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Structure-activity relationship of gramicidin S analogues on membrane permeability

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The previous study of the action of gramicidin S on bacteria (Katsu, T., Kobayashi, H. and Fujita, Y. (1986) Biochim. Biophys. Acta 860, 608–619) prompted us to investigate further the structure-activity relationship of the gramicidin S analogues on membrane permeability. Two types of the gramicidin S analogues were used in the present study: (1) *cyclo*(-X-D-Leu-D-Lys-D-Leu-L-Pro-)$_2$, where X = Gly, D-Leu and D-cyclohexylalanine (D-*c*HxAla); (2) *N*,*N'*-diacetyl derivative of gramicidin S (diacetyl-gramicidin S) which lacks a cationic moiety of gramicidin S. All the analogues have a β-sheet conformation as gramicidin S. The following cellular systems were used: *Staphylococcus aureus* as Gram-positive bacteria, *Escherichia coli* as Gram-negative bacteria, human erythrocytes, rat liver mitochondria and artificial liposomal membranes. It was found that gramicidin S and one of the type 1 analogues having X = D-*c*HxAla induced the efflux of K^+ through the cytoplasmic membrane of all types of the cells. In addition, these two peptides had the ability to lower the phase transition temperature of dipalmitoylphosphatidylcholine. Accordingly, it was concluded that, if peptides can expand greatly the membrane structure of neutral lipids which constitute main parts of the biological membrane, they can stimulate the permeability of cells without any selectivity. The action of the type 2 peptide, diacetyl-gramicidin S, was strongly cell dependent. Although this peptide stimulated the efflux of K^+ from mitochondria, it did not do so efficiently, if at all, from *S. aureus*, *E. coli* and erythrocytes. In experiments using liposomes, diacetyl-gramicidin S increased markedly the permeability of liposomes composed of egg phosphatidylcholine. The presence of egg phosphatidylethanolamine or cholesterol reduced its activity. These results on liposomes explained well the low sensitivity of diacetyl-gramicidin S against *E. coli* and erythrocytes in terms of lipid constituents of the membranes. The mechanism of action of diacetyl-gramicidin S was discussed from the formation of a boundary lipid induced by this peptide.

Abbreviations: Mops, 4-morpholinepropanesulphonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine.

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Introduction

Gramicidin S, a cyclic decapeptide of (-Val-Orn-Leu-D-Phe-Pro-)$_2$, is interesting from the viewpoint of the structure-activity relationship

[1,2]. Its relatively simple primary structure and high antimicrobial activity prompted many workers to correlate the activity with the secondary structure of this peptide. It is now well established that gramicidin S has a β-sheet structure with two cationic ornithine residues on one side of the molecular plane and hydrophobic residues on the other side (Fig. 1) [1,2]. Izumiya and colleagues discussed the structure-activity relationship of many gramicidin S analogues and proposed the 'sidedness hypothesis' [2,3]. This hypothesis describes the importance of 'sidedness' of the hydrophobic and hydrophilic parts of the gramicidin S molecule attained by the β-sheet conformation for antimicrobial activity: in other words, if the peptide cannot form the β-sheet conformation, the antimicrobial activity is markedly reduced. The hypothesis further emphasizes that the existence of cationic moieties of gramicidin S is essential for the activity. A typical example is the *N,N'*-diacetyl derivative of gramicidin S (diacetyl-gramicidin S). This peptide retains the β-sheet conformation, similar to gramicidin S [4,5], but it lacks cationic amino groups. It is well known that the antimicrobial activity of diacetyl-gramicidin S decreases markedly [5].

In spite of extensive studies of the structure-activity relationship of the gramicidin S analogues, the mechanism of antimicrobial action has not yet been completely elucidated. Gramicidin S can increase the permeability of sensitive microorganisms, and thus it is supposed that the antimicrobial activity is due to the increased permeability of membrane [1,2,6].

Gramicidin S is also known as an effective uncoupler of the oxidative phosphorylation in mitochondria [7]. It has been reported that the uncoupling effect is due to the induced permeability enhancement of membrane [8,9], similar to the case of bacteria. However, in mitochondria, diacetyl-gramicidin S also increased the permeability [9]. Accordingly, Sholtz et al. [9] have concluded that although the β-sheet conformation of gramicidin S is important for the uncoupling effect, the presence of positive charge on gramicidin S is not essential for the effect. This conflicted entirely with the sidedness hypothesis proposed for bacteria.

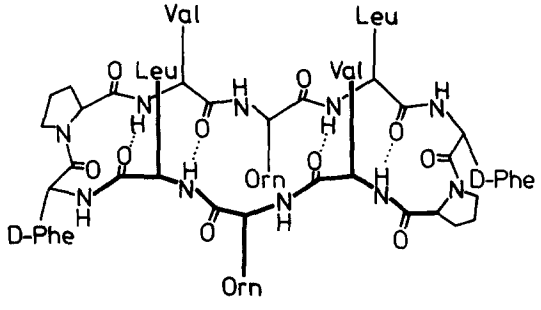

Fig. 1. β-Sheet conformation of gramicidin S.

These difference in the action of diacetyl-gramicidin S on both mitochondria and bacteria prompted us to elucidate the mechanism of action of the gramicidin S analogues on various membranes more clearly. The major purposes of the present work were to clarify (1) whether the antimicrobial activity of the gramicidin S analogues is attributable to an increase in membrane permeability, and (2) whether the sidedness hypothesis holds for other membranes than those of bacteria. By investigating the structure-activity relationship, we intended to elucidate the mechanism of action of gramicidin S and diacetyl-gramicidin S at molecular level. Fig. 2 shows the primary structure of gramicidin S, along with that of the analogues used in the present study. Diacetyl-gramicidin S has a structure that the two amino groups in ornithine residues of gramicidin S are acetylated (not shown). We used abbreviation X = (amino acid) * to indicate an individual gramicidin S analogue shown in Fig. 2b. The following cellular systems were used: *Staphylococcus aureus* as Gram-positive bacteria, *Escherichia coli* as Gram-negative bacteria, human red blood cells, rat liver mitochondria and artificial liposomal membranes. It was found that the antimicrobial activity of peptides on *S. aureus* coincided well with their ability to increase the permeability of the cell. Among peptides, gramicidin S and one of the analogues, X = D-cyclohexylalanine (X = D-*c*HxAla), markedly increased the permeability. These two peptides further had the ability to stimulate the permeability of mitochondria, red

* In addition to the usual amino acids, X = D-cyclohexylalanine which was synthesized by the hydrogenation of D-phenylalanine was used in the present study. This was abbreviated to X = D-*c*HxAla.

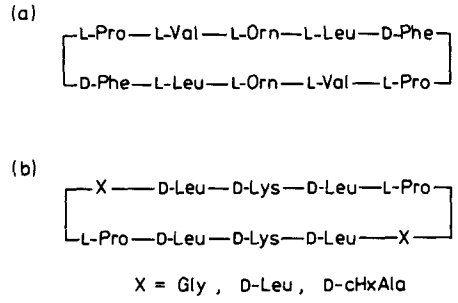

Fig. 2. Primary structure of (a) gramicidin S and (b) gramicidin S analogues used in the present study.

blood cells and artificial liposomes. Diacetyl-gramicidin S enhanced the permeability of mitochondrial membrane as reported previously [9], but it did not increase the permeability of the membranes of bacteria and erythrocytes. It was found that diacetyl-gramicidin S enhanced greatly the K^+ permeability of liposomes prepared from egg PC. The existence of egg PE and cholesterol reduced the action of diacetyl-gramicidin S, which explained the low activity of this peptide against *E. coli* and erythrocyte in terms of lipid constituents of the membranes. We discussed the mechanism of permeability change in connection with changes in membrane fluidity induced by the gramicidin S analogues.

Materials and Methods

Materials. The sources of materials were as follows: gramicidin S, compound 48/80, DPPC and cholesterol from Sigma; Triton X-100 and diphenylhexatriene from Tokyo Kasei Kogyo; cetyltrimethylammonium bromide from Nakarai Chemicals; egg PC from The Green Cross Corporation, Japan; egg PE and egg PG from Lipid Products, U.S.A. Diacetyl-gramicidin S was prepared according to the literature [10] and recrystallized twice from diluted ethanol. This peptide was hygroscopic: colourless needles; mp. 283–284 °C (uncorrected; Ref. 4: 305–306 °C); $[\alpha]_D^{27.5} - 292°$, $c = 1.0$ in 70% (v/v) ethanol (Ref. 10: $[\alpha]_D^{22} - 313°$, $c = 1.5$ in 70% (v/v) ethanol).
Analysis for $C_{64}H_{96}N_{12}O_{12} \cdot 3H_2O$:
Calcd.: C, 60.07; H, 8.03; N, 13.13.
Found: C, 59.81; H, 7.96; N, 12.90.
The gramicidin S analogues were synthesized by the conventional solution method [11]. Other chemicals used were all of analytical reagent grade.

Preparation of ion-selective electrode. Changes in the K^+ permeability of cells were measured by using a K^+ ion-selective electrode. The K^+ ion-selective electrode was constructed by the use of poly(vinyl chloride)-based membrane [6,12]. We applied a dialysis membrane to cover the sensor membrane of the ion-sensitive electrode to avoid the interference of peptides and surfactants as reported previously [6].

Growth and preparation of bacteria. E. coli K12 strain W3110 and *S. aureus* 209P were used. *E. coli* cells were grown at 37 °C in a minimal salt medium supplemented with 1% polypeptone [13], while *S. aureus* cells were grown at 37 °C in a medium containing 1.5% polypeptone, 0.5% bovine extract, 0.5% NaCl and 0.5% K_2HPO_4. The cells were harvested in the late exponential phase of growth, washed twice with buffer (50 mM Mops-Tris/100 mM choline chloride, pH 7.2) and suspended in this buffer at 10 mg protein/ml [6,13]. Protein content was determined by the method of Lowry et al. [14].

Preparation of erythrocytes, mitochondria and liposomes. Human erythrocytes were washed twice with buffer (50 mM Mops-Tris/100 mM choline chloride, pH 7.2) and suspended in this buffer at a concentration of 0.5% (v/v). Mitochondria were isolated from rat liver [15,16] and suspended in 0.25 M sucrose/5 mM Tris-HCl (pH 7.4) at 15 mg protein/ml. Lipids of *E. coli* cells and mitochondria were extracted according to the methods in the literature [17,18]. Liposomes were prepared by the method of reverse-phase evaporation [6,19]. The inner aqueous phase of liposomes contained 50 mM Mops-Tris (pH 7.2)/100 mM KCl. Washed liposomes were suspended in 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride [6].

Fluorescence polarization. Peptide-induced changes in the phase transition temperature of DPPC liposomes were measured by the fluorescence polarization technique [6,20]. Diphenylhexatriene (1 mol% of lipid) was used as a fluorescence probe. The multilamellar liposome of DPPC containing diphenylhexatriene was prepared as reported previously [6]. In brief, DPPC containing diphenylhexatriene was swollen in buffer (50 mM Mops-Tris/100 mM choline chlo-

ride, pH 7.2) at 55 °C and suspended at a final concentration of 0.1 μmol/ml of lipid. Then a peptide dissolved in ethanol was added and sonicated briefly (bath-type, Bransonic B-220, 125W) for 10 s to obtain a homogeneous dispersion of the peptide. Fluorescence polarization was measured by excitation at 360 nm and emission at 430 nm. The degree of polarization was calculated as reported previously [6]. A small amount of ethanol used as the solvent of the peptides did not affect the phase transition temperature of DPPC at all.

Differential scanning calorimetry. A Rigaku-Denki DSC-10A was used at a scanning rate of 2 Cdeg/min. Only heating scans were recorded. Samples were prepared as follows. The dried thin films of DPPC and DPPC/diacetyl-gramicidin S were swollen in buffer solution comprising 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride at 55 °C. The resulting liposome suspensions were centrifuged (105 000 × *g*, 30 min) at 25 °C, and the pellets were resuspended in 50 μl of buffer. The suspension (20 μl) was pipetted and sealed in an aluminium pan. Each pan contained 2 μmol of lipid.

Results

Action of gramicidin S analogues on biological membranes

The structure-activity relationship of the gramicidin S analogues has been extensively studied using Gram-positive bacteria such as *S. aureus* [1,2]. Thus, first of all, the effect of the gramicidin S analogues on the permeability of *S. aureus* cells was investigated. As shown in Fig. 3, it was observed that gramicidin S and one of the analogues, X = D-*c*HxAla, increased greatly the efflux of K^+, while other analogues showed a rather weak action. The efflux increased in the order: X = Gly < diacetyl-gramicidin S < X = D-Leu < X = D-*c*HxAla ≈ gramicidin S. This order corresponded well with the minimum inhibitory concentrations of peptides on *S. aureus* reported previously [11]. The greater the amount of K^+ efflux showed the lower values of the minimum inhibitory concentration of the peptide: X = Gly (> 100 μg/ml) > X = D-Leu (25 μg/ml) > X = D-*c*HxAla (3 μg/ml) > gramicidin S (1.5 μg/ml). Diacetyl-gramicidin S, which barely increased the permeability, is also known to be inactive on bacteria [5]. This good correlation between the permeability increase and the antimicrobial activity indicates that the primary target of the gramicidin S analogues on *S. aureus* cells is the membrane structure. Here, we tried to estimate the percentage of K^+ efflux induced by peptides. To determine the total amount of K^+ in cells, we measured the efflux of K^+ induced by a surfactant. Two types of a cationic and a neutral surfactants, cetyltrimethylammonium bromide and Triton X-100, were used. These surfactants were added in a cell suspension at concentrations above their critical micelle concentrations (Fig. 3f and 3g). It was observed that cetyltrimethylammonium bromide caused the efflux of K^+ more efficiently than Triton X-100. This result was consistent with those of the earlier studies where a cationic surfactant showed bactericidal action due to an increase in the permeability of membrane [21,22], while a neutral surfactant acted poorly on bacteria [23]. Probably, Triton X-100 would have rather a weak ability to solubilize the bacterial membrane. We regarded the amount of K^+ efflux induced by cetyltrimethylammonium bromide as the total quantity of K^+ in *S. aureus* cells. It was calculated that gramicidin S and X = D-*c*HxAla caused an almost complete efflux of K^+ from cells within 1 min. In experiments described below, cetyltrimethylammonium bromide was used to determine the level of 100% efflux from bacteria, whereas Triton X-100 was utilized for erythrocytes, mitochondria and liposomes, because it is known

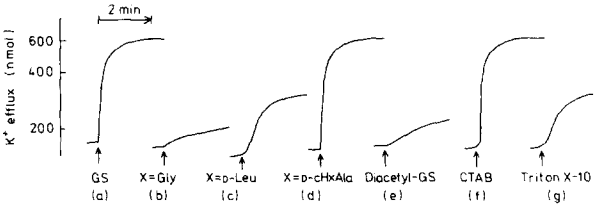

Fig. 3. The efflux of K^+ from *S. aureus* cells. Cell suspension (0.05 ml, 0.5 mg cell protein) was added to 1 ml 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride at 28 °C. (a)–(e) At the arrow, peptide (2 μl ethanol solution, final concentration: 20 μM) was added. Gramicidin S, GS. (f) Cetyltrimethylammonium bromide (CTAB, 50 μl, final concentration: 0.25 mg/ml) was added at the arrow. (g) Triton X-100 (50 μl, final concentration: 0.5 mg/ml) was added at the arrow. The efflux of K^+ was monitored with a K^+-ion selective electrode.

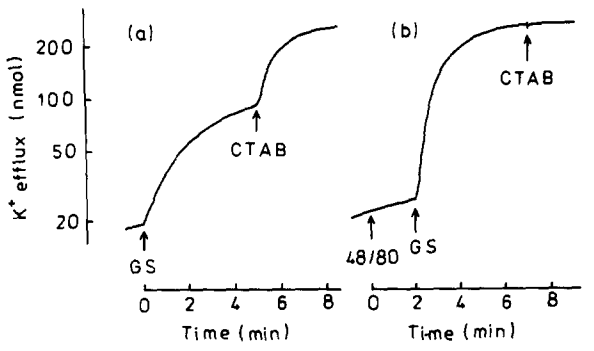

Fig. 4. The efflux of K^+ from *E. coli* cells. Cell suspension (0.05 ml, 0.5 mg cell protein) was added to 1 ml 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride at 28°C. (a) At the first arrow, gramicidin S (GS, 2 μl ethanol solution, final concentration: 20 μM) was added. The second arrow indicates the time when cetyltrimethylammonium bromide (CTAB, 50 μl, final concentration: 0.25 mg/ml) was added. (b) The first arrow indicates the time when compound 48/80 (2 μl, final concentration: 10 μg/ml) was added; second arrow indicates the time when gramicidin S (2 μl ethanol solution, final concentration: 20 μM) was added. At the third arrow, cetyltrimethylammonium bromide (50 μl, final concentration: 0.25 mg/ml) was added.

that Triton X-100 increases effectively the permeability of the cytoplasmic membranes composed of phospholipids with unsaturated fatty acids alone and of the phospholipids/cholesterol system [24].

Then, we investigated the action on *E. coli* cells. In contrast to Gram-positive bacteria, Gram-negative bacteria such as *E. coli* have an outer membrane in their cell structure [25,26]. It is well known that the outer membrane prevents the penetration of hydrophobic antibiotics or macromolecular proteins into cells [25,26]. We have recently reported that gramicidin S is able to disrupt the *E. coli* outer membrane structure and reaches the cytoplasmic membrane to cause a change in the K^+ permeability [6]. Fig. 4a shows a typical result of K^+ efflux from *E. coli* cells after addition of gramicidin S. In this case, however, it was observed that addition of cetyltrimethylammonium bromide caused further efflux of K^+. This indicates that gramicidin S alone can not efficiently increase the K^+ permeability of *E. coli* cells. The higher value of the minimum inhibitory concentration of gramicidin S against *E. coli* was consistent with this result [1,2]. We speculated that the weak action of gramicidin S on *E. coli* cells might be due to its rather poor ability to disrupt the outer membrane structure. To evaluate this possibility, cells were treated with compound 48/80 before addition of gramicidin S, since 48/80 disrupted more effectively the barrier function of the outer membrane [27,28]. As shown in Fig. 4b, gramicidin S induced completely the efflux of K^+ from *E. coli* cells after treatment with 48/80. As for the action of the gramicidin S analogues, X = D-*c*HxAla showed a similar behaviour, while other analogues did not increase the K^+ permeability of *E. coli* cells even in the presence of 48/80. These results indicate that gramicidin S and X = D-*c*HxAla bring a great increase in the permeability of bacterial cytoplasmic membrane irrespective of Gram-positive and -negative bacteria.

Next, we investigated the action of peptides on rat liver mitochondria *. Also, gramicidin S and X = D-*c*HxAla increased the K^+ permeability, while X = D-Leu and X = Gly did not. However, with mitochondria, it was found that diacetyl-gramicidin S caused the efflux of K^+ to the same level of gramicidin S within a few minutes (compare Fig. 5a with b). Such a great increase in the K^+ efflux was not observed with bacteria. We further measured the efflux of K^+ from human red blood cells. Although gramicidin S and X = D-*c*HxAla similarly increased the permeability of red blood cells, other analogues including diacetyl-gramicidin S did not.

Table I summarizes the percentage of K^+ efflux induced by the peptides. As can be seen from Table I, gramicidin S and one of the analogues, X = D-*c*HxAla, increased the permeability of all types of the cytoplasmic membrane of cells non-selectively. These two peptides, however, acted rather weakly on the outer membrane of *E. coli*. Diacetyl-gramicidin S increased the permeability of only the mitochondrial membrane.

Action of gramicidin S analogues on liposomal membranes

In order to examine whether the action was dependent on the lipid portions of membrane, we

* Most experiments in this work were made at 28°C. However, in mitochondria, experiments were carried out at the lower temperature of 20°C, since a great deal of spontaneous efflux of K^+ was observed at 28°C.

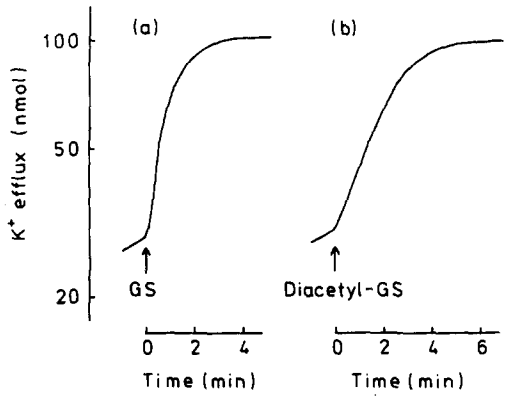

Fig. 5. The efflux of K^+ from rat liver mitochondria upon addition of (a) gramicidin S (GS) and (b) diacetyl-gramicidin S. Cells (0.05 ml, 0.75 mg cell protein) were suspended in 1 ml 50 mM Mops-Tris (pH 7.2)/ 100 mM choline chloride at 20 °C. At the arrow, peptide (2 μl ethanol solution, final concentration: 20 μM) was added.

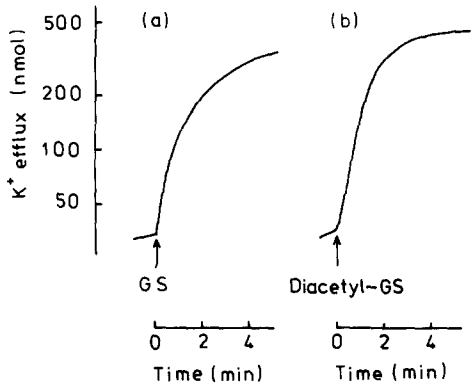

Fig. 6. The efflux of K^+ from liposomes prepared with egg PC upon addition of (a) gramicidin S (GS) and (b) diacetyl-gramicidin S. Liposomes (0.3 mg lipid/ml) were suspended in 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride at 28 °C. At the arrow, peptide (2 μl ethanol solution, final concentration: 20 μM) was added.

extracted lipids from *E. coli* cells and mitochondria to prepare liposomes by the method of reverse-phase evaporation. Table II shows the efflux of K^+ induced by peptides from the liposomes containing K^+ ion. In both liposome systems, the efflux increased in the order: X = Gly ≈ X = D-Leu < X = D-*c*HxAla ≈ gramicidin S. Diacetyl-gramicidin S elicited greatly the efflux of K^+ from liposomes prepared with mitochondrial lipid, but it acted weakly on liposomes prepared from *E. coli* lipid. These results corresponded well with the permeability change occurring on intact cells, though X = D-Leu and X = Gly stimulated the efflux of K^+ more significantly from liposomes than from whole cells.

Then, we considered why diacetyl-gramicidin S could increase the permeability of only mitochondrial membrane. We analysed the action of diacetyl-gramicidin S in connection with the lipid composition of membranes of mitochondria and bacteria. It is well known that the mitochondrial membrane is composed of PC (45 mol%), PE (30 mol%) and cardiolipin (15 mol%) [29], while the cytoplasmic membrane of *E. coli* consists of PE (80 mol%) and cardiolipin and/or PG (20 mol%) [30,31]. The essential difference seems to be the existence of PC in mitochondrial membrane. Thus we prepared the liposomes composed of egg PC and compared the action of diacetyl-gramicidin S with that of gramicidin S. It was found that diacetyl-gramicidin S induced the efflux of K^+ more markedly than gramicidin S (Fig. 6). Here we were interested in whether all of the action of diacetyl-gramicidin S on biological membranes can be reproduced by using egg yolk phospholipids. Although we were unable to prepare liposomes composed of egg PE alone, further addition of egg PG (10 mol%) formed liposomes [30]. With this

TABLE I

PERCENTAGE OF PEPTIDE-INDUCED K^+ EFFLUX FROM CELLS

Cells were suspended in 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride at the following concentrations: *S. aureus* (0.5 mg cell protein/ml); *E. coli* (0.5 mg cell protein/ml); rat liver mitochondria (0.75 mg cell protein/ml); human erythrocytes (0.5% (v/v)). The concentration of peptide added was 20 μM. Percentage effused within 5 min was calculated. Measurements were made at 28 °C, except for the case of mitochondria which was performed at 20 °C. GS, gramicidin S.

	GS	X = Gly	X = D-Leu	X = D-*c*HxAla	Diacetyl-GS
S. aureus	100	10	30	100	20
E. coli	30	0	0	20	0
E. coli [a]	100	0	0	100	0
Mitochondria	100	0	0	100	100
Erythrocytes	100	0	0	100	0

[a] Pre-treated with compound 48/80 to disrupt the outer membrane structure.

TABLE II

PERCENTAGE OF PEPTIDE-INDUCED K^+ EFFLUX FROM LIPOSOMES

Lilposomes were suspended at the following lipid concentrations: *E. coli* lipid (0.3 mg/ml); mitochondrial lipid (0.3 mg/ml); egg PC (0.3 mg/ml); egg PE/egg PG (0.3/0.03 mg/ml); egg PC/cholesterol (0.3/0.15 mg/ml). The concentration of peptide added was 20 μM. Percentage effused within 5 min was calculated. Measurements were made at 28° C. –, not determined. GS, gramicidin S.

	GS	X = Gly	X = D-Leu	X = D-*c*HxAla	Diacetyl-GS
E. coli lipid	80	20	30	80	35
Mitochondrial lipid	100	25	15	100	80
Egg PC	80	20	25	75	100
Egg PE/egg PG	100	–	–	–	35
Egg PC/cholesterol	60	–	–	–	10

liposomes, the action of diacetyl-gramicidin S decreased greatly as in the case of *E. coli* lipid (Table II). We further prepared liposomes composed of egg PC/cholesterol. It was observed that the presence of cholesterol reduced greatly the action of diacetyl-gramicidin S, which reflected the low sensitivity of this peptide on human erythrocytes. These results gave a good explanation of why diacetyl-gramicidin S acted selectively on the mitochondrial membrane rich in PC.

Changes in the membrane fluidity of liposomes induced by gramicidin S analogues

The above results indicated that the gramicidin S analogues acted on lipid bilayer of membrane, resulting in changes in the permeability. The next problem is the mechanism of action on lipid bilayer. We classified the peptides used in the present study into two types: (1) gramicidin S and the analogues, *cyclo*(-X-D-Leu-D-Lys-D-Leu-L-Pro-)$_2$, where X = Gly, D-Leu and D-*c*HxAla; (2) diacetyl-gramicidin S, because diacetyl-gramicidin S alone did not contain a cationic group in the molecule.

At first, we considered the mechanism of the type 1 peptides. We have recently reported that gramicidin S decreases the phase transition temperature of neutral phospholipid DPPC [6]. This indicates that gramicidin S is able to penetrate into neutral lipids which constitute the main part of biological membrane and can expand the distance between neighbouring phospholipid molecules to decrease the phase transition temperature. Thus, we have speculated that gramicidin S, as a result of such a strong membrane disturbance, increases the permeability of various kinds of membranes non-selectively [6]. It is of interest to investigate whether the type 1 analogues decrease the phase transition temperature of DPPC liposomes, depending on their ability to increase the permeability. It was found that X = D-*c*HxAla decreased the phase transition temperature of DPPC liposomes, while X = D-Leu and X = Gly did not do so to a significant extent (Fig. 7). These

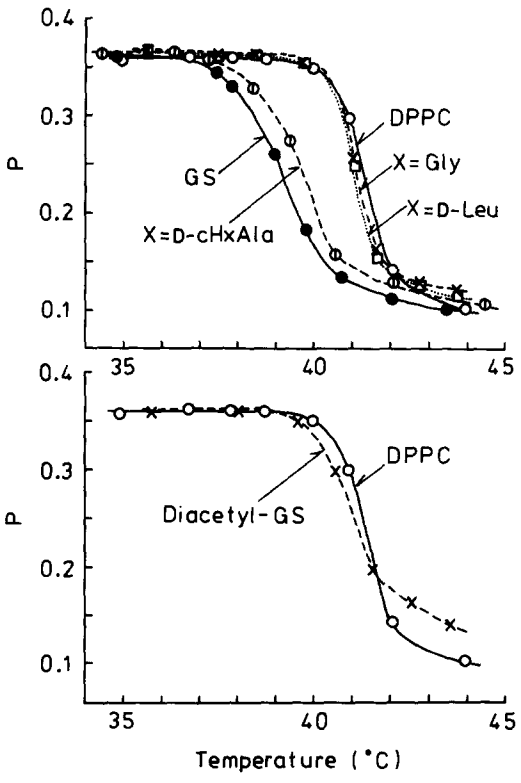

Fig. 7. Changes in the degree of polarization (P) of DPPC liposomes before and after addition of gramicidin S (GS) analogues. The results of the type 1 and the type 2 peptides are shown separately. In these figures, 'DPPC' shows the result of DPPC liposomes alone (100 μM) in buffer solution comprised of 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride. Each result after addition of a peptide (40 μM) is shown by describing the abbreviated name of a peptide used in the text.

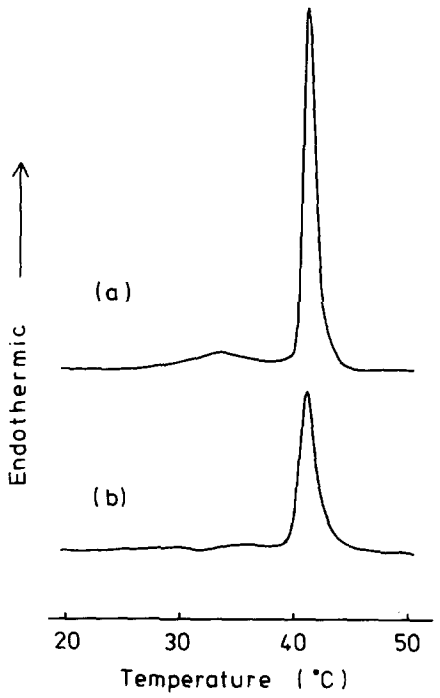

Fig. 8. Changes in the enthalpy of the phase transition of DPPC liposomes in the presence of diacetyl-gramicidin S. (a) DPPC liposomes alone. (b) In the presence of diacetyl-gramicidin S (20 mol%).

data show that there is a common tendency between the ability of peptides to decrease the phase transition temperature of DPPC liposomes and to increase the permeability of cells.

The result of the type 2 analogue, diacetyl-gramicidin S, is shown in the lower part of Fig. 7. This peptide rendered the degree of polarization (P) of the fluorescence probe significantly high at the temperature region above the phase transition of DPPC. This indicates that the mobility of DPPC at liquid phase was rather restricted by diacetyl-gramicidin S. Furthermore, a calorimetric study showed that diacetyl-gramicidin S decreased markedly the enthalpy of the phase transition of DPPC liposomes (Fig. 8). The presence of 20 mol% diacetyl-gramicidin S reduced the enthalpy of DPPC alone to about 60%. Such a pronounced decrease in the enthalpy of the phase transition is one of the characteristics of an intrinsic protein embedded into membrane [32,33]. We shall discuss the mechanism of permeability increase by diacetyl-gramicidin S in connection with the recent studies on the interaction between the intrinsic protein and membrane.

Discussion

The present study showed that the antimicrobial activity of the gramicidin S analogues was derived from the induced permeability enhancement of membrane. The order of the efflux of K^+ from *S. aureus* cells coincided well with that of the minimum inhibitory concentrations of peptides. Among the peptides examined, gramicidin S and one of the analogues, X = D-*c*HxAla, increased greatly the permeability of *S. aureus* cells. However, these two peptides acted on *E. coli* cells less effectively than on *S. aureus* cells. After *E. coli* cells were treated with compound 48/80 which is known to disrupt effectively the barrier function of the outer membrane [27,28], gramicidin S and X = D-*c*HxAla induced completely the efflux of K^+ from cells within a few minutes. Thus, it was concluded that the lower antimicrobial activity against *E. coli* was due to the low ability of peptides to disrupt the outer membrane structure existing in Gram-negative bacteria. Furthermore, it was observed that gramicidin S and X = D-*c*HxAla enhanced the efflux of K^+ from rat liver mitochondria, human erythrocytes and liposomes. These data indicated that these two peptides were able to increase the permeability of various kinds of the cytoplasmic membrane of cells without any selectivity. One of the analogues, diacetyl-gramicidin S, showed interesting behaviour. This peptide increased the permeability of mitochondria, while it did not increase the permeability of other biological membranes.

According to the classification as mentioned in the Results, the peptides used in the present study were devided into the following two types: (1) gramicidin S and its analogues, *cyclo*(-X-D-Leu-D-Lys-D-Leu-L-Pro-)$_2$, where X = Gly, X = D-Leu and X = D-*c*HxAla; (2) diacetyl-gramicidin S which lacks a cationic moiety of gramicidin S.

At first, the mechanism of action of the type 1 peptides is discussed. The present results showed a good correlation between the ability of the peptides to increase the K^+ permeability of the cytoplasmic membranes and to decrease the phase transition temperature of DPPC liposomes. The decrease in

the phase transition temperature can be explained as follows. As already mentioned in the Introduction, gramicidin S has the β-sheet structure with two cationic ornithine residues on one side of the molecular plane and hydrophobic residues on the other side (Fig. 1). In phospholipid bilayer, two cationic ornithine residues of gramicidin S are exposed in an aqueous phase, while the hydrophobic side is penetrated into the phospholipid molecules; the existence of the hydrophilic parts prevents the embedding of the peptide into the lipid bilayer [34]. The partial penetration of the peptide makes a certain space in the interior of membrane, resulting in an increase in the movement of the acyl chains of phospholipid molecules to decrease the phase transition temperature [32,33]. We depict such gramicidin S-phospholipid interaction in Fig. 9. The circles indicate the charged head groups of phospholipid and the zigzag lines represent the two fatty-acid chains. Although a similar illustration of gramicidin S in bilayer membrane has recently been presented in the study of nuclear magnetic resonance [34], the present figure is slightly revised to illustrate the induced disturbance. It seems likely that the appearance of the disordered regions in an ordered membrane triggers the efflux of K^+ [35,36]. Papahadjopoulos and co-workers [32,33] have indicated that there is a correlation between the ability of peptides to decrease the phase transition temperature and to increase the permeability of membrane.

Now, the effect of gramicidin S on membrane fluidity is compared with those of various polycationic compounds. So far, peptide-induced changes in the phase transition temperature have been investigated by many workers [37]. It has been reported that cationic peptides with an appropriate lipophilic character (e.g., basic myelin protein and cytochrome *c*) lower the phase transition temperature of acidic phospholipid, dipalmitoylphosphatidylglycerol, but they do not affect the phase transition temperature of neutral phospholipid DPPC at all [32,33]. Amphipathic polycations such as polymyxin B and compound 48/80 also decrease the phase transition temperature of acidic phospholipids only [20,36]. There is a tendency that charged molecules do not act on neutral lipids. In contrast, gramicidin S and one of

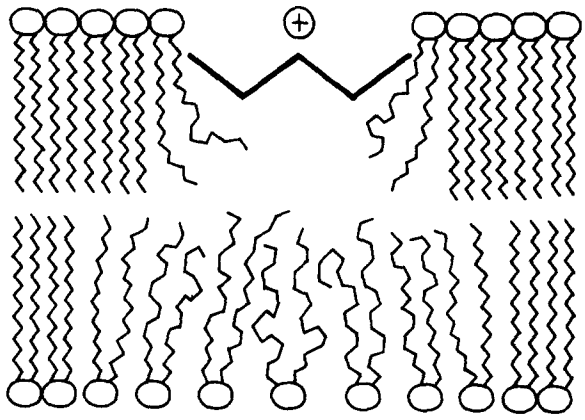

Fig. 9. Schematic illustration of gramicidin S-phospholipid interaction.

the analogues having X = D-*c*HxAla decrease the phase transition temperature of neutral lipid DPPC. The ability of gramicidin S to lower the phase transition temperature of DPPC or of another neutral lipid, dimyristoylphosphatidylcholine, is also detected by means of the differential scanning calorimetry [38,39]. These surveys indicate that gramicidin S and X = D-*c*HxAla, differing from many polycationic compounds, can penetrate into even neutral lipids constituting the main part of the biological membrane. It is tempting to speculate that such a strong membrane disturbance of gramicidin S arises from its characteristic 'sidedness' structure.

The previous study [11] has indicated that all the gramicidin S analogues of the type 1 used in the present study have the mirror image conformation of gramicidin S-like β-sheet. Fig. 10 shows a schematic illustration of the conformation of the gramicidin S analogues, compared with that of gramicidin S. It is understandable that the bulkiness and hydrophobic characters of the gramicidin S analogues increase in the order: X = Gly < D-Leu < D-*c*HxAla, depending on the substituent X at the both ends of the β-sheet structure. Thus, the packing structure can be expanded in this order upon the penetration of the peptides into the membrane, and this is the reason why X = D-*c*HxAla has the strong ability to decrease the phase transition temperature of DPPC membrane. It is reasonable to expect that, if peptides can expand greatly the membrane structure of neutral lipids which constitute the main parts of the bio-

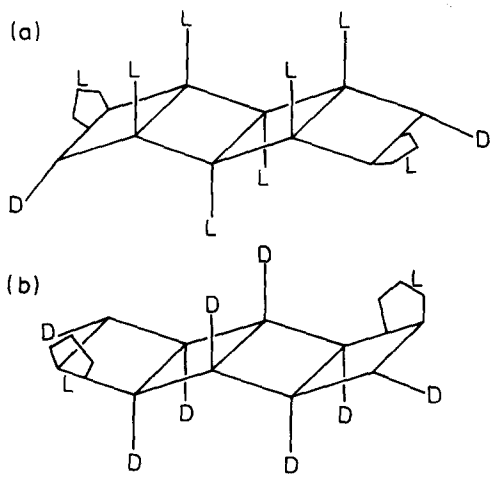

Fig. 10. β-Sheet conformations of (a) gramicidin S and (b) gramicidin S analogues.

logical membrane, they can stimulate the permeability of cells without any selectivity. Here, the phospholipid composition of the cytoplasmic membrane of *S. aureus* cells should be mentioned. It has been reported that the composition of phospholipids of *S. aureus* varies greatly with the pH of the medium [40–42]. At neutral pH, as in the present case, the phospholipids were composed of *O*-lysylphosphatidylglycerol (15–20 mol%), phosphatidylglycerol (60–70 mol%) and cardiolipin (15–25 mol%) [42], and therefore the membrane is rich in negatively charged lipids. Thus, *S. aureus* cells might be more sensitive to cationic peptides, and even the analogues having X = D-Leu and X = Gly stimulated the efflux of K^+ to some extent, although they scarcely acted on other biological membranes (Table I).

These considerations do not conflict with the 'sidedness hypothesis' [1,2] which points out the importance of the 'sidedness' of hydrophobic and hydrophilic parts of the gramicidin S molecule for the antimicrobial activity. We emphasize in the present study that the existence of the β-sheet conformation and appropriate hydrophobicity in gramicidin S causes the expansion of the membrane structure, resulting in an increase in the permeability of the membrane, and this brings about various biological activities of gramicidin S, including the antimicrobial activity.

The next problem is the mechanism of action of the type 2 peptide, diacetyl-gramicidin S. Although this peptide enhanced the permeability of the mitochondrial membrane, it did not increase the permeability of membranes of bacteria and erythrocytes. It was found that diacetyl-gramicidin S enhanced markedly the permeability of liposomes composed of PC. The presence of PE or cholesterol reduced the action of diacetyl-gramicidin S. The fluorescence polarization experiment showed that diacetyl-gramicidin S restricted slightly the motion of acyl chains of DPPC liposomes in the liquid phase of the membrane. The calorimetric study showed that the peptide reduced greatly the enthalpy of the phase transition of DPPC. It has been reported that an intrinsic protein embedded into membrane (such as myelin proteolipid) did not affect the phase transition temperature of liposomes, but reduced the enthalpy of the phase transition [32,33], this being similar to the present case of diacetyl-gramicidin S. There is also much evidence indicating the inhibition of molecular motion of the acyl chains of phospholipid adjacent to intrinsic protein [43]. In the calorimetric study, the restricted motion of lipid induced by diacetyl-gramicidin S was not directly detected; however, the decrease in the enthalpy implied that fewer phospholipid molecules participate in the cooperative melting of the bulk lipid. The fluorescence polarization measurement using diphenylhexatriene indicated more directly the evidence that the peptide formed a tight domain of lipid. It is reasonable to assume that the efflux of K^+ occurred markedly through the boundary between such a tight lipid and the bulk lipid. It is well known that the permeability of membrane is most enhanced through the interface between solid and liquid phases of membrane [36,44–46].

Then the question arose why diacetyl-gramicidin S did not markedly increase the permeability of PE membrane. In this connection, it is worthwhile to refer the recent study [47] on the permeability characteristics of PE membrane itself. In contrast with PC membrane, the permeability of PE membrane did not increase at its phase transition temperature from gel to liquid-crystal. This has been explained by the strong hydrogen bonding between the PE molecules, which would minimize the formation of a defect structure at the boundary between solid and liquid

phases [47]. It seems likely that such a strong intermolecular bonding of PE rejects the penetration of diacetyl-gramicidin S into membrane. The existence of cholesterol may also weaken the penetration of this peptide into membrane. On the other hand, it has been reported that another intrinsic protein, glycophorin, acts on the DPPC membrane in a different way [48]. This protein formed a fluid-like domain in PC membrane [48,49] differently from the intrinsic protein already mentioned. Nevertheless, the effects of glycophorin and diacetyl-gramicidin S on the permeability of membrane resembled each other well; that is, glycophorin markedly affected the permeability of PC membrane, but not PE membrane; the presence of cholesterol in PC membrane also decreased the permeability [50]. At the present stage, however, we can not say with confidence whether a common mechanism exists in the permeability changes induced by the intrinsic proteins.

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