at the 26th Symposium on Peptide Chemistry of Japan, 1988 [10].

Materials and Methods

Chemicals. Gramicidin S and melittin (product number M2272; lot number 95F-4024) were purchased from

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

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Sigma, U.S.A. A surfactant, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulphonate (Zwittergent) was obtained from Calbiochem, U.S.A. Other chemicals used were all of analytical reagent grade.

Erythrocytes. Human erythrocytes were used. Cells were washed twice with buffer (0.15 M NaCl/5 mM Hepes-NaOH (pH 7.4)) and suspended in this buffer at a concentration of 10% hematocrit.

Growth and preparation of bacteria. S. aureus 209P and *E. coli* K12 strain W3110 were used. *S. aureus* cells were grown at 37°C in a medium containing 1.5% polypeptone/0.5% bovine extract/0.5% NaCl/0.5% K_2HPO_4, while *E. coli* cells were grown at 37°C in a minimal salt medium supplemented with 1% polypeptone [8,11]. The cells were harvested in the late exponential phase of growth, washed twice with buffer (100 mM choline chloride/50 mM Mops-Tris (pH 7.2)) and suspended in this buffer at 10 mg protein/ml [8]. Protein content was determined by the method of Lowry et al. [12]. To disrupt the outer membrane structure of *E. coli*, cells were treated with 150 mM Tris-HCl (pH 7.2)/1 mM EDTA at 37°C for 2 min [13,14]. Because EDTA was toxic to cells [13], about 30% of cells was killed by this procedure.

Measurements of K^+ efflux and hemolysis. Erythrocytes were suspended in 2 ml 150 mM NaCl/5 mM Hepes-NaOH (pH 7.4) at the final concentration of 1% hematocrit. The number of cells was $1 \cdot 10^8$ cells/ml. The total amount of phospholipids contained in this cell suspension was 60 μM in egg phosphatidylcholine equivalent. *S. aureus* cells were suspended in 100 mM choline chloride/50 mM Mops-Tris (pH 7.2) at $5 \cdot 10^9$ cells/ml (0.6 mg cell protein/ml), whose phospholipid concentration was the same as that of erythrocytes. *E. coli* cells were suspended in the same buffer used in *S. aureus* at $5 \cdot 10^9$ cells/ml (0.7 mg cell protein/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 [8,15]. The total amount of K^+ was determined by disrupting cells with a surfactant Triton X-100 or cetyltrimethylammonium bromide [8]. Hemolysis was estimated by measuring the absorbance at 540 nm [9]. All experiments on melittin were carried out by using plastic tubes instead of the usual glass tubes, because the reproducibility of data was significantly improved.

Release of phospholipids and lipopolysaccharides from cells. Assay conditions were the same as in the case of K^+ permeability measurements, except that a cell suspension of large volume (50 ml) was used. After being incubated with a peptide at 37°C for 30 min, erythrocytes were centrifuged at $14000 \times g$ for 1 min [9,16], while bacteria at $14000 \times g$ for 3 min. For bacteria, a longer centrifuging time was chosen to sediment cells adequately. Lipids in supernatants were extracted with chloroform/methanol [17] and assayed for phospholipid phosphorus [18,19]. Lipopolysaccharides in the supernatants were determined as reported previously [20,21], except that samples were hydrolyzed in 0.25 M H_2SO_4 at 90°C for 20 min.

Measurement of cell viability. Cell viability was measured under the same conditions of the K^+ permeability measurements [15]. The cell suspension, treated with a peptide at 37°C for 30 min, was diluted with physiological saline and dispersed on an agar plate prepared with 1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar (pH was adjusted to 7 by adding 1 M KOH). The viability of cells was determined by counting colonies after standing for 15 h at 37°C.

Morphological observation of erythrocytes. To 0.2 ml of the peptide-treated or non-treated (control) erythrocyte suspension was added 0.2 ml of 2% glutaraldehyde dissolved in 1/30 M NaH_2PO_4/Na_2HPO_4 (pH 7.4) for the fixation of cells. After the sedimentation of cells, 20 μl of samples enriched with cells were pipetted onto glass plates and observed under an optical microscope at a magnification of 1000 × [10].

Results

Peptide-induced permeability change and phospholipid release

Fig. 1 shows the gramicidin S-induced K^+ efflux and the phospholipid release from erythrocytes, *S. aureus* and *E. coli*. In the case of erythrocytes, hemolysis was also determined (Fig. 1a). Hemolysis occurred in almost the same concentration range of the K^+ efflux. In experiments using *E. coli* cells, the liberation of lipopolysaccharides was detected as well (Fig. 1c). Both phospholipids and lipopolysaccharides were released in the same concentration range. The dose–response of the K^+ efflux and the release of phospholipids coincided well, except for the case of *E. coli* cells, where the release of lipid components was markedly suppressed at higher concentrations of gramicidin S (Fig. 1c). Probably, gramicidin S released massive membrane fragments which sedimented during a centrifugal procedure. It was observed that the amount of K^+ efflux from *E. coli* cells was not so dominant as those from *S. aureus* cells and erythrocytes. Then, a question might arise why *E. coli* showed a small efflux of K^+ in spite of marked release of lipid components. Here, it should be noted that *E. coli* belonging to Gram-negative bacteria has an outer membrane in the cell structure. In the previous study [15], it had been shown that gramicidin S formed a rather small-size lesion in the outer membrane. Thus, the peptide found it difficult to intrude deeper into the cytoplasmic membrane of *E. coli*, resulting in a small efflux of K^+. The fact that the amount of the release of lipopolysaccharides was markedly higher than that of phospholipids suggested that gramicidin S induced the predominant release of lipopolysaccharide rich in the

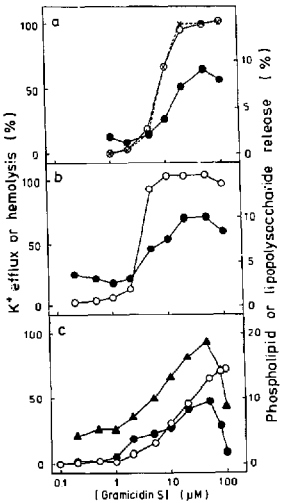

Fig. 1. The dose-response curves of K^+ efflux (○), hemolysis (×) and phospholipid (●) or lipopolysaccharide (▲) release from (a) erythrocytes, (b) *S. aureus*, and (c) *E. coli* cells induced by gramicidin S. Erythrocytes were suspended in 150 mM NaCl/5 mM Hepes-NaOH (pH 7.4) at $1 \cdot 10^8$ cells/ml. *S. aureus* and *E. coli* cells were suspended in 100 mM choline chloride/50 mM Mops-Tris (pH 7.2) at $5 \cdot 10^9$ cells/ml. Cells were incubated with gramicidin S at 37 °C for 30 min. The amounts of K^+ efflux were estimated by using a K^+ ion-selective electrode. Phospholipids and lipopolysaccharides released from cells were analysed after cells had been removed at 14000 × *g* for 1 min (erythrocytes) or at 14000 × *g* for 3 min (bacteria).

outer membrane. Then, we measured the viability of cells in order to confirm the view that the antimicrobial activity of gramicidin S is due to enhancement of permeability. As shown in Fig. 2, gramicidin S killed *S. aureus* cells efficiently, but acted weakly on *E. coli* cells, in proportion to the degree of the efflux of K^+ from cells. Furthermore, we treated *E. coli* cells with Tris-EDTA to disrupt the outer membrane structure intensively [13,14]. *E. coli* cells treated thus became markedly sensitive to gramicidin S (Fig. 3). This result supported strongly the notion that the weakness of antimicrobial activity of gramicidin S against *E. coli* cells was due to a lack of ability to make a large-size lesion in the outer membrane as described above.

Next we examined the action of melittin. In the case of erythrocytes, hemolysis, K^+ efflux and phospholipid

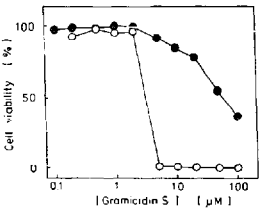

Fig. 2. Changes in the viability of *S. aureus* (○) and *E. coli* (●) cells after treatment with gramicidin S. Cell viability was measured under the same conditions of the K^+ permeability measurements.

release occurred in the same concentration range of melittin (Fig. 4a). However, such coincidence was not observed when cells were replaced with *S. aureus* (Fig. 4b). In spite of a marked efflux of K^+, the release of phospholipids could not be detected. When the cells had been sedimented in a shorter centrifuging time, (14000 × *g* for 1 min), the release of phospholipids could be clearly detected (6% at 10 μM melittin). This indicates that melittin also released massive membrane fragments from *S. aureus* cells as in the case of gramicidin S against *E. coli* cells. Melittin acted poorly on *E. coli* cells (Fig. 4c). Fig. 5 shows changes in the viability of cells. Melittin decreased markedly the viability of *S. aureus* cells, but scarcely acted on *E. coli* cells. Also, in this case, a correlation between changes in the permeability and viability of cells was clearly observed. Melittin exerted a strong effect on *E. coli* cells treated with Tris-EDTA (Fig. 6), indicating that melittin is able to act on bacterial cytoplasmic membranes irrespective of Gram-positive and -negative bacteria such as gramicidin S.

We further found that gramicidin S and melittin stimulated the release of a membrane protein, acetyl-

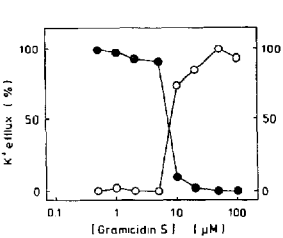

Fig. 3. Action of gramicidin S on Tris-EDTA-treated *E. coli* cells. The dose-response relations of cell viability (●) and K^+ efflux (○) are shown. Cells were incubated with gramicidin S at 37 °C for 30 min.

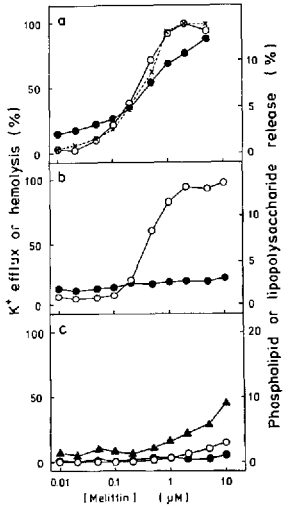

Fig. 4. Melittin-induced K^+ efflux, hemolysis and lipid release from (a) erythrocytes, (b) *S. aureus* and (c) *E. coli* cells. Meanings of symbols used in this figure are the same as in Fig. 1.

cholinesterase [16], from erythrocytes in the same concentration range of phospholipid release (data not shown). This indicates that both peptides have an ability to pluck off various constituents of plasma membrane.

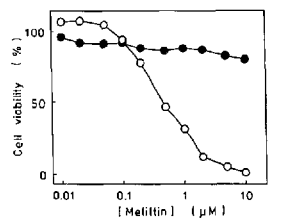

Fig. 5. Changes in the viability of *S. aureus* (○) and *E. coli* (●) cells after treatment with melittin.

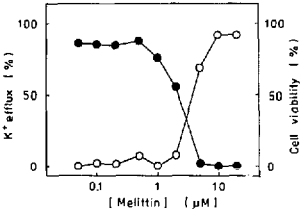

Fig. 6. Action of melittin on Tris-EDTA-treated *E. coli* cells. Cell viability (●) and K^+ efflux (○) are shown.

Shape transformation of erythrocytes

Next, we observed morphological changes in erythrocytes induced by peptides. Gramicidin S caused a change in the shape of erythrocytes from normal discoid to crenated form. Such a change occurred at around 5 μM gramicidin S (Fig. 7b), just prior to the liberation of membrane components, and became remarkable with an increase in the concentration of gramicidin S (Fig. 7c). A similar transformation to crenated form was observed after addition of melittin (Fig. 7d). Tosteson and co-workers [22,23] have also observed a change in the erythrocyte shape to spiculate form at the lytic concentration of melittin.

We were interested in the fact that both peptides induced hemolysis immediately after an appearance of crenated cells. Although many amphipathic molecules are known to alter the shape of erythrocytes [24,25], hemolysis does not occur at the stage of crenated cells (named echinocytes). One example is seen in a case of a surfactant, Zwittergent [26,27]. This surfactant forms echinocytes at a lower concentration region (Fig. 7e); however, further increases in the concentration lead to the formation of spherical cells (named spherocytes) as shown in Fig. 7f. Fig. 8 shows the results of hemolysis, K^+ efflux and phospholipid release from erythrocytes. Although phospholipids began to release at around 50 μM of Zwittergent, where the transformation of shape proceeded to echinocyte II according to the literature [25], hemolysis was not induced at all until a large amount of phospholipid was stripped from erythrocyte membrane. Hemolysis occurred after the formation of spherocytes. These differences in the action between peptides and surfactant will be discussed later.

Discussion

The present study shows that amphipathic peptides gramicidin S and melittin stimulate the release of membrane phospholipids from various types of cells in the same concentration range of the K^+ efflux. This indi-

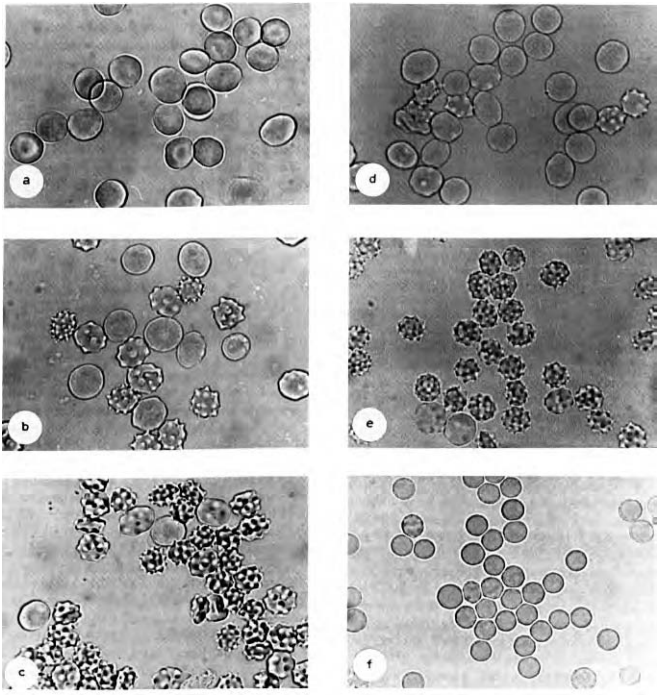

Fig. 7. Changes in the morphology of erythrocytes. (a) Intact cells. Erythrocytes were incubated at 37 °C for 30 min with (b) 5 μM and (c) 10 μM gramicidin S, (d) 0.05 μM melittin, or (e) 50 μM and (f) 500 μM Zwittergent. They were observed under an optical microscope after being fixed with glutaraldehyde.

cates that both peptides are able to pluck off membrane components from various biological membranes, increasing the permeability of membrane simultaneously. Several characteristics of peptide actions should be mentioned. Firstly, when we examined the action of melittin on *S. aureus* cells, the release of membrane phospholipids could not be detected until cells were sedimented under gentle centrifugal conditions. Such a precipitation of membrane fragments during a hard centrifugal procedure was also observed in the case of *E. coli* cells using gramicidin S at high concentrations. These results show that amphipathic peptides released rather heavy membrane fragments from cells. Secondly, it was observed that gramicidin S caused the K^+ efflux less effectively from *E. coli* cells than from *S. aureus* cells and erythrocytes. Among these three cells, only *E. coli* has an outer membrane in the cell structure. Because gramicidin S could not make a large size of lesion in the outer membrane [15], it was difficult for the peptide to intrude deeper into the cytoplasmic membrane, causing a small efflux of K^+. Thirdly, the action of melittin on *E. coli* cells was much weaker than that of gramicidin S. Melittin induced only a small amount of lipopolysaccharide release from the outer membrane,

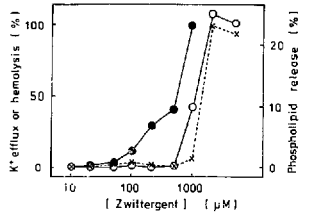

Fig. 8. The dose-response curves of K^+ efflux (○), hemolysis (×) and phospholipid release (●) from erythrocytes induced by Zwittergent. Assay conditions were the same as in Fig. 1.

indicating that the peptide had poor ability to disrupt the outer membrane structure. At present, however, we can not explain why melittin acted poorly on the outer membrane. After *E. coli* cells were treated with Tris-EDTA, the cells became strongly sensitive to gramicidin S and melittin, showing that both peptides can act on bacterial cytoplasmic membranes regardless of Gram-positive and -negative bacteria.

We consider that peptide molecules accumulate predominantly in the outer half of lipid bilayer, which render erythrocyte cells crenated. It is generally accepted that when gramicidin S interacts with phospholipid membrane, two cationic ornithine residues of gramicidin S are exposed to an aqueous phase, while the hydrophobic side of the molecule penetrates into the lipid bilayer; the existence of hydrophilic parts prevents the embedding of the peptide deeper into the lipid bilayer [8,28]. Such a situation may inhibit the transbilayer movement of gramicidin S, leading to the unbalanced accumulation of peptide molecules in the bilayer. Melittin will also accumulate in the outer half of bilayer as gramicidin S, though this peptide is known to form a single channel through the lipid bilayer under certain conditions [1]. It is supposed that the membrane was broken and some membrane fragments were expelled from the cells as the amount of the peptides penetrated into membrane increased, and the K^+ permeability was enhanced simultaneously. These considerations are illustrated in Fig. 9. The peptide molecules anchor in the

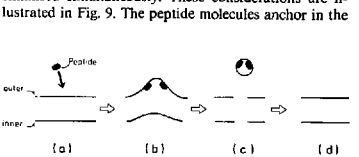

Fig. 9. Schematic illustration of action mechanism of gramicidin S and melittin on biomembranes.

vicinity of the polar head of lipids, deforming greatly the lipid packing (Fig. 9b). A large accumulation of peptide liberates membrane fragments and enhances the permeability simultaneously (Fig. 9c). As has been discussed in the previous paper [9], there are several possibilities for the form of the membrane fragments, namely, mixed micelles with peptides, lamellar lipid structure surrounded by peptides [29], vesicles [16,30], or a non-specific cluster [31].

Then, we discuss differences in the action of peptide and surfactant on biomembrane. It has been shown that many amphipathic compounds can alter the shape of erythrocyte from normal discoid to spiculate form and to stimulate the release of membrane vesicles [16,30]. However, these compounds, differently from the present peptides, did not enhance the permeability of cells even when a large amount of membrane fragments was plucked off. This was also confirmed by the present example, Zwittergent. We supposed that if incorporated amphipathic molecules existed stably as bilayer constituents, the membrane did not break even when a large amount of lipids was released. However, gramicidin S or melittin was unable to exist as a stable membrane constituent, resulting in a break of membrane.

After membrane fragments have been expelled outside cells, a pore would be formed at this place (Fig. 9c). While a large amount of fragment release was observed upon addition of peptide, the size of pore detected was extremely small [9]. This fact indicates that the pore would rapidly be annealed by a lateral diffusion of remaining lipid molecules (Fig. 9d). If membrane fragments or remaining amphipathic peptide molecules can not disrupt cells further, the efflux of K^+ or hemolysis will stop. Recently, two detailed kinetic studies on hemolysis by melittin have been reported [23,32]. Unfortunately, the experimental findings differ from each other. Tosteson and co-workers [23] have shown that melittin causes hemolysis quite transiently, indicating that further disruption of erythrocyte membrane was not induced by membrane fragments or remaining peptides. On the other hand, DeGrado et al. [32] have reported that the action of melittin was not so transient and gradual hemolysis took place after rapid hemolysis. The discrepancy of the results may partly be attributed to differences in the experimental conditions (e.g., the ionic strength of solution). It seems likely that different environments induce different activity of the reactants against cells [32].

We now suppose that many amphipathic peptides, which separate well the hydrophobic and hydrophilic amino acid residues within the molecule upon interaction with biomembrane, will increase the permeability of cells by essentially the same mechanism. These peptides are, for example, staphylococcal δ-toxin [1,33], pardaxins [34,35] and some analogues of mitochondrial protein precursors [4].

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