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Dication and trication which can increase the permeability of *Escherichia coli* outer membrane

Takashi Katsu *, Mie Shibata and Yuzaburo Fujita

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

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Recent success in the preparation of the monomer, dimer and trimer in compound 48/80 prompted us to investigate the action of these compounds on *Escherichia coli* cells. It was found that compound 48/80 inhibited growth of *E. coli* cells, while the monomer, dimer and trimer in 48/80 did not. However, the following experiments showed that the dimer and trimer disrupted the permeability barrier of the outer membrane of *E. coli*. First, addition of the dimer or trimer in cell suspension stimulated the uptake of tetraphenylphosphonium cation. Second, the synergistic effect of the dimer on the action of gramicidin caused the efflux of K^+. In experiments using isolated cytoplasmic membrane vesicles, addition of gramicidin alone caused the efflux of K^+. Thus, it was speculated that, with whole cells, the dimer formed some defect structure in the outer membrane, through which gramicidin reached the cytoplasmic membrane and increased the K^+ permeability. The temperature dependence of efflux of K^+ showed that the dimer in 48/80 rendered the outer membrane permeable to gramicidin at temperatures above the phase transition of the outer membrane.

Introduction

Compound 48/80 is commonly used as a drug to cause histamine release from mast cells [1]. This compound is synthesized by condensing *p*-methoxy-*N*-methylphenethylamine with formaldehyde [2] and is thought to be a mixture of linear copolymers. Dialysis and gel filtration [3] and ^{13}C-nuclear magnetic resonance (NMR) studies [4] have shown that the most active polymer is probably the hexamer (Fig. 1).

It is known that various polycations including compound 48/80, polylysine, protamine and histone inhibit growth of Gram-negative bacteria [5–7]. These polycations have the ability to disrupt the permeability barrier of the outer membrane of bacteria [7–9]. It has been reported that the action of polycations on bacteria is dependent on the degree of polymerization (n) [7,10]. For example, the action of polylysine on *Escherichia coli* varied as follows [7,10]. A polylysine with low degree of polymerization (around $n = 20$) only produced an increase in permeability of the outer membrane [7,10]. Highly polymerized polylysines ($n > 100$) dissipated the membrane potential of the cytoplasmic membrane to inhibit cell growth [10], suggesting that these polylysines attacked the cytoplasmic membrane after disruption of the permeability barrier of the outer membrane. Tetralysine ($n = 4$) did not affect the permeability of the outer membrane at all [7]. Protamine which consisted of

* To whom correspondence should be addressed.

Abbreviations: TPP^+, tetraphenylphosphonium ion; Mops, 4-morpholinepropanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

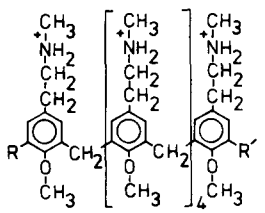

Fig. 1. Proposed structure of compound 48/80.

21 arginine residues increased the permeability of the outer membrane and inhibited bacterial growth, while its hydrolysate with 4–6 arginine residues did not affect the permeability and cell growth [7,8]. There was a tendency that polycations with a few basic amino acids did not increase the membrane permeability, except for the limited examples such as polymyxin analogs [7,11].

We were interested in the fact that compound 48/80 inhibited bacterial growth in spite of the hexamer [5]. It was expected that lower degrees of polymerization of 48/80 might cause only an increase in the permeability of the bacterial outer membrane, as in the case of polylysine with $n = 20$. Recently, we succeeded in the preparation of the monomer, dimer and trimer in 48/80 [12]. Fig. 2 shows the structure of these compounds. The present study examined the action of these isolated components of 48/80 on *E. coli* cells. It was found that the dimer and trimer increased the uptake of TPP^+, but the monomer had no effect. Treatment of cells with the dimer sensitized gramicidin-induced K^+ permeation through the cytoplasmic membrane. These dimer and trimer in 48/80 are new dication and trication which can increase the

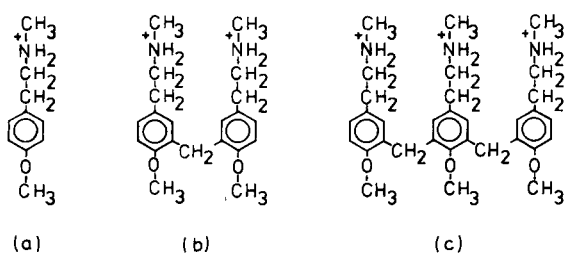

Fig. 2. Structure of the monomer (a), dimer (b) and trimer (c) in compound 48/80.

permeability of the bacterial outer membrane. The mode of the permeability-increasing action was investigated by measuring the temperature dependence of the synergistic effect of the dimer on the action of gramicidin. A preliminary report on this work has been communicated [13].

Materials and Methods

Chemicals. The monomer, dimer and trimer in compound 48/80 were prepared as reported previously [12]. Compound 48/80 and gramicidin (a mixture of gramicidin A, B and C) were purchased from Sigma. FCCP was a product of Fluka. Other chemicals used were all of analytical reagent grade.

Measurements of TPP^+ uptake and K^+ efflux. The amounts of TPP^+ uptake and K^+ efflux were measured with ion-selective electrodes (ISEs). These ISEs were constructed by the use of poly(vinyl chloride)-based membranes as reported previously [9,11,14].

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 [9–11]. Cells were harvested in the late exponential phase of growth, washed 2 times with buffer (50 mM Mops-Tris and 100 mM choline chloride, pH 7.2) and suspended in this buffer at 10 mg of protein/ml [9–11]. Protein content was determined by the method of Lowry et al. [15]. Isolated cytoplasmic membrane vesicles were prepared by the EDTA-lysozyme method described by Kaback and co-workers [16,17]. Loading of high concentrations of K^+ into the vesicles was achieved as described previously [11,18].

Measurement of growth rate. Growth rate was determined by measuring the absorbance at 650 nm after inoculation of a medium consisting of a minimal salt supplemented with 1% polypeptone and 25 μg/ml of a test compound with 10^7 cells/ml [13]. Cells were grown under aerobic conditions at 37°C.

Results

Action of the monomer, dimer and trimer in 48/80 on E. coli outer membrane

We measured the uptake of TPP^+ to test whether

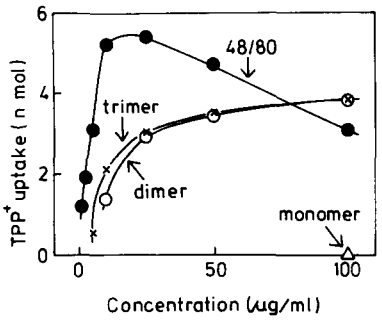

Fig. 3. The uptake of TPP^+ caused by addition of compound 48/80 and the monomer, dimer and trimer in 48/80. Cell suspension (0.05 ml, 0.5 mg cell protein) was diluted in 1 ml of assay mixture containing 50 mM Mops-Tris (pH 7.2), 100 mM choline chloride, 10 mM sodium lactate and 10 μM TPPCl at 28°C. Then, a test compound was added at various concentrations, and 2 min later FCCP (1 μl of ethanol solution, final concentration: 1 μM) was added. The uptake of TPP^+ was estimated from the amount effluxed by FCCP addition.

the monomer, dimer and trimer in 48/80 disrupted the permeability barrier of the outer membrane. It is known that TPP^+, a lipophilic cation, diffuses passively across the membrane, depending on the membrane potential [19,20]. Such diffusion does not occur efficiently in Gram-negative bacteria, due to the presence of the outer membrane [21]. Disruption of the permeability barrier of the outer membrane stimulates the uptake of TPP^+ [9,11]. We measured the uptake of TPP^+

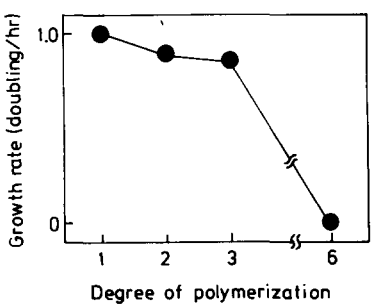

Fig. 4. Effect of the degree of polymerization of 48/80 on growth rate of *E. coli* cells. A medium containing a minimal salt supplemented with 1% polypeptone and 25 μg/ml of a test compound was inoculated with 10^7 cells/ml. Cells were grown under aerobic conditions at 37°C. Growth rate was determined by measuring the absorbance at 650 nm. The growth rate in a control experiment without addition of drugs was 0.95 doubling per hour.

upon addition of compound 48/80 and the monomer, dimer and trimer in 48/80. Fig. 3 shows the dose-response relations. It was found that the dimer and trimer enhanced the uptake of TPP^+, while the monomer did not at all. Compound 48/80 most enhanced the uptake at lower concentration regions, but reduced largely at high concentrations. Although the correlation between the amount of TPP^+ uptake and the membrane potential remains still obscure [22,23], we supposed that large dose of 48/80 affected the membrane potential of the cytoplasmic membrane to decrease the uptake of TPP^+, as in the case of polylysines with high degrees of polymerization [10]. We measured the growth rate of cells in the presence of compound 48/80, because the highly polymerized polylysines inhibited cell growth in accordance with the decrease in the TPP^+ uptake [10]. It was observed that compound 48/80 inhibited cell growth, while the monomer, dimer and trimer did not affect cell growth (Fig. 4). These results suggested that compound 48/80 affected both the outer and cytoplasmic membranes, but the dimer and trimer showed only the action increasing the permeability of the outer membrane alone.

Synergistic effect of the dimer in 48/80 on the action of gramicidin

To clarify the mode of the permeability-increasing action, we examined the synergistic effect of the dimer on the action of gramicidin, a channel-forming ionophore [24]. Addition of gramicidin alone to cell suspension was less effective due to the barrier function of the outer membrane. If gramicidin molecules reach the cytoplasmic membrane, pores of diameter of 4 Å are formed [25], and thus the efflux of K^+ will be caused through the cytoplasmic membrane. Fig. 5a shows an increase in the gramicidin-induced K^+ permeability upon addition of the dimer in 48/80. Without the dimer, the efflux of K^+ did not take place at this concentration of gramicidin. The efflux took place with a time lag, and this delay was prolonged by lowering the temperature. The synergism was not an energy-dependent process, since addition of 10 mM NaCN did not affect the efflux of K^+. Fig. 5b shows the efflux of K^+ from isolated cytoplasmic membrane vesicles upon ad-

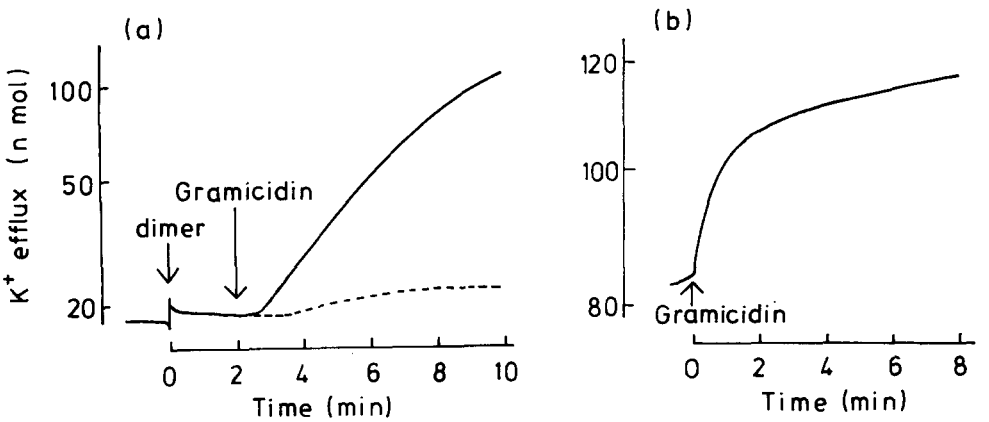

Fig. 5. Gramicidin-induced K^+ permeability change in (a) whole cells and (b) membrane vesicles. (a) Cells (50 μl, 0.5 mg cell protein) were suspended in 1 ml of 50 mM Mops-Tris (pH 7.2) and 100 mM choline chloride at 27°C. At the time indicated by the first arrow, 50 μl of the dimer in 48/80 (final concentration: 50 μg/ml) was added. Gramicidin (1 μl of ethanol solution, final concentration: 1 μM) was added at the time indicated by the second arrow. The dashed line indicates the result of addition of gramicidin alone. (b) Membrane vesicles (50 μl, 0.5 mg cell protein) were suspended in 1 ml of buffer solution (50 mM Mops-Tris (pH 7.2) and 0.4 M sucrose) at 27°C. At the time indicated by the arrow, 1 μl of gramicidin in ethanol solution (final concentration: 1 μM) was added.

dition of gramicidin. Membrane vesicles did not need the dimer to cause the efflux of K^+, and the efflux of K^+ was induced without a time lag.

Fig. 6a shows the temperature dependence of efflux of K^+ from whole cells induced by the synergistic effect. The amount of K^+ efflux decreased at temperatures below about 30–35°C. The temperature dependence of gramicidin-induced efflux of K^+ from membrane vesicles is shown in Fig. 6b. The amount of efflux declined at temperatures below about 15–20°C. This temperature range differed largely from that of whole cells.

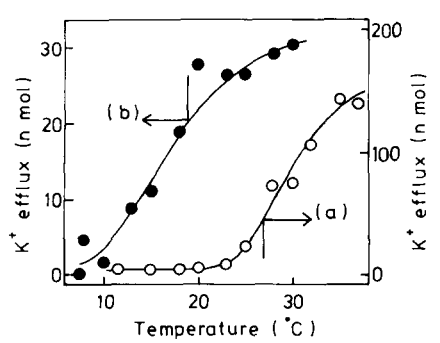

Fig. 6. The temperature dependence of efflux of K^+ from (a) whole cells and (b) membrane vesicles. Assay conditions were the same as in Fig. 5. The efflux of K^+ was determined from the amount of K^+ effluxed within 4 min after gramicidin addition.

Discussion

The present study showed that the dimer and trimer in compound 48/80 disrupted the permeability barrier of the outer membrane. To investigate this effect, we measured the uptake of TPP^+ at first. It is known that the uptake of TPP^+ is stimulated when the permeability barrier of the outer membrane is disrupted [9,11]. We monitored the uptake by using a TPP^+-ISE, because the potentiometric measurement with ISE was fast, easy, and continuous. This method made it easy to evaluate the drug-induced disruption of the permeability barrier of the outer membrane.

The synergistic effect of the dimer in 48/80 on the action of gramicidin caused the efflux of K^+ from *E. coli* cells. The efflux took place with a time lag. In an experiment using isolated cytoplasmic membrane vesicles, addition of gramicidin alone caused the efflux of K^+ without delay, indicating that gramicidin rapidly induced a change in the K^+ permeability of the cytoplasmic membrane. We considered that the dimer might form some defect structure in the outer membrane, permitting the penetration of gramicidin. The observed time

lag of the efflux with whole cells can be ascribed to the time required for permeation of gramicidin through the outer membrane. Gramicidin which passed through the outer membrane reached the cytoplasmic membrane, in which it induced a permeability change.

We compared the action of the dimer and trimer in 48/80 with that of other polycations [6–11]. Although various polycations can increase the permeability of the outer membrane [7–9,11], basic oligopeptides or polyamines with a small number of basic charges do not usually show such action [7]. In this respect, the dimer and trimer in 48/80 are new cations. 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 delay was not observed with membrane vesicles [11]. This situation accords with the present case of gramicidin action. It has been suggested that the interaction of polymyxin B with the outer membrane is the rate-determining step in the action of polymyxin, and the phase transition temperature of the outer membrane affects the permeation of polymyxin to the cytoplasmic membrane [11,26]. It is reasonable to consider that the dimer also renders the outer membrane permeable to gramicidin at temperatures above the phase transition of the outer membrane, because the temperature dependence of efflux of K^+ induced by the synergistic effect of the dimer on the action of gramicidin is similar to that of polymyxin action [11]. It was observed that gramicidin caused the efflux of K^+ from membrane vesicles at much lower temperatures (Fig. 6b). This difference of the temperature range is attributable to the difference in the membrane fluidity of the outer and cytoplasmic membranes. It is well known that the phase transition temperature of the cytoplasmic membrane is lower than that of the outer membrane [27,28]. The fact that, with membrane vesicles, gramicidin decreased the efflux of K^+ at temperatures below the phase transition of the cytoplasmic membrane coincides with the previous observation that the gramicidin-induced permeability change was suppressed by appearance of the solid phase of membrane [29].

We supposed that the dimer and trimer acted on the outer membrane as follows. It is now well established that lipopolysaccharide is located at the outside of the outer membrane [27], and divalent cations (such as Mg^{2+} and Ca^{2+}) maintain the outer membrane structure [27,30]. It seems likely that the divalent cations at the binding site of lipopolysaccharide are replaced by the dimer and trimer. The fact that the monomer did not increase the permeability of the outer membrane is understandable since two amino groups must attack the divalent binding site. In this connection, it is worthwhile to compare the action of the dimer with that of EDTA. It is well known that treatment of *E. coli* cells with EDTA increases the permeability of the outer membrane [31]. EDTA eliminates divalent cations from the binding site, apparently triggering disruption of the barrier function of the outer membrane. It is probable that dications and trications can remove efficiently the divalent cation from the binding site, leading to disruption of the outer membrane structure. It is known, however, that dications and trications such as cadaverine and spermidine do not disrupt the barrier function of the outer membrane [7], though these cations can bind isolated lipopolysaccharide [7,32]. Why the dimer and trimer in 48/80 had the ability to disrupt the outer membrane structure cannot be explained exactly at present; however, since the dimer and trimer are relatively bulky molecules, binding of these to lipopolysaccharide will lead to separate adjacent lipopolysaccharide molecules, loosening the outer membrane packing and increasing the permeability against various drugs. Such an increase in permeability will not efficiently be induced at temperatures below the phase transition of the outer membrane, because of tightness of the membrane structure surrounding the dimer and trimer, similarly to the case of polymyxin action on the outer membrane [11].

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