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Mode of action of gramicidin S on *Escherichia coli* membrane

Takashi Katsu *, Hideki Kobayashi and Yuzaburo Fujita

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700 (Japan)

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The action of a cationic antibiotic gramicidin S on the outer and cytoplasmic membranes of *Escherichia coli* was studied. It was found that gramicidin S disrupted the permeability barrier of the outer membrane, permitting the permeation of an antibiotic ionophore, this being similar to the action of the dimer in compound 48/80 (Katsu, T., Shibata, M. and Fujita, Y. (1985) Biochim. Biophys. Acta 818, 61–66). However, differently from the dimer, gramicidin S further stimulated the efflux of K^+ through the cytoplasmic membrane of *E. coli*. The time course of K^+ permeability change accorded well with that of change in the viability of *E. coli* cells. These changes occurred at temperatures above the phase transition of the cytoplasmic membrane. This temperature range differed greatly from the case of polymyxin B, a polycationic antibiotic acting at temperatures above the phase transition of the outer membrane. We discuss the mode of gramicidin S action on the cytoplasmic membrane of *E. coli*, in comparison with the results on red blood cells and liposomes.

Introduction

Gramicidin S, a cyclic decapeptide of (-Val-Orn-Leu-D-Phe-Pro-)$_2$, shows the antimicrobial action [1]. The conformation 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 amino residues on the other side (Fig. 1) [2,3]. Although various analogues of gramicidin S have been prepared in order to clarify the relationship between structure and biological activity, the mechanism of antibiotic action of gramicidin S is not yet completely elucidated. Gramicidin S is known to release various intracellular components, and thus it is speculated that the antimicrobial activity is due to the induced permeability of bacterial membranes [2,3].

As a part of our project to elucidate the mechanism of action of polycationic compounds on bacteria, the mode of action of gramicidin S on *Escherichia coli* was examined in the present study. The cell envelope of Gram-negative bacteria such as *E. coli* consists of three essential layers, namely the cytoplasmic membrane, the peptidoglycan layer and the outer membrane. The peptidoglycan layer contributes mechanical rigidity. The outer membrane is characteristic of Gram-negative bacteria [4–6]. Many hydrophobic antibiotics or macromolecular proteins (such as lysozyme) cannot permeate through the outer membrane [4–6]. It is now well established that a lipopolysaccharide is located at the outside of the outer membrane, and divalent cations (such as Mg^{2+} and Ca^{2+}) maintain the outer membrane structure [5,6]. Vaara and Vaara [7,8] and we [9,10] have

* To whom correspondence should be addressed.

Abbreviations: Mops, 4-morpholinepropanesulphonic acid; ANS, 8-anilino-1-naphthalenesulphonic acid; DPPC, dipalmitoylphosphatidylcholine; CTAB, cetyltrimethylammonium bromide.

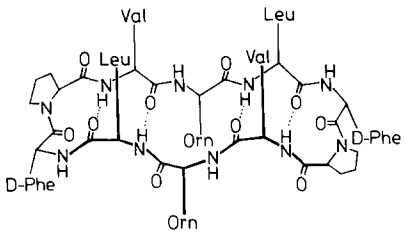

Fig. 1. β-sheet conformation of gramicidin S.

reported that polycationic compounds such as polylysine and polymyxin analogues are able to increase the permeability of the outer membrane of Gram-negative bacteria. These polycations bind lipopolysaccharide molecules in the outer membrane to trigger disruption of the permeability barrier of membrane [6,11–13], though the mode of disruption differs significantly from one compound to another [6–8]. It has also been reported, however, that polycations with a small number of basic charges do not disrupt the barrier function of the outer membrane [7], though these cations can bind isolated lipopolysaccharide [7,14]. The exact structure-activity relationship remains obscure at present. In the screening of various polycations, we found a dication having the ability to increase the permeability of the outer membrane of *E. coli* [15,16]. The dication, referred to as the 'dimer' in compound 48/80, bis[2-methoxy-5-(2-methylaminoethyl)phenyl]methane dihydrochloride, has two cationic amino and two hydrophobic phenyl groups in the molecule [17]. We were interested in the structural resemblance between the dimer and gramicidin S; both are dications with a lipophilic character. We expected that gramicidin S would disrupt the barrier function of the outer membrane, similarly to the action of the dimer [15,16]. As was expected, gramicidin S was able to increase the permeability of the outer membrane of *E. coli*. However, differently from the dimer, gramicidin S further elicited a change in the K^+ permeability of the cytoplasmic membrane of *E. coli*. The permeability change occurred at temperatures above the phase transition of the cytoplasmic membrane. This temperature range differed greatly from the case of a polycationic antibiotic polymyxin B reported previously [10,18]. In this communication we discuss the mechanism of the gramicidin S-induced permeability change of the cytoplasmic membrane of *E. coli*, in comparison with the results on red blood cells and liposomes.

Materials and Methods

Chemicals. The sources of chemicals used in this work were as follows: gramicidin S, gramicidin D (a mixture of gramicidin A, B and C) and DPPC from Sigma; nigericin from Calbiochem-Behring; valinomycin from Boehringer Mannheim; di(*n*-octyl)phthalate, ammonium salt of ANS and diphenylhexatriene from Tokyo Kasei Kogyo; poly(vinyl chloride) (degree of polymerization = 1020) and CTAB from Nakarai Chemicals; tetraphenylphosphonium chloride and sodium tetraphenylborate from Dojindo Laboratories. The dimer in 48/80 was prepared as reported previously [17]. Other chemicals used were all of analytical reagent grade.

Growth and preparation of bacteria. The bacterial strain used in this study was *E. coli* W3110, a derivative of K 12. Cells were grown at 37°C in a minimal salt medium supplemented with 1% polypeptone [9,10]. 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 [9,10]. Protein content was determined by the method of Lowry et al. [19]. Isolated cytoplasmic membrane vesicles were prepared by the EDTA-lysozyme method described by Kaback and co-workers [20,21]. Loading of high concentrations of K^+ into the vesicles was achieved as described previously [10,22].

Measurement of minimum inhibitory concentration. Minimum inhibitory concentrations were measured as reported earlier [7]. A liquid medium (250 μl) containing a minimal salt supplemented with 1% polypeptone [9,10] and a drug to be tested was pipetted into wells of a microtitre plate (Sumitomo-Bakelite Co. Ltd., Tokyo, Japan; multiplate MS-3396U and cover MS-39961). In each well, 50 μl of cell suspension (final concentration: 10^4 cells/ml) were added. The plates were allowed to stand for 18 h at 37°C. The lowest concentration of drug that completely inhibited visible growth was recorded and regarded as the mini-

mum inhibitory concentration.

Measurement of critical micelle concentration. The critical micelle concentrations of gramicidin S and the dimer in 48/80 were determined by the fluorescence probe method using ANS [10]. Solutions of various amounts of gramicidin S were prepared by adding ethanol solution of gramicidin S to a buffer solution comprising 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride. Because the presence of ethanol affects the critical micelle concentration [23], we always added a constant amount of ethanol (5% (v/v)). Solutions containing various amounts of the dimer could be prepared without addition of ethanol. Fluorescence measurements were performed at 28°C by adding 10 μM ANS to the solutions. Fluorescence intensity was measured by excitation at 375 nm and emission at 480 nm.

Preparation of ion-selective electrode and measurement of K^+ efflux from cells. A K^+-sensitive electrode was constructed by the use of poly(vinyl chloride)-based membranes as reported previously [9,10,24]. The poly(vinyl chloride) membrane had the following composition: 1 mg valinomycin, 60 μl di(*n*-octyl)phthalate and 25 mg poly(vinyl chloride). The materials were dissolved in 2–3 ml tetrahydrofuran. The solution was poured into a flat petri dish of 30 mm diameter, then the solvent was evaporated off slowly at room temperature. The resulting poly(vinyl chloride) membrane was cut and stuck on poly(vinyl chloride) tube (outer diameter: 4 mm, inner diameter: 3 mm) with tetrahydrofuran. The sensor membrane was soaked in 10^{-2} M solution of KCl overnight. It was observed, however, that the K^+-sensitive electrode thus prepared responded to gramicidin S, and the measurements of efflux of K^+ from cells were greatly interfered with. We applied a dialysis membrane (cellophane tubing-seamless, Union Carbide Co.) to cover the sensor membrane of the ion sensitive electrode. This procedure was successful for the measurement of the gramicidin S-induced changes in K^+ permeability, free from interference. Fig. 2a shows a schematic illustration of the present K^+-sensitive electrode. A polyethylene tube (outer diameter: 3mm, inner diameter: 2 mm) was inserted into a poly(vinyl chloride) tube to support the dialysis membrane with a rubber O-ring. An Ag/AgCl electrode was prepared by anodic chlorination of silver wire (0.3 mm in diameter, 30 mm in length) in an aqueous solution containing 0.1 M NaCl and 0.1 M HCl at 0.5 mA for 30 min. Fig. 2b shows a reference electrode constructed in our laboratory. To make a solution junction, the end of a glass tube was sealed with a piece of platinum wire (0.8 mm in diameter), in which pinholes afforded ionic transport. A salt bridge was filled with an aqueous solution of 1 M

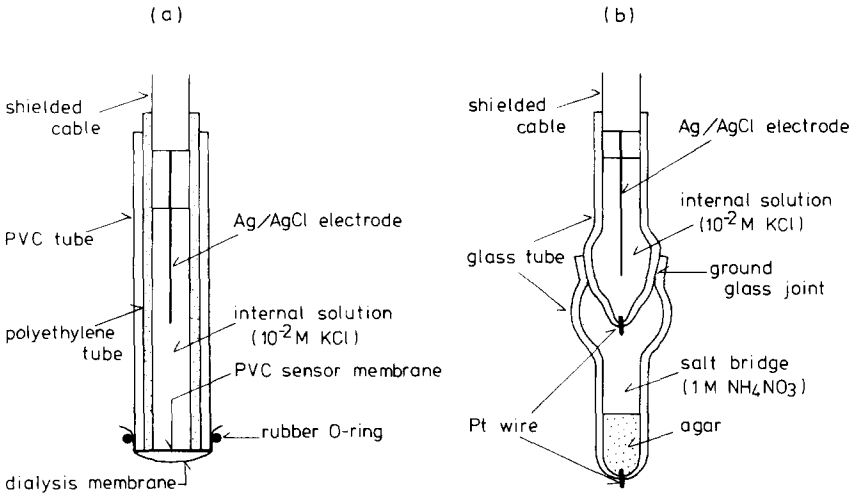

Fig. 2. Construction of (a) K^+-sensitive electrode and (b) reference electrode.

NH_4NO_3 and inserted between a reference and a sample solution. Agar was filled at the lower part of the salt bridge to minimize the leakage of a high concentration of ions into the sample solution. The electrochemical cell in the present study can be represented as follows: Ag,AgCl/0.01 M KCl/poly(vinyl chloride) sensor membrane attached to dialysis membrane/sample solution/1 M NH_4NO_3/0.01 M KCl/Ag,AgCl. Electromotive force between a pair of Ag/AgCl electrodes was measured with an appropriate field effect transistor-operational amplifier (input resistance: $> 10^{12}$ Ω) and recorded. The sample solution was stirred with a magnetic stirrer. Fig. 3 shows the calibration curve of K^+-sensitive electrode in buffer solution comprising 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride. The electrode exhibited a Nernstian response (57 mV/decade) from 10^{-5} M to 10^{-2} M of K^+. A typical measurement of efflux of K^+ from cells was performed as follows. 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. Then 2.5 μl gramicidin S in ethanol solution (final concentration: 25 μg/ml) was added. The concentration of K^+ in the assay media was calculated from the calibration curve of the K^+-sensitive electrode. The amount of efflux at various temperatures was determined from the calibration curve measured at the corresponding temperatures. In this work, we also prepared a tetraphenylphosphonium ion-

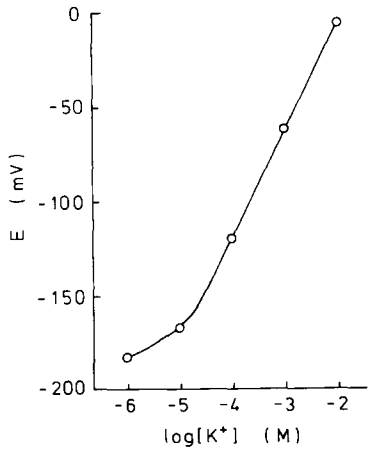

Fig. 3. Response of K^+-sensitive electrode in 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride at 28°C.

(TPP^+-) sensitive electrode. The TPP^+-sensitive electrode had the following membrane composition: 0.1 mg sodium tetraphenylborate, 60 μl di(*n*-octyl)phthalate and 25 mg poly(vinyl chloride) [9,10]. A cover of dialysis membrane was omitted because the TPP^+-sensitive electrode did not respond to gramicidin S.

Measurement of cell viability. The viability of *E. coli* cells was determined by counting colonies. After gramicidin S was added to the cell suspension, samples were taken at intervals, 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). Colonies were counted after standing for 15 h at 37°C.

Preparation of red blood cells and liposomes. Preserved sheep blood was purchased from Nishinippon Sheep Farm. Cells 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 1% (v/v). Liposomes were prepared by the method of reverse-phase evaporation [25]. Egg phosphatidylcholine (7.7 mg, purchased from The Green Cross Corporation, Osaka, Japan) was dissolved in 1.5 ml diethyl ether, followed by addition of 1 ml of aqueous solution containing 50 mM Mops-Tris (pH 7.2)/100 mM KCl. The mixture was sonicated (Tomy Seiko Co., Ltd., Tokyo, Japan; UR-200P) for 2 min at 0°C to obtain a homogeneous emulsion. The diethyl ether solvent was then removed by using a conventional rotary evaporator under reduced pressure (by water aspirator) at 25°C. After the diethyl ether was completely removed, a homogeneous suspension of liposomes was formed. The liposomes were centrifuged (105 000 × *g*, 30 min) and washed twice to remove the untrapped K^+. The final pellet was suspended in 10 ml 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride.

Fluorescence polarization. Drug-induced changes in the phase transition temperature of DPPC liposomes were measured by the fluorescence polarization technique [10,26]. Diphenylhexatriene (1 mol% of DPPC) was used as the fluorescence probe. DPPC containing diphenylhexatriene was swollen in buffer (50 mM Mops-Tris (pH 7.2)/100 mM choline chloride) at 55°C, resulting in the formation of multilamellar liposomes. A small

aliquot of the liposomes was pipetted and suspended in the same buffer at a final concentration of 0.1 μmol/ml of lipid. Then a drug was added to this liposome suspension, and the suspension was briefly sonicated (for about 10 s at 55°C) to make a homogeneous dispersion. Fluorescence polarization was measured by using a Hitachi MPF-4 fluorospectrophotometer equipped with polarizers and thermoregulated cells. The degree of polarization was calculated according to the following equation:

$$P = \frac{I_{VV} - C_f I_{VH}}{I_{VV} + C_f I_{VH}}$$

where I is the fluorescence intensity, and subscripts V and H refer respectively to the vertical and horizontal orientations of the excitation (first) and emission (second) polarizers. $C_f (= I_{HV}/I_{HH})$ is a correction factor [27]. The temperature in the cell was determined with a thermistor. A small amount of ethanol used as the solvent of gramicidin S did not affect the phase transition temperature of DPPC at all.

Results

Minimum inhibitory concentrations and critical micelle concentrations of gramicidin S and the dimer in 48/80

We examined the minimum inhibitory concentrations of gramicidin S and the dimer in 48/80 on *E. coli* W3110 used in the present study. Gramicidin S inhibited cell growth at 6 μg/ml, while the dimer did not inhibit even at a concentration above 200 μg/ml. Although the dimer did not affect the growth of cells at all, this compound had the ability to stimulate the permeability of the outer membrane [15,16], as mentioned in the Introduction. The critical micelle concentration of gramicidin S and the dimer were also examined by the fluorescent probe method using ANS [10]. In the presence of ANS, there was an abrupt increase in the fluorescence intensity of ANS at concentrations of gramicidin S above 400 μg/ml and of the dimer above 800 μg/ml. We regarded these concentrations as the critical micelle concentrations of gramicidin S and the dimer. The critical micelle concentrations of gramicidin S and the dimer were comparable; however, only gramicidin S inhibited the growth of *E. coli* cells. Furthermore, the critical micelle concentration of gramicidin S was much higher than its minimal inhibitory concentration. These data seemed to suggest that the mechanism of growth inhibition by gramicidin S was not simply due to the detergent action. In the present study, we usually used 25 μg/ml gramicidin S. At this concentration, it was expected that gramicidin S would show its bactericidal action, because the previous study [1] had indicated that the bactericidal concentration of gramicidin S rose to about 5–10-times its minimum inhibitory concentration.

Gramicidin S-induced changes in permeability and cell viability

Katchalski and co-workers reported in 1956 that gramicidin S released inorganic phosphate from bacteria [1], indicating that it increased membrane permeability. We reinvestigated the change in permeability by measuring the efflux of K^+ from *E. coli* cells. As shown in Fig. 4a, the efflux of K^+ occurred rapidly upon addition of gramicidin S. We also investigated the gramicidin S-induced change in cell viability (Fig. 4b); it reduced cell viability within a few minutes, in agreement with the rapid increase in permeability, indicating that the bactericidal activity of

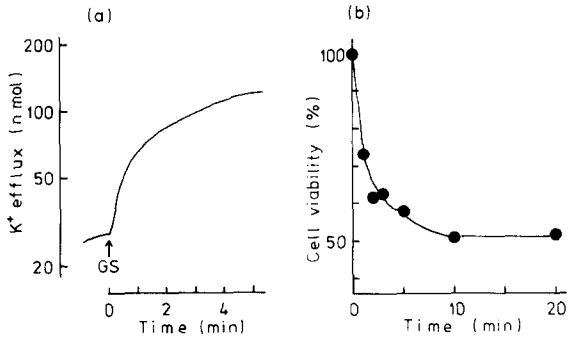

Fig. 4. The time course of (a) efflux of K^+ and (b) cell viability upon addition of gramicidin S. Cell suspension (0.05 ml, 0.05 mg cell protein) was added to 1 ml 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride at 28°C. At time zero, 2.5 μl gramicidin S in ethanol solution (final concentration: 25 μg/ml) was added. The efflux of K^+ was monitored with a K^+-sensitive electrode. Cell viability was measured under the same conditions of the K^+ permeability measurements. Samples (50 μl) were taken at intervals, diluted with physiological saline and dispersed on an agar plate. The viability of cells was determined by counting colonies.

gramicidin S was caused by the increase in the permeability of the cytoplasmic membrane. It was also observed that increasing the concentration of gramicidin S increased permeability and decreased cell viability (data not shown) in accordance with the previous report [1]. Here, we tried to estimate the percentage of K^+ efflux induced by gramicidin S. We added a cationic surfactant CTAB at concentration above its critical micelle concentration, because it is known that cationic surfactants have a bactericidal action on Gram-negative bacteria, due to an increase in membrane permeability [28,29]. It was observed that CTAB induced the efflux of K^+ within a few minutes to a level of 260 nmol under the same conditions as shown in Fig. 4a (data not shown). We regarded this amount as the total quantity of efflux of K^+ from cells. It was thus calculated that gramicidin S caused 30% efflux within 5 min. This percentage seemed to be consistent with the result of 50% for cell viability (Fig. 4b). Fig. 5 shows the temperature dependence of K^+ efflux and cell viability. At around 20°C, the degrees of both K^+ efflux and cell viability changed markedly. In the absence of gramicidin S, no such remarkable change was observed. These data indicate that gramicidin S acts on *E. coli* cells only at temperatures above the phase transition of the cytoplasmic membrane [10,16].

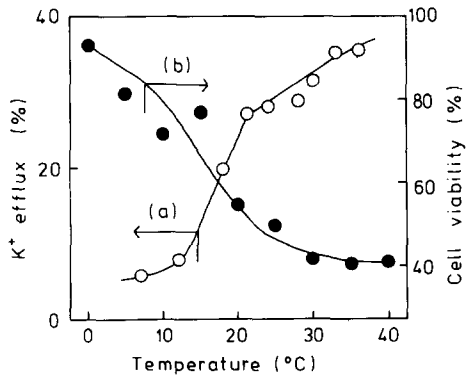

Fig. 5. The temperature dependence of (a) percentage of K^+ efflux and (b) cell viability. Assay conditions were the same as in Fig. 4. The K^+ efflux and cell viability were recorded at 5 min after addition of gramicidin S. The total amount of K^+ in the cells was determined by addition of a surfactant, CTAB.

Action of gramicidin S on the outer membrane

We speculated that gramicidin S disrupted the barrier function of the outer membrane of *E. coli*, and then increased the K^+ permeability of the cytoplasmic membrane to decrease cell viability. At first, we examined the mode of action of gramicidin S on the outer membrane, in comparison with that of the dimer in 48/80 [15,16].

We have recently shown that the dimer enables a hydrophobic ionophore gramicidin D (a mixture of A, B and C) to pass through the outer membrane, and that the ionophore reaching the cytoplasmic membrane of *E. coli* can cause the efflux of K^+ [16]. Without addition of the dimer, gramicidin D alone could not induce the efflux of K^+. As mentioned in the Introduction, a hydrophobic antibiotic can not permeate through the outer membrane of Gram-negative bacteria. Here, it should be remembered that, although the name of gramicidin D resembles that of gramicidin S, the chemical structure and characteristics of gramicidin D are quite different from those of gramicidin S. Gramicidin D is known to form a channel in the cytoplasmic membrane of cells, resulting in a change in the permeability of alkali metal and H^+ ions [30]. The temperature dependence of the synergistic effect of the dimer and gramicidin D showed that the efflux of K^+ was induced at temperatures above the phase transition of the outer membrane [16]. In the present study, we examined the synergistic effect of the dimer and another ionophore nigericin. It is well known that nigericin can exchange alkali metal ion for H^+ across the cytoplasmic membrane [30,31]. We used 0.25 μM nigericin, because such a large dose caused the efflux of K^+ to a significant extent. Fig. 6 shows the synergistic effect of the dimer on the action of nigericin, along with that of gramicidin D reported previously [16]. Nigericin elicited the efflux of K^+ much more rapidly than gramicidin D. Fig. 7 shows the effect of concentration of the dimer on the synergism. Nigericin required a smaller amount of the dimer to induce the efflux of K^+ from cells. An increase in the concentration of gramicidin D (5 μM) could not greatly stimulate the efflux of K^+ by synergism (data not shown). These results indicate that the dimer rendered the outer membrane permeable to nigericin more efficiently than to

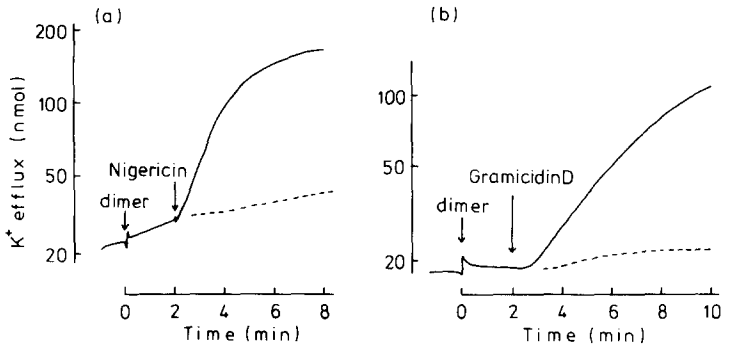

Fig. 6. The synergistic effects of the dimer in 48/80 on the action of ionophores (a) nigericin and (b) gramicidin D. Cells (50 μl, 0.5 mg cell protein) were suspended in 1 ml 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride. At time zero, 25 μl of the dimer (final concentration: 50 μg/ml) was added, and 2 min later (a) nigericin (0.5 μl ethanol solution, final concentration: 0.25 μM) or (b) gramicidin D (1 μl ethanol solution, final concentration: 1 μM) was added. The dashed lines indicate the results of addition of ionophores alone. The measurements were made at 27°C.

gramicidin D. Fig. 8a shows the temperature dependence of efflux of K^+ induced by the synergistic effect of the dimer and nigericin. It was observed that nigericin increased remarkably the efflux of K^+ at temperatures above 20°C, which corresponded to the temperatures above the phase transition of the cytoplasmic membrane [10,16]. This temperature range differed largely from the result for gramicidin D reported previously [16] (Fig. 8b). Nigericin caused the efflux of K^+ at much lower temperatures. We also investigated the synergistic effect of the dimer on the action of gramicidin S. Although addition of gramicidin S alone caused the efflux of K^+ as shown in Fig. 4a, the efflux was greatly enhanced by synergism at temperatures above 20°C, similarly to the case of nigericin (data not shown). These results indicate that the dimer can promote the permeation of drugs even at temperatures below the phase transition of the outer membrane.

Then we investigated the permeability-increasing action of gramicidin S on the outer membrane. We added 5 μg/ml gramicidin S, since large doses of gramicidin S induced high leakage of K^+ and interfered with observation of the efflux of K^+ induced by the synergistic effects. As shown in Fig. 9, synergism enhanced the efflux of K^+, and nigericin increased it to a greater extent than

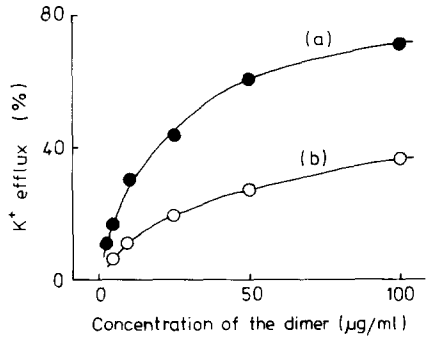

Fig. 7. The effect of concentration of the dimer in 48/80 on synergism. (a) Nigericin and (b) gramicidin D. Assay conditions were the same as in Fig. 6, except that the concentration of the dimer was varied. The percentage of K^+ efflux was calculated from the amount of K^+ effused within 4 min after addition of each ionophore. Measurements were made at 28°C.

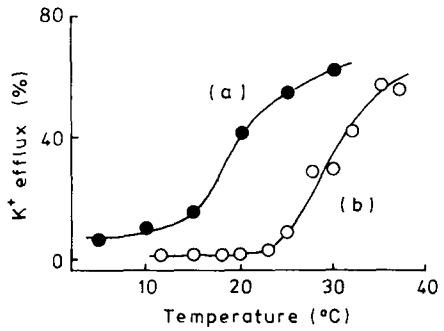

Fig. 8. The temperature dependence of efflux of K^+ induced by the synergistic effects of the dimer in 48/80 on the action of ionophores (a) nigericin and (b) gramicidin D. Assay conditions were the same as in Fig. 6. The percentage of K^+ efflux was calculated from the amount of K^+ effused within 4 min after addition of each ionophore.

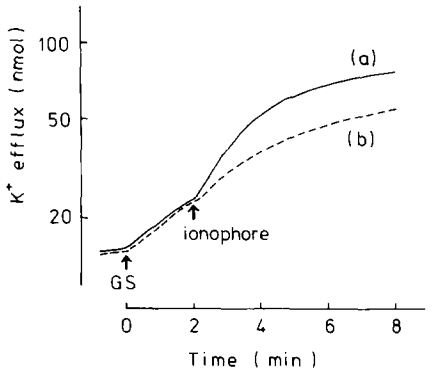

Fig. 9. The synergistic effects of gramicidin S on the action of ionophores (a) nigericin and (b) gramicidin D. Assay conditions were the same as in Fig. 6, except that gramicidin S was added instead of the dimer in 48/80. At the first arrow, gramicidin S (0.5 μl ethanol solution, final concentration: 5 μg/ml) was added. At the second arrow, (a) nigericin (0.5 μl ethanol solution, final concentration: 0.25 μM) or (b) gramicidin D (1 μl ethanol solution, final concentration: 1 μM) was added. Measurements were made at 28°C.

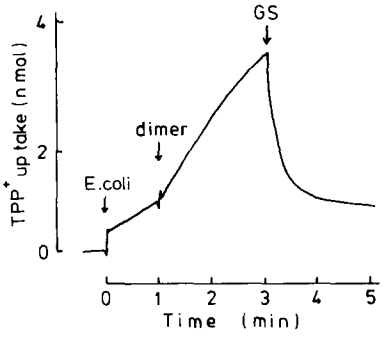

Fig. 10. The efflux of tetraphenylphosphonium ion (TPP^+) induced by addition of gramicidin S. Assay mixtures consisted of 1 ml 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride/10 mM sodium lactate/10 μM tetraphenylphosphonium chloride. At the first arrow, 50 μl of cell suspension (0.5 mg cell protein) was added. The second arrow indicates the time when 25 μl of the dimer in 48/80 (final concentration: 50 μg/ml) was added, and at the third arrow gramicidin S (2.5 μl ethanol solution, final concentration:25 μg/ml) was added. The measurements were made at 28°C.

gramicidin D, as seen for the dimer, indicating that gramicidin S also rendered the outer membrane permeable to nigericin more easily than to gramicidin D. We also examined the uptake of tetraphenylphosphonium ion (TPP^+) upon addition of gramicidin S. It is well known that the uptake of TPP^+ is enhanced when the permeability barrier of the outer membrane is disrupted [9,10,15,16,32]. It was observed, however, that gramicidin S did not increase the uptake of TPP^+ at all. We considered that gramicidin S dissipated the membrane potential of the cytoplasmic membrane instantly, in accordance with a rapid change in the permeability of the cytoplasmic membrane (Fig. 4a). We treated cells with the dimer in 48/80, making the outer membrane permeable to TPP^+, and then gramicidin S was added. If gramicidin S could abolish the membrane potential, it should cause the efflux of the accumulated TPP^+. As was expected, gramicidin S caused the efflux of TPP^+ (Fig. 10).

Action of gramicidin S on the cytoplasmic membrane

The above results seemed to indicate that after gramicidin S disrupted the permeability barrier of the outer membrane, it reached the cytoplasmic membrane to induce the permeability change. However, there is another possibility that gramicidin S interacting only with the outer membrane leads to the efflux of K^+, because we have recently observed that polylysine increases the K^+ permeability of whole cells of *E. coli*, but it can not increase the permeability of the isolated cytoplasmic membrane vesicles of *E. coli* at all [33]. Such behaviour of polylysine is unexplainable at present. In contrast with the action of polylysine, polymyxin B increased the permeability of both whole cells and membrane vesicles of *E. coli* [10], indicating that polymyxin B increased the permeability of the cytoplasmic membrane of *E. coli* after disrupting the outer membrane structure. Fig. 11a shows that gramicidin S can increase the K^+ permeability of membrane vesicles, this being similar to the action of polymyxin B. Fig. 11b and c shows that gramicidin S can elicit the efflux of K^+ from sheep red blood cells and liposomes prepared with egg phosphatidylcholine. These indicate that gramicidin S is able to stimulate the permeability of various kinds of membranes without selectivity. Such behaviour of gramicidin S is different from that of polymyxin B, because polymyxin B is known to act selectively on the

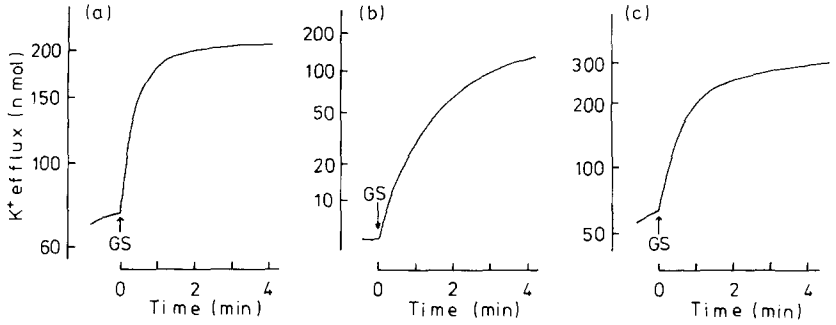

Fig. 11. Gramicidin S-induced efflux of K^+ from (a) isolated cytoplasmic membrane vesicles of *E. coli*, (b) sheep red blood cells and (c) liposomes prepared with egg phosphatidylcholine. (a) Membrane vesicles (50 μl, 0.5 mg cell protein) were diluted in 1 ml 50 mM Mops-Tris (pH 7.2)/0.4 M sucrose. (b) Sheep red blood cells (1% (v/v)) and (c) liposomes (0.77 mg lipid/ml) were suspended in 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride. Gramicidin S (2.5 μl ethanol solution, final concentration: 25 μg/ml) was added at time zero. The measurements were made at 28°C.

negatively charged membrane of Gram-negative bacteria such as *E. coli* [10,34–36].

We also investigated the action of the dimer on various membrane systems. It was observed that the dimer did not increase the permeability of the isolated cytoplasmic membrane vesicles of *E. coli*, the red blood cells and the liposomes at 50 μg/ml (data not shown), indicating that the dimer could not affect the permeability of the cytoplasmic membrane of cells. The dimer was able to increase selectively the permeability of only the outer membrane of bacteria.

Finally, we measured changes in the phase transition temperature of DPPC liposomes induced by gramicidin S and the dimer. It was observed that gramicidin S decreased the phase transition temperature of liposomes, while the dimer did not to a significant extent (Fig. 12). These differences in the effects on the phase transition temperature will be discussed in connection with the permeability change of the cytoplasmic membrane.

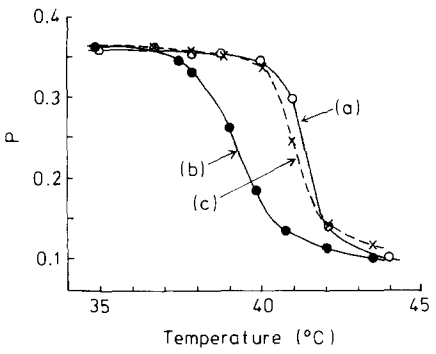

Fig. 12. Changes in the degree of polarization of DPPC liposomes before and after addition of gramicidin S or the dimer in 48/80. (a) DPPC liposomes alone (100 μM) in buffer solution containing 50 mM Mops-Tris (pH 7.2)/100 mM choline chloride. (b) After addition of gramicidin S (40 μM). (c) After addition of the dimer (40 μM).

Discussion

The present study showed that gramicidin S increased the permeability of both the outer and cytoplasmic membranes of *E. coli*. Firstly, we shall discuss the action of gramicidin S on the outer membrane. We have recently reported that the dimer in 48/80, bis[2-methoxy-5-(2-methylaminoethyl)phenyl]methane dihydrochloride, stimulates the permeability of the outer membrane of *E. coli* [15,16]. This compound is an unusual dication having the ability to increase the permeability of the outer membrane. In general, polycationic compounds with a small number of basic charges do not show such activity [6,7]. In the present study, we showed that gramicidin S also enhanced the permeability of the outer membrane. We consider that the increase in permeability is caused by the binding of gramicidin S with lipopolysaccharide molecules existing at the outside of the outer membrane. Since divalent cations (such as Mg^{2+} and Ca^{2+}) are necessary to hold lipo-

polysaccharides stable in the outer membrane [5,6,37], substitution at this binding site with an amphiphatic dication of large size will destabilize the outer membrane to cause an increase in permeability. However, diamines of a small size may fit to the inherent space at the binding site of lipopolysaccharide and maintain a stable outer membrane structure. It is conceivable that after a gramicidin S molecule had disrupted the outer membrane structure, probably another gramicidin S molecule reached the cytoplasmic membrane and caused the efflux of K^+.

It was observed that gramicidin S elicited the efflux of K^+ without a time lag at temperatures above the phase transition of the cytoplasmic membrane. We have recently reported that polymyxin B, a cationic polypeptide with five basic charges, causes the efflux of K^+ from *E. coli* cells after 30 s, and this efflux decreased at temperatures below about 30°C [10]. In an experiment using isolated cytoplasmic membrane vesicles of *E. coli*, the delay of the efflux of K^+ was not observed with polymyxin B and the efflux decreased at temperatures below 20°C [10]. Thus we have concluded that the interaction of polymyxin B with the outer membrane is the rate-determining step in the action, and the phase transition temperature of the outer membrane affects the penetration of polymyxin to the cytoplasmic membrane [10]. In contrast with the action of polymyxin B, gramicidin S increased the efflux of K^+ from *E. coli* cells without a time lag at temperatures above 20°C. It is conceivable that, because gramicidin S is a smaller molecule with two basic charges, it can readily penetrate the outer membrane and can increase the K^+ permeability even at temperatures below the phase transition of the outer membrane.

We showed that the dimer permitted the permeation of the ionophores nigericin and gramicidin D. Dose-response curves of the dimer (Fig. 7) showed that nigericin passed through the outer membrane more easily than gramicidin D. Temperature dependence (Fig. 8) data showed that nigericin and gramicidin D increased the efflux at temperatures above the phase transition of the cytoplasmic and outer membranes, respectively. In addition, nigericin increased the efflux rapidly, while gramicidin D increased it slowly, with considerable delay (Fig. 6). The differences in temperature dependence and time course of action of nigericin and gramicidin D closely resemble the difference between gramicidin S and polymyxin. As in the case of polymyxin action, the outer membrane became the barrier to the permeation of gramicidin D at temperatures below the phase transition of the outer membrane.

Here, we compared the degree of disruption of the outer membrane structure caused by polymyxin B and the dimer. Although polymyxin acted on *E. coli* cells at temperatures above the phase transition of the outer membrane, previous study [10] has shown that this antibiotic rendered the outer membrane permeable to tetraphenylphosphonium ion (TPP^+) at temperatures below the phase transition of the outer membrane, showing that polymyxin enables the smaller TPP^+ molecules to permeate at lower temperatures, as in the synergistic effect of the dimer with nigericin. It has also been observed that polymyxin led to a greater amount of uptake of TPP^+ than the dimer (compare Fig. 4 in Ref. [10] with Fig. 10 in this paper). These facts indicate that polymyxin formed a defect structure of rather larger size than the dimer, though polymyxin itself could not efficiently permeate the outer membrane at temperatures below the phase transition of the outer membrane, probably due to its large and highly charged molecular characteristics. Furthermore, it was supposed that the dimer and gramicidin S formed a defect structure of a similar size, because the concentration dependence of the dimer and gramicidin S on synergism seemed to be rather similar. These comparisons imply that the size of a defect structure increases in the order: the dimer $\approx$ gramicidin S < polymyxin B.

Next we consider the mechanism of action of gramicidin S on the cytoplasmic membrane of *E. coli*. Several authors have investigated gramicidin S-induced changes in the permeability of membrane. Gramicidin S increased the permeability of both Gram-negative and -positive bacterial membranes [1–3], liposome [38] and planar bilayer membrane [39]. However, quite opposite results have been reported on mitochondrial membrane [40–42]; a more recent work showed that gramicidin S increased permeability [41,42], while the earlier study indicated that it did not affect

permeability [40]. Gramicidin S did not enhance the permeability of sheep red blood cells in an earlier study [43]; nevertheless, the present study showed that permeability was increased by the compound. It is reasonable to suppose that gramicidin S can increase the permeability of various kinds of membranes, though a few exceptions exist as described above.

Physicochemical methods including X-ray analysis revealed that the conformation of gramicidin S is a β-sheet structure [2,3,44]. This structure is retained in solvents with a wide range of polarity [45]. The compact folding of the gramicidin S molecule precludes the possibility for a carrier-type ionophore [46]. It has been reported that a diacetyl derivative of gramicidin S reduced antimicrobial activity [46], indicating that the amino group of gramicidin S is essential to the activity. This result is understandable in the case of Gram-negative bacteria, since amino groups of gramicidin S may attack the divalent cation site of lipopolysaccharide molecules in the outer membrane.

It was observed that gramicidin S decreased the phase transition temperature of neutral phospholipid DPPC (Fig. 12), in agreement with the previous result obtained by differential scanning calorimetry [47]. Gramicidin S is also known to decrease the phase transition temperature of another neutral lipid, dimyristoylphosphatidylcholine [48]. However, the dimer in 48/80 did not affect the phase transition temperature of DPPC to a significant extent. It has also been reported that polycationic compounds such as compound 48/80 [49] or polymyxin B [26] do not affect the phase transition temperature of neutral phospholipids, though these compounds can decrease the phase transition temperature of acidic phospholipids. This indicates that gramicidin S, differing from many polycations, can penetrate into even neutral lipids constituting the main part of biological membranes and can expand the distance between neighbouring phospholipid molecules to decrease the phase transition temperature. It seems likely that such a strong membrane disturbance leads to an increase in the permeability of various biological and artificial membranes without selectivity. Papahadjopoulos and coworkers [50,51] have reported that there is a correlation between the ability to decrease the phase transition temperature and to increase the permeability of membrane.

Finally, we compared the action of gramicidin S with that of other polycations. It is known that basic oligopeptides or polyamines with a small number of basic charges do not inhibit bacterial growth [6,7], except for the limited examples such as polymyxin B described above [10,34]. Various analogues of gramicidin S have been prepared and their antimicrobial activities have been investigated; however, the structure-activity relationships are still obscure [2,3,46]. We are now considering these relationships. Besides gramicidin S, it has been shown that another type of diamine, irehdiamine A, is bactericidal to *E. coli* [52]. Since irehdiamine A is a dication with a bulky steroidal ring, it is conceivable that this compound can also increase the permeability of both the outer and cytoplasmic membranes of *E. coli* to reduce cell viability, similarly to the action of gramicidin S. Methylamino derivatives of irehdiamine A were also prepared, and it was observed that increased methyl substitution decreased the antimicrobial activity [52]. As yet, this substitution effect is unexplainable.

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