Mode of action of a bactericidal protein induced in the haemolymph of *Sarcophaga peregrina* (flesh-fly) larvae

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The mode of action of a bactericidal protein (sarcotoxin I) purified from the haemolymph of *Sarcophaga peregrina* (flesh-fly) larvae was studied, focusing attention on its effect on the function of the membrane of *Escherichia coli*. Sarcotoxin I almost completely blocked the uptakes of tetraphenylphosphonium ion and proline, which are known to be driven by a membrane potential, indicating that this protein disrupts the membrane potential of bacteria.

It is known that, in some insects, humoral antibacterial substances are induced on injection of dead or live bacteria (Whitcomb *et al.*, 1974; Chadwick, 1975; Boman, 1981; Chadwick *et al.*, 1982). This induction is thought to be a defence reaction of the insects for preventing invasion of various parasites and pathogens. Little is yet known about the molecular natures of these antibacterial substances, because they are difficult to purify. Of these substances, cecropins, which are antibacterial proteins of the giant silk moth *Hyalophora cecropia*, have been most extensively studied and have been found to be a group of small proteins with minor differences in amino acid sequences (Hultmark *et al.*, 1980, 1982; Steiner *et al.*, 1981; Qu *et al.*, 1982). Studies on these inducible antibacterial proteins in invertebrates seem to be important from the viewpoint of comparative immunology.

Previously we reported the induction of bactericidal proteins in the haemolymph of third-instar larvae of *Sarcophaga peregrina* (flesh-fly) when their body wall was pricked with a hypodermic needle (Natori, 1977). Recent studies revealed that there are three bactericidal proteins in the haemolymph of injured larvae, and we purified one of these proteins to homogeneity (Okada & Natori, 1983). This protein (hereafter called 'sarcotoxin I') is a small basic protein with M_r 5000. Since sarcotoxin I is cytotoxic to both Gram-negative and Gram-positive bacteria at a concentration of

less than 0.5 $\mu\text{g/ml}$, we examined the mechanism of its cytotoxicity to bacteria.

The present paper describes the effect of sarcotoxin I on *Escherichia coli*. We found that cells became susceptible to a lower concentration of detergent and that their transportations of TPP^+ and proline were blocked immediately by treatment with sarcotoxin I, suggesting that the primary target of this protein is the cytoplasmic membrane of bacteria.

Materials and methods

Sarcotoxin I

A bactericidal protein (sarcotoxin I) was purified from the haemolymph of *Sarcophaga peregrina* larvae as described previously (Okada & Natori, 1983). About 50 μg of pure protein was obtained from 120 ml of haemolymph, which was collected from about 15000 larvae.

Bacterial strains and media

Most experiments were done with *E. coli* K-12 594(str^r), but for assays of proline transport and uptake of TPP^+ we used *E. coli* K-12 JE 2133(str^r) (Motojima *et al.*, 1978) and AS-1, which is an *acrA* mutant derived from *E. coli* K-12 W3110 (Imae, 1968), respectively. The media used for growth of bacteria were antibiotic medium (Bacto Penassay Broth; Difco) and minimal medium A (Davis & Mingioli, 1950), consisting of 1.5 mM-sodium citrate, 7.6 mM-(NH_4)₂SO₄ and 8.3 mM-MgSO₄ in 55 mM-potassium phosphate buffer, pH 7.4, supplemented with 0.4% (w/v) casamino acids and 54 mM-glycerol.

Abbreviations used: TPP^+ , tetraphenylphosphonium ion; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PMS, phenazine methosulphate; SDS, sodium dodecyl sulphate.

Scanning electron microscopy

E. coli K-12 594(str^r) cells were incubated with sarcotoxin I (2 µg/ml) for 40 min at 30°C in 10 mM-phosphate buffer, pH 6.0, containing 130 mM-NaCl and 0.1% (w/v) bovine serum albumin. The cells were collected on a Millipore filter (GSPW, 0.22 µm pore size) by filtration, washed with the buffer, and fixed by dipping the filter successively into 100 mM-phosphate buffer, pH 7.5, containing 2.5% (w/v) glutaraldehyde and 1% (w/v) tannic acid, and the same phosphate buffer, containing 1% (w/v) osmic acid. Filters were dried by the method of Klainer *et al.* (1974), coated with platinum in an ion coater, and examined by scanning electron microscopy on an Hitachi S430 instrument.

Measurement of TPP⁺ uptake

The effect of sarcotoxin I on the membrane potential was examined by measuring the uptake of TPP⁺ by the method of Hirota *et al.* (1981). *E. coli* *acrA* mutant AS-1 was suspended at a density of 10⁹ cells/ml in 10 mM-Hepes buffer, pH 7.5, containing 5 mM-glucose and 0.1% bovine serum albumin, and preincubated at 30°C for an appropriate period. Then 0.31 µCi of [¹⁴C]TPP⁺ (31.4 mCi/mmol) was added and the final concentration of TPP⁺ was adjusted to 10 µM. Samples were taken at intervals and filtered on a Sartorius membrane filter (SM 111, 0.45 µm pore size) and radioactivity taken up into the cells was measured. The effect of sarcotoxin I was examined by adding it to the medium during the preincubation period.

Measurement of proline uptake

Proline uptake by *E. coli* was measured by the method of Anraku *et al.* (1973). *E. coli* K-12 JE2133(str^r) cells were suspended at a density of 10⁹ cells/ml in minimal medium A containing chloramphenicol (40 µg/ml) and 0.1% bovine serum albumin, and preincubated at 30°C for an appropriate period. Then sodium ascorbate and PMS were added at final concentrations of 35 mM and 0.15 mM respectively. After incubation for 1.5 min, 0.11 µCi of [¹⁴C]proline (0.1 mM) was added with thorough mixing. Then samples were taken at intervals and filtered on a Millipore membrane filter (HAWP, 0.45 µm pore size) and the radioactivity taken up by the cells was measured. The effect of sarcotoxin I was examined by adding it to the medium during the preincubation period.

Results

Effect of sarcotoxin I on resting and growing bacteria

We first examined the effect of sarcotoxin I on the viability of *E. coli* under both resting and

growing conditions by counting colony-forming cells. As shown in Figs. 1(a) and 1(b), viability rapidly decreased irrespective of the growth state of the bacteria, indicating that growth of bacteria is not essential for the bactericidal action of sarcotoxin I. However, as is evident from Fig. 1(a), no loss of viability was observed when the bacteria were kept at 0°C in the presence of sarcotoxin I. When plating the bacteria after their incubation with sarcotoxin I, the reaction mixture was diluted many-thousand-fold. Thus, if sarcotoxin I did not bind to *E. coli* at 0°C, it should be diluted out during this process and the bacteria should be free from sarcotoxin I and form colonies.

Therefore these results suggest that once sarcotoxin I binds to *E. coli*, the bacteria lose viability irrespective of their cellular metabolism, and that the binding of sarcotoxin I to *E. coli* depends on the temperature.

In order to gain a more direct insight into the interaction of sarcotoxin I with *E. coli*, we examined the bacteria treated with sarcotoxin I by scanning electron microscopy. When specimens were prepared after treatment with sarcotoxin I for 10 min, when bacterial viability had decreased more than 80%, the *E. coli* cells appeared intact and showed no significant morphological change. However, specimens of cells treated with sarcotoxin I for 40 min showed clear morphological change, as seen in Fig. 2. Although the shape of the *E. coli* cells had not changed, string-like substances, which may have been derived from the bacteria, were seen stacked around the cells. These

Fig. 1. Loss of viability of *E. coli* cells in the presence of sarcotoxin I

E. coli K-12 594(str^r) was incubated with sarcotoxin I (1 µg/ml) in insect saline (130 mM-NaCl, 5 mM-KCl and 1 mM-CaCl₂) or in antibiotic medium. Samples were taken at intervals and the number of viable cells was counted. ●, Incubation at 30°C with sarcotoxin I; ○, incubation at 30°C without sarcotoxin I; ⊙, incubation at 0°C with sarcotoxin I.

Fig. 2. Scanning-electron-microscopic appearance of *E. coli* treated with sarcotoxin I. *E. coli* K-12 594(str^r) was incubated in 10mM-phosphate buffer, pH 6.0, containing 130mM-NaCl and 0.1% bovine serum albumin with or without sarcotoxin I (2 μ g/ml) at 30°C, and then specimens were prepared for scanning electron microscopy. (a) Intact bacteria; (b) bacteria treated with sarcotoxin I. The bars represent 0.5 μ m.

substances had probably burst out of the bacteria as a result of damage induced by sarcotoxin I.

Induction of damage of the membrane by sarcotoxin I

Since *E. coli* lose viability very rapidly on treatment with sarcotoxin I, and the substances inside the cells seem to burst out subsequently, the first target of sarcotoxin I is likely to be the membrane. It is known that when colicin K or T₄-phage ghosts bind to the surface of *E. coli*, the cells become susceptible to a lower concentration of SDS, resulting in rapid lysis (Cavard *et al.*, 1974). Therefore we tested the effect of sarcotoxin I on the stability of *E. coli* in the presence of a low concentration of SDS. As shown in Fig. 3, when *E. coli* was preincubated with sarcotoxin I (2 μ g/ml) for a short period, the cells became susceptible to 0.13% SDS and rapidly lysed on addition of the detergent, whereas untreated cells did not lyse under these conditions.

Since *E. coli* became susceptible to SDS in a few minutes on pretreatment with sarcotoxin I, the effect of sarcotoxin I seems to be similar to that of colicin K or phage-T₄ ghosts, which are known to bind to the cell surface of *E. coli*. Thus it is likely that sarcotoxin I affects the membrane in some way. At the moment, the primary target of sarcotoxin I on the surface of *E. coli* is unknown.

However, we found that the function of the inner membrane was significantly affected by treatment of the cells with sarcotoxin I, and, in particular, the membrane potential seems to be lost.

The effect of sarcotoxin I on the uptake of TPP⁺ was studied by using an *acrA* mutant of *E. coli* that has a defect in lipid A, which is a hydrophobic region of lipopolysaccharide and thus a highly permeable outer membrane to lipophilic cations such as TPP⁺. Accumulation of TPP⁺ is a good indicator of the membrane potential, since uptake of such cations depends on the membrane potential (Rottenberg, 1979). As shown in Fig. 4, uptake of TPP⁺ was significantly inhibited by pretreatment of the cells with sarcotoxin I for 20 min. Furthermore, no significant incorporation of TPP⁺ was detected even when sarcotoxin I was added simultaneously with TPP⁺. These results strongly suggest that sarcotoxin I in some way causes loss of the preformed membrane potential.

To confirm this conclusion, we examined the effect of sarcotoxin I on the uptake of proline, since it is well known that proline is taken up via a membrane potential in *E. coli* (Berger, 1973). As shown in Fig. 5, uptake of proline was almost completely blocked by preincubation of *E. coli* with sarcotoxin I for 20 min. A shorter preincubation period was also effective, and the uptake of proline was

Fig. 3. Change in susceptibility of *E. coli* cells to SDS. *E. coli* K-12 594(str^r) was suspended at a density of 10^8 cells/ml in 10 mM-phosphate buffer, pH 6.0, containing 130 mM-NaCl and 0.1% bovine serum albumin, and incubated with sarcotoxin I ($2 \mu\text{g/ml}$) at 30°C for various periods. Then SDS was added to a final concentration of 0.13% (w/v) and incubation was continued. The change in turbidity was monitored by measuring the A_{650} . (a) Treated bacteria; the preincubation periods were: 1, 0 min; 2, 3 min; 3, 6 min; 4, 10 min; and 5, 15 min respectively. (b) Control. Arrows indicate the time of addition of SDS.

significantly suppressed even when sarcotoxin I was added to the suspension of *E. coli* simultaneously with sodium ascorbate and PMS, which are essential for formation of a membrane potential, as shown in Table 1.

Thus it is possible that sarcotoxin I not only causes loss of a preformed membrane potential, but also inhibits formation of the membrane potential itself.

Discussion

Sarcotoxin I is a bactericidal protein induced in the haemolymph of *Sarcophaga peregrina* larvae when their body wall is injured (Okada & Natori, 1983). It is thought to be a kind of defence protein,

Fig. 4. Effect of sarcotoxin I on TPP⁺ uptake in *E. coli*. *E. coli* *acrA* mutant AS-I was preincubated with or without sarcotoxin I ($2 \mu\text{g/ml}$) for 5 min at 30°C . Then [^{14}C]TPP⁺ was added and its uptake was measured with time. ●, Preincubation in the presence of sarcotoxin I; ○, preincubation in the absence of sarcotoxin I; ⊗, sarcotoxin I ($2 \mu\text{g/ml}$) was added simultaneously with [^{14}C]TPP⁺.

Table 1. Relation between preincubation time with sarcotoxin I and proline uptake in *E. coli*

Preincubation time with sarcotoxin I (min)	$10^{-4} \times$ Uptake of proline (c.p.m./ 10^8 cells)
20	48 ± 6
10	48 ± 15
6	43 ± 2
3	89 ± 11
0*	104 ± 10
Control†	318 ± 37

* Sarcotoxin I was added simultaneously with sodium ascorbate and PMS.

† Without sarcotoxin I.

preventing infection by pathogens invading the larvae through the wound. Since insects do not have a humoral defence mechanism such as the antibody or complement systems of vertebrates, antibacterial proteins may be important in protection from infection. Of these proteins, cecropins of *Hyalophora cecropia* and sarcotoxin I have been

Fig. 5. Effect of sarcotoxin I on proline uptake in *E. coli*. *E. coli* K-12 JE2133 was preincubated with (●) or without (○) sarcotoxin I (2 µg/ml) for 20 min at 30°C. Then sodium ascorbate and PMS were added to form a membrane potential. After 1.5 min incubation, [¹⁴C]proline was added and its uptake was measured with time.

purified to homogeneity, and complete amino acid sequences of cecropins have been determined (Hultmark *et al.*, 1982). However, little is known about the mode of action of these proteins. The antibacterial spectrum of sarcotoxin I is rather broad, since this protein is effective towards both Gram-negative and Gram-positive bacteria. Therefore sarcotoxin I probably kills various bacteria by attacking a common site.

In the present paper we suggest that the primary target of sarcotoxin I is the membrane, and we demonstrate that the uptakes of TPP⁺ and proline, both of which depend on a membrane potential, were markedly blocked by treating *E. coli* with sarcotoxin I. The exact mechanism of the effect of sarcotoxin I on the membrane potential is unknown, but it is possible that this protein penetrates the inner membrane of *E. coli* and makes channels that permit the influx of cations into the cells, including H⁺, which is driven out during respiration. This reaction should occur even when the bacteria are in a resting state, so this could explain the decrease in viability of non-growing *E. coli* when incubated with sarcotoxin I. It is

interesting that sarcotoxin I was not cytotoxic to bacteria at 0°C. This could be explained by supposing that the state of the membrane is different at 0°C and 30°C, and that sarcotoxin I can penetrate the inner membrane at 30°C, but not at 0°C. Since the state of phospholipids is known to change markedly with temperature (Uehara *et al.*, 1977), the state of phospholipids in the bacterial membrane may be a critical factor for expression of cytotoxicity by sarcotoxin I.

At the moment it is unknown whether sarcotoxin I affects other membrane functions. In the experiment on proline uptake, the membrane potential was generated by H⁺ produced during respiration driven by ascorbate and PMS. Since it is technically difficult to wash out sarcotoxin I after generation of the membrane potential, the possibility that sarcotoxin I inhibits formation of the membrane potential itself cannot be excluded, although it is quite clear from the experiment on TPP⁺ uptake that sarcotoxin I causes loss of a preformed membrane potential. Probably sarcotoxin I causes some fatal damage to the membrane of bacteria over a short period. Its effect in inducing increased sensitivity to SDS was also a very rapid reaction. These results clearly indicated that the state of the membrane changes on treatment with sarcotoxin I. Increase in detergent-sensitivity has been observed when *E. coli* cells are treated with colicin K or T₄-phage ghosts, but not T₄-phage itself (Cavard *et al.*, 1974). The mechanism by which this increase occurs is unknown, but the composition of phospholipids was found to change on treatment with T₄-phage ghosts (Cavard *et al.*, 1974). Possibly the peptide/glycan network is partly broken by an enzyme that is activated by treatment with sarcotoxin I, resulting in increase in detergent-sensitivity.

It is interesting that insects produce a protein with these pleiotropic functions when their body wall is injured. The haemolymph of injured *Sarcophaga peregrina* larvae also contains two other bactericidal proteins besides sarcotoxin I, and these three proteins may form a strong defence against invasion of various parasites and pathogens.

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