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Action mechanism of amphipathic peptides gramicidin S and melittin on erythrocyte membrane

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Amphipathic peptides gramicidin S and melittin caused a characteristic colloid-osmotic hemolysis on human erythrocytes; that is, the peptides produced initially a small membrane lesion in erythrocyte membrane, followed by the release of hemoglobin. The size of membrane lesion increased with an increase in the concentration of peptide. Under the conditions causing membrane lesion, we observed the release of membrane fragments containing phospholipids. The present results show that both the peptides have the ability to stimulate the release of membrane fragments out of the cells and this brings about the perforation of molecules of small size, leading to a colloid-osmotic hemolysis.

Introduction

Amphipathic peptides gramicidin S and melittin enhance the permeability of various kinds of cells, and the mechanism of action is discussed in connection with the conformation of peptides [1–11]. The primary structure of gramicidin S is *cyclo*(-Val-Orn-Leu-D-Phe-Pro-)$_2$, and that of melittin is:

NH_2-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-

Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-

Arg-Lys-Arg-Gln-Gln-$CONH_2$.

The secondary structure of gramicidin S is well characterized as a β-sheet structure with two cationic ornithine residues on one side of the molecular plane and hydrophobic residues on other side [3,4]. This β-sheet structure is retained in membrane [5]. Melittin interacting with membrane forms a bent α-helical rod facing hydrophobic and hydrophilic sides in opposite direction [6–9,11]. It has been discussed that both peptides, penetrating into membrane, make a disordered region in lipid packing and this may trigger the permeability enhancement of membrane [1,7].

In the present study, we examined the size of membrane lesion formed in human erythrocytes in order to obtain further insight into the mechanism of permeability enhancement by these peptides. It was found that the size of lesion increased with an increase in the concentration of peptide. Moreover, the release of membrane fragments containing phospholipids was detected under the conditions of membrane lesion. The present results

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; ANS, 8-anilino-1-naphthalenesulphonic acid.

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indicate that an increase in the permeability of erythrocytes is not brought about only by the penetration of peptide, but is induced by disruption of the membrane structure causing the release of membrane fragments.

Materials and Methods

Chemicals. The sources of chemicals were as follows: gramicidin S and melittin (product number M2272; lot number 95F-4024) from Sigma; D-mannitol, sucrose, raffinose and polyethylene glycols (average molecular weights 400, 600, 1000 and 2000) from Wako Pure Chemical Industries; ammonium salt of ANS from Tokyo Kasei Kogyo. 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 concentration of 5% (v/v).

Measurements of K^+ efflux and hemolysis. The efflux of K^+ from erythrocytes was measured with a K^+ ion-selective electrode as reported previously [2]. In brief, erythrocytes were suspended in 1 ml 0.15 M NaCl/5 mM Hepes-NaOH (pH 7.4) at the final concentration of 0.5% (v/v). The 2 μl of gramicidin S in ethanol solution (final concentration: 20 μM) was added. The amount of K^+ efflux was determined from the calibration curve of the K^+ ion-selective electrode. The total amount of K^+ was determined by disrupting cells with a surfactant Triton X-100 [1]. Hemolysis was estimated by measuring the absorbance at 540 nm. The total amount of hemoglobin was determined after erythrocytes were lysed by adding water.

Osmotic protection experiments. Erythrocytes (0.5% (v/v)) were suspended in 0.135 M NaCl/5 mM Hepes-NaOH (pH 7.4)/30 mM of one of the following substances: D-mannitol; sucrose; raffinose; polyethylene glycols whose molecular weights are 400, 600, 1000 and 2000. Then, peptide was added and hemolysis was determined after incubation for 30 min at 28° C. The following molecular diameters of substances were used [12–14]: mannitol, 7 Å; sucrose, 9 Å; raffinose, 11 Å; polyethylene glycols 400, 600, 1000 and 2000, 12 Å, 16 Å, 20 Å and 29 Å, respectively.

Fluorescence measurements. After peptide and erythrocytes (0.5% (v/v)) were incubated at 28° C for 30 min in a buffer solution comprising 0.15 M NaCl/5 mM Hepes-NaOH (pH 7.4), cells were centrifuged at $14000 \times g$ for 1 min [15]. In the supernatant, ANS was added at the final concentration of 10 μM. Fluorescence measurements were performed using a Hitachi MPF-4 fluorospectrophotometer. Excitation wavelength was 375 nm. Fluorescence spectra were corrected by using standard compounds, *N,N*-dimethyl-*m*-nitroaniline and quinine sulphate [16,17].

Phosphorus assay. Lipid extracts [18] from supernatants were assayed for phospholipid phosphorus [19,20].

Results

Apparent size of gramicidin S-induced membrane lesion

Fig. 1 shows the comparison of time courses of K^+ efflux and hemolysis induced by gramicidin S. Although gramicidin S caused a rapid efflux of K^+, hemolysis occurred gradually, suggesting that the peptide produced a rather small size of membrane lesion, and a colloid-osmotic process might be involved in hemolysis [21,22]. If the colloid-osmotic process is concerned, hemolysis will be protected by a solute of an appropriate size added in an outer aqueous solution [23–25]. This consideration is based on the fact that, if the osmotic pressure of intracellular hemoglobin is balanced with that of the solute added in the outer solution,

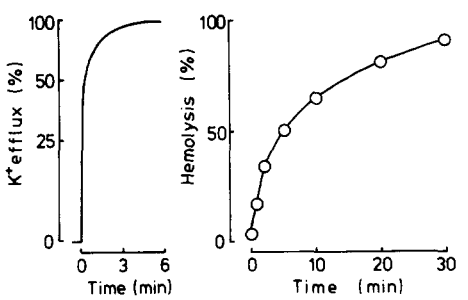

Fig. 1. Time courses of (a) K^+ efflux and (b) hemolysis upon addition of gramicidin S. Erythrocytes (0.5% (v/v)) were suspended in 0.15 M NaCl/5 mM Hepes-NaOH (pH 7.4) at 28° C. At time zero, gramicidin S (final concentration: 20 μM) was added. The efflux of K^+ was monitored with a K^+ ion-selective electrode. Hemolysis was determined by measuring the absorbance at 540 nm.

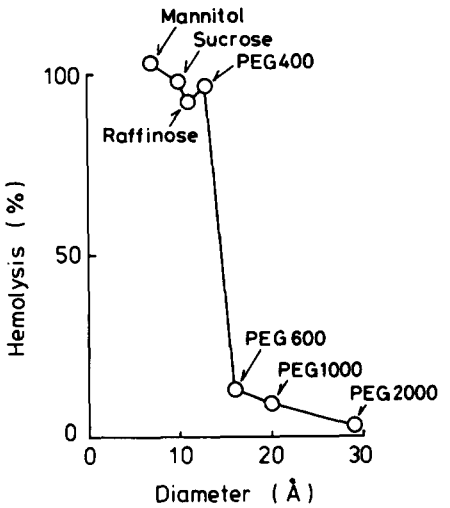

Fig. 2. Gramicidin S-induced hemolysis in the presence of various colloid-osmotic protectants. Erythrocytes (0.5% (v/v)) were suspended in 0.135 M NaCl/5 mM Hepes-NaOH (pH 7.4)/30 mM protectant. Subsequently gramicidin S (final concentration: 20 μM) was added and hemolysis was determined after incubation for 30 min at 28°C. The diameters of protectants are described in Materials and Methods. PEG, polyethylene glycol.

hemolysis is not induced. Thus, the size of membrane lesion can be determined by examining whether the substances added can protect hemolysis. Fig. 2 shows the gramicidin S-induced hemolysis in the presence of various protectants. In this figure, the degree of hemolysis was plotted as a function of the diameters of the protectants. It was found that hemolysis was protected by substances having bigger diameters than polyethylene glycol 600. This indicates that the apparent size * of membrane lesion produced by gramicidin S is nearly equal to 16 Å, corresponding to the diameter of polyethylene glycol 600.

The above experiments were performed at 20 μM of gramicidin S. Then we tried the osmotic protection experiments at various concentrations of gramicidin S (Fig. 3). It was observed that mannitol, raffinose and polyethylene glycol 600, 1000 and 2000 protected hemolysis occurring at 5, 10, 15, 40 and 60 μM of gramicidin S, respectively. We further observed that the threshold concentration of gramicidin S causing the efflux of K^+ was 5 μM (data not shown). From these data, we could depict the change in apparent sizes of membrane lesion depending on the concentration of gramicidin S as in Fig. 4. In this figure, the diameter of hydrated K^+ was taken as 5 Å [26]. It was found that the size of lesion increased greatly with increases in the concentration of gramicidin S.

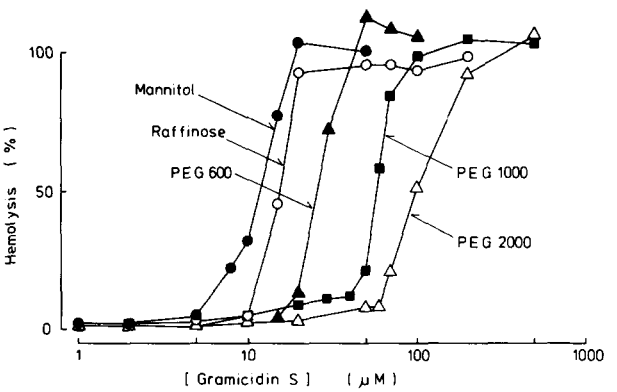

Fig. 3. Protection of hemolysis induced at various concentrations of gramicidin S. Assay conditions were the same as in Fig. 2, except that the concentration of gramicidin S was changed. PEG, polyethylene glycol.

Detection of membrane fragments

We supposed that gramicidin S might release

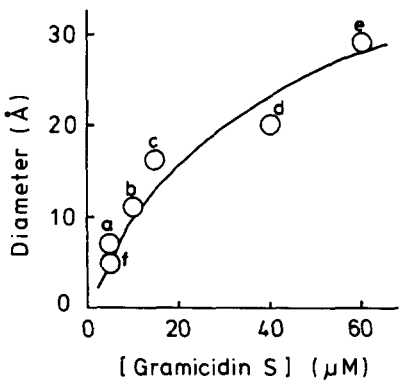

Fig. 4. Plot of the apparent size of membrane lesions vs. the concentration of gramicidin S. These data were obtained from the osmotic protection experiments using (a) mannitol, (b) raffinose, (c) polyethylene glycol 600, (d) polyethylene glycol 1000 and (e) polyethylene glycol 2000, and from the measurement of K^+ efflux (f).

* The term 'apparent' size was used, since the size of membrane lesion increased with longer incubation periods and at high temperatures. The present results were obtained with incubation at 28°C for 30 min.

membrane fragments from erythrocytes resulting in large membrane lesions. To detect such fragments we applied the fluorescence probe method using ANS at first. The fluorescence intensity of ANS is well known to increase in the presence of phospholipids [27]. Thus, if the membrane fragments contained phospholipids, they can easily be detected by monitoring an increase in the fluorescence intensity of ANS. To test this possibility, being incubated with gramicidin S, cells were centrifuged and ANS was added in the supernatant. Fig. 5a shows the fluorescence spectrum. As a control experiment, the fluorescence spectrum of ANS added in solution of gramicidin S alone was measured (Fig. 5b). Compared with the control, the fluorescence intensity in the supernatant was strongly enhanced and its emission maximum shifted to around 490 nm. These are features of ANS dissolved in phospholipid membrane [27]. The contamination of intracellular proteins such as hemoglobin did not affect significantly the fluorescence intensity. When the supernatant was further centrifuged at 20000 × *g* for 10 min, the fluorescence intensity of ANS decreased markedly, indicating that the released membrane fragments were rather large. Fig. 6 depicts changes in the fluorescence intensity of ANS as a function of the concentration of gramicidin S. It should be

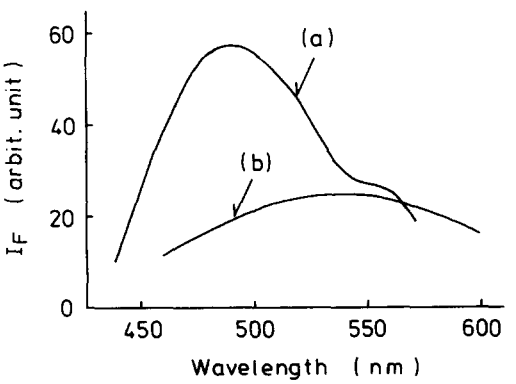

Fig. 5. Changes in the fluorescence spectra of ANS. (a) After erythrocytes (0.5% (v/v)) and 60 μM gramicidin S were incubated for 30 min at 28°C in a buffer solution comprising 0.15 M NaCl/5 mM Hepes-NaOH (pH 7.4), cells were centrifuged at 14000 × *g* for 1 min and ANS was added in the supernatant. (b) As a control experiment, ANS was added in 60 μM gramicidin S alone in the same buffer solution. The final concentration of ANS was 10 μM. Excitation wavelength was 375 nm.

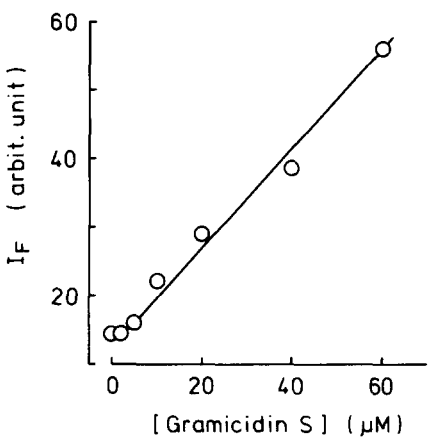

Fig. 6. Plot of fluorescence intensity of ANS vs. the concentration of gramicidin S. After erythrocytes (0.5% (v/v)) and gramicidin S were incubated for 30 min at 28°C in a buffer solution comprising 0.15 M NaCl/5 mM Hepes-NaOH (pH 7.4), cells were centrifuged at 14000 × *g* for 1 min and ANS was added in the supernatant at the final concentration 10 μM. Fluorescence intensity was measured with excitation and emission wavelengths of 375 nm and 480 nm, respectively.

emphasized that the intensity began to increase at around 5 μM gramicidin S, where the initial membrane lesion was induced. Furthermore, the fluorescence intensity of ANS increased remarkably with the concentration of gramicidin S, suggesting that the amount of fragments increased with an increase in the concentration of gramicidin S. However, this fluorescence probe method was rather indirect. We used the phosphorus assay to detect more directly the existence of phospholipids. At 60 μM of gramicidin S, it was found that 20% of the phospholipids were released from the erythrocytes.

Experiments on melittin

Then, we examined the action of another amphipathic peptide melittin. It is well known that this peptide penetrated into and disrupted both natural and synthetic phospholipid bilayers [6,28–30]. Among many studies on the action of melittin, we were particularly interested in the recent experiment that melittin caused a colloid-osmotic hemolysis [31]. This seemed to suggest that melittin acted on the erythrocytes by the same mechanism as gramicidin S. We performed the colloid-osmotic protection experiments at various concentrations of melittin (Fig. 7). It was

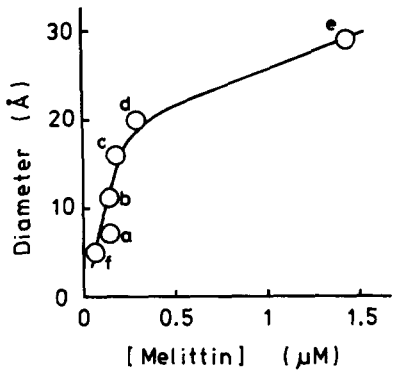

Fig. 7. Plot of the apparent size of membrane lesion vs. the concentration of melittin. The size was determined as in the case of gramicidin S; (a) mannitol, (b) raffinose, (c) polyethylene glycol 600, (d) polyethylene glycol 1000 and (e) polyethylene glycol 2000, and (f) K^+ efflux.

observed that an increase in the concentration of melittin caused larger membrane lesions as in the case of gramicidin S. Furthermore, fluorescence enhancement of ANS was observed at the concentration range of the membrane lesion (Fig. 8). Such increases in fluorescence intensity were not observed at all without erythrocytes. The release of phospholipids was also detected (30% at 0.6 μM of melittin). These results on gramicidin S and melittin indicate strongly that there is a common process of action of amphipathic peptides on the erythrocyte membrane.

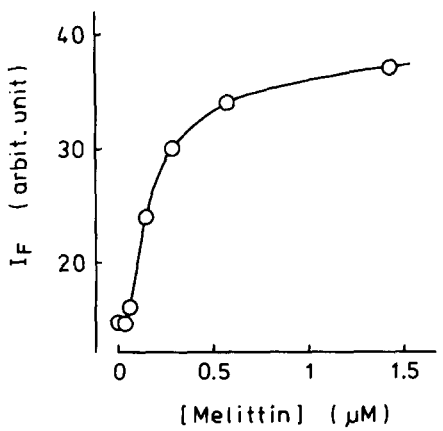

Fig. 8. Plot of the fluorescence intensity of ANS vs. the concentration of melittin. Assay conditions were the same as in Fig. 6, except that melittin was used instead of gramicidin S.

Discussion

The present study shows that amphipathic peptides gramicidin S and melittin caused a characteristic colloid-osmotic hemolysis on human erythrocytes; that is, the peptides produced initially a small membrane lesion in the membrane, following the release of hemoglobin. The size of membrane lesion increased with an increase in the concentration of peptides. We have recently discussed the mechanism of gramicidin S-induced permeability enhancement of membrane in connection with a change in the fluidity of membrane [1]. So far, many workers have shown that gramicidin S decreases the phase transition temperature of zwitterionic phospholipids such as dimyristoylphosphatidylcholine [32,33], dipalmitoylphosphatidylcholine [1,32,34] and dipalmitoylphosphatidylethanolamine [34], indicating that gramicidin S is able to penetrate into zwitterionic phospholipids to stimulate the movement of the acyl chains of the lipids. Because many amphipathic peptides do not affect the phase transition temperature of zwitterionic lipids [1,35–37], we have considered that, gramicidin S, as a result of such a membrane disturbance to zwitterionic lipids which constitute the main part of the biological membrane, increases the permeability of various kinds of membranes without any selectivity [1]. However, there existed an ambiguity whether only the penetration of gramicidin S into the membrane, making a disordered region in the ordered membrane, was adequate to induce a great increase in the permeability of biological membrane, though it is well known that the permeability of membrane is enhanced through the packing mismatch of different membrane phases [38–40]. We considered that, in the previous mechanism [1], the size of the membrane lesion could not exceed the molecular size of gramicidin S. However, we found in the present study that the peptide produced a larger membrane lesion in the erythrocyte membrane. Thus, the mechanism of action of gramicidin S had to be reconsidered. Basically, we believe that gramicidin S penetrated into membrane triggers the permeability enhancement. However, as the amount of gramicidin S penetrated into the erythrocyte membrane increased, the membrane was broken and some membrane frag-

ments were expelled out of the cells. This was confirmed by the detection of released phospholipids. It was observed that the fluorescence intensity of ANS increased at the concentration range of gramicidin S and melittin causing the membrane lesion (Figs. 6 and 8). Furthermore, the phosphorus assay clarified more directly the release of phospholipids. However, we can not report at present the precise form of the membrane fragments released from erythrocytes. There are several possibilities of whether the fragments are mixed micelles with peptides, lamellar lipid structure surrounded by peptides [11], vesicles [15,41] or non-specific cluster [42]. It is interesting that small fragments have been observed with the interaction between melittin and liposomes [11,28,43].

Here, the action of melittin on planar lipid bilayers should be mentioned. Several workers [44,45] have observed single-channel openings in melittin-doped bilayers. The channel has been observed at high ionic strength (e.g., 1 M NaCl). Inouye [46] has proposed a model that a channel-forming structure is produced by the association of some α-helices of amphipathic peptides. A tubular hydrophilic channel will be formed, when each α-helix is arranged so that all side chains facing outside are hydrophobic, while those on the inside are hydrophilic [46,47]. Ions of small size will pass through this channel. It is probable that some α-helices of melittin associate to form a channel in this way [48]. On the other hand, Kempf et al. [49] have observed that melittin produces a broad range of conductance increases rather than discrete channel type openings at more physiological conditions. The latter experimental conditions were similar to the present case, and thus this result was more appropriate for interpreting our results.

In conclusion, the present study showed that the amphipathic peptides gramicidin S and melittin, which segregated well the hydrophobic and hydrophilic amino acid residues upon interaction with the membrane, increased the permeability of erythrocytes by releasing membrane fragments. We now consider that the action of many amphipathic peptides on various biological membranes will proceed basically in a similar way. These peptides are, for example, staphylococcal δ-toxin [6,30,50], mastoparan [51] and some analogues of mitochondrial protein precursors [52,53].

Acknowledgements

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