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Ionophore activity of sarcotoxin I, a bactericidal protein of *Sarcophaga peregrina*

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When *Escherichia coli* was treated with sarcotoxin I, a potent bactericidal protein of *Sarcophaga peregrina* (fleshfly), K^+ inside of the cells leaked out rapidly and the ATP pool of the cells rapidly decreased. These results suggested that the bactericidal effect of sarcotoxin I was due to its ionophore activity, and that it blocked the generation of ATP by inhibiting formation of the proton gradient essential for oxidative phosphorylation. This was confirmed by use of an *uncA* mutant, which was much less susceptible than the wild-type strain to sarcotoxin I under fixed ionic conditions.

It is known that in some insects antibacterial activity in the haemolymph is induced by injection of live or dead bacteria (Whitcomb *et al.*, 1974; Boman, 1981; Chadwick *et al.*, 1982). This phenomenon is interesting from the viewpoint of comparative immunology, since this activity is believed to participate in the defence mechanism of invertebrates that have no immune network. The only antibacterial components of insects that have been well characterized are the cecropins of *Hyalophora cecropia* (cecropia moth), which have been shown to be a series of peptides consisting of 35 or 37 amino acid residues (Boman & Hultmark, 1981; Hultmark *et al.*, 1982). More recently, cDNA clones of attacins, another group of antibacterial proteins of cecropia, have been characterized (Engström *et al.*, 1984; Kockum *et al.*, 1984). However, the mechanism of the antibacterial action of these proteins is not clear. However, Steiner *et al.* (1982) have described the action of cecropins on the disruption of the outer layer of bacteria.

We have identified three antibacterial proteins that are induced in the haemolymph of *Sarcophaga peregrina* larvae when their body wall is injured with a hypodermic needle, and we have purified one of these proteins, termed 'sarcotoxin I', with an M_r of about 5000, to homogeneity (Natori, 1977, Okada & Natori, 1983, 1984). Since this peptide is toxic to both Gram-positive and Gram-negative bacteria at a concentration of less than 0.5 μg/ml, we investigated the mechanism of its bactericidal action and found that its primary target is the cytoplasmic membrane of bacteria (Okada & Natori, 1984). On treatment of *Escherichia coli* with sarcotoxin I, the uptakes of proline and tetraphenylphosphonium ion immediately stopped, suggesting that the membrane potential essential for the transport of these substances was disrupted by sarcotoxin I. Further studies on the effect of sarcotoxin I on *E. coli* showed that sarcotoxin I had an ionophore action. When *E. coli* cells were treated with sarcotoxin I, their ATP content decreased drastically, although their O_2 consumption was not affected.

Materials and methods

Sarcotoxin I

The bactericidal protein sarcotoxin I was purified from the haemolymph of *Sarcophaga peregrina* larvae as described previously (Okada & Natori, 1983). The purified sample was dissolved in distilled water and stored at −20°C.

Bacterial strains and media

Most experiments were done with *E. coli* K-12 594 (str^r). The uncoupler action of sarcotoxin I was examined by using *E. coli* K-12 AN120 (str^r, *uncA*), an *uncA* mutant, and its parental strain *E. coli* K-12 AN180 (str^r). All bacteria were grown in antibiotic medium M3 (Bacto Penassay Broth; Difco Laboratories) and used at the exponential phase.

For measurement of viable-cell numbers, nutrient agar (Difco Laboratories), LBNa agar and LBK agar were used. LBNa agar contained 100 mM-NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract 1.5% (w/v) agar and 100 μg of streptomycin/ml to inhibit growth of bacteria contaminating the reaction mixture during the experiment. LBK agar contained 100 mM-KCl instead of NaCl.

Measurement of ATP

E. coli cells were suspended at a density of 5×10^8 cells/ml in 100mM-sodium phosphate buffer, pH6.0, containing 130mM-NaCl, 0.1% (w/v) bovine serum albumin and 0.4% (w/v) glucose, and preincubated at 30°C for 10min. Then sarcotoxin I was added at a concentration of 10μg/ml and incubation was continued. Samples (40μl) were taken at intervals and diluted 10-fold with distilled water, and ATP was extracted by boiling them for 15min. Samples (200μl) of the resulting extract was added to 400μl of 0.05M-KH_2AsO_4 solution, pH7.4, containing 0.02M-$MgSO_4$ with thorough mixing. Then 10μl of firefly lantern extract (Sigma FLE-50) was added with shaking and the fluorescence was measured in an ATP photometer (CHENM-GLOW). The amount of ATP in the extract was calculated from a standard curve.

Measurement of O_2 consumption

E. coli K-12 594 (str^r) was suspended at a density of 10^8 cells/ml in 10mM-sodium phosphate buffer, pH6.0, containing 130mM-NaCl, 0.1% (w/v) bovine serum albumin and 0.4% (w/v) glucose, and the mixture was preincubated at 30°C for 5min with shaking. Then sarcotoxin I was added at a concentration of 10μg/ml, and the change in concentration of dissolved O_2 was monitored with time with an oxygen electrode (type LN 50u; Yellow Spring Instrument Co.).

Measurement of K^+ leakage

E. coli K-12 594 (str^r) was suspended in 150mM-NaCl solution at a density of 5×10^8 cells/ml and preincubated at 30°C for 10min. Then sarcotoxin I was added at a concentration of 10μg/ml and incubation was continued. Samples (100μl) were taken at intervals, and promptly filtered through a Millipore membrane filter (HV, pore size 0.45μm), with a 1ml plastic syringe. The concentration of K^+ in the filtrate was measured with an atomic absorption flame photometer (Perkin–Elmer model 370). For measurement of the intracellular K^+ concentration, *E. coli* cells were suspended in 100μl of 150mM-NaCl solution containing 5% (w/v) trichloroacetic acid and heated for 5min at 100°C, and the extracted K^+ was measured as described above. The water used in these experiments was supplied from a water purifier (Yamato PURELINE WE-21), and contained an insignificant amount of K^+.

Results

Effect of sarcotoxin I on the ATP pool in E. coli

As reported before, when *E. coli* was treated with sarcotoxin I, the uptake of proline and tetraphenylphosphonium ion immediately stopped (Okada & Natori, 1984). Since these substances are known to be taken up into cells via a membrane potential, sarcotoxin I seems to disrupt the membrane potential and proton gradient in some way. In *E. coli*, most ATP is synthesized in the cytoplasmic membrane by F_1-ATPase at the expense of the proton gradient produced by respiration (Futai & Kanazawa, 1983). Therefore, if sarcotoxin I disrupts the membrane potential and proton gradient, the amount of ATP in the cells should decrease rapidly. When *E. coli* was incubated in the presence of glucose, the intracellular ATP concentration was maintained constant, probably because the synthesis and degradation of ATP were in equilibrium. However, on addition of sarcotoxin I to this system, the amount of ATP decreased with time and almost disappeared in a few minutes, as shown in Fig. 1. These results suggest that sarcotoxin I disrupted the proton gradient in the membrane that is essential for generation of ATP, resulting in rapid decrease of the ATP pool in the cells.

Next, the effect of sarcotoxin I on respiration was investigated by measuring O_2 consumption. The proton gradient necessary for ATP generation is believed to be formed during this process. As evident from Table 1, O_2 consumption of *E. coli*

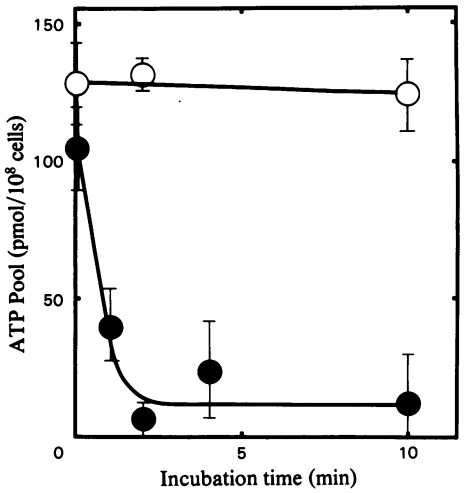

Fig. 1. *Effect of sarcotoxin I on the ATP pool of E. coli*
E. coli K-12 597 (str^r) was suspended in 10mM-phosphate buffer, pH6.0, containing 130mM-NaCl, 0.1% bovine serum albumin and 0.4% glucose, and preincubated for 10min at 30°C. Then sarcotoxin I was added at a final concentration of 10μg/ml, and incubation was continued. Samples (40μl) were taken at intervals and ATP was measured by luciferin/luciferase assay. ○, Control cells treated with distilled water; ●, cells treated with sarcotoxin I.

Table 1. *Effect of sarcotoxin I on O_2 consumption by E. coli*

E. coli K-12 594 (str^r) cells were suspended in 10mM-phosphate buffer, pH6.0, containing 130mM-NaCl, 0.1% bovine serum albumin and 0.4% glucose. Then sarcotoxin I was added at a final concentration of 10μg/ml, and the change in dissolved O_2 concentration was monitored with time. Simultaneously, samples were taken to determine viable cell numbers. The O_2 concentration of control cells was about 30nmol/min per 10^8 cells. As a positive control, KCN (50mM) was used as inhibitor.

Conditions		O_2 consumption (%)	Viability (%)
Control		100	100
+Sarcotoxin I	0 min	94	97
	3 min	109	5
	6 min	98	1
	12 min	98	0
+KCN		0	0

did not change on treatment with sarcotoxin I, under conditions where the viability of the cells was almost completely lost. When KCN was added as a control, both O_2 consumption and viability were lost. Therefore sarcotoxin I acted as an uncoupler as a consequence of its ionophore activity; it apparently inhibited the coupling between respiration and ATP generation by disrupting formation of the proton gradient.

Induction of K^+ leakage by sarcotoxin I

One possible mechanism by which sarcotoxin I might inhibit formation of a proton gradient is by changing the permeability of the membrane so that ionic conditions inside the cells equilibrate with those in the medium. To test this possibility, we investigated whether K^+ leaked from cells on treatment with sarcotoxin I. As shown in Fig. 2, K^+ in the cells rapidly leaked out when sarcotoxin I was added to the medium, and the amount of K^+ in the medium reached a plateau in about 5min. The overall leakage amounted to about 70% of the total K^+ in the cells. As reported previously (Okada & Natori, 1984), no significant morphological change was observed under these conditions, suggesting that sarcotoxin I makes channels in the surface membrane of *E. coli* and ions such as K^+ and H^+ leak out through these channels. Probably this is the main reason for loss of membrane functions such as active transport of proline and ATP generation on treatment with sarcotoxin I.

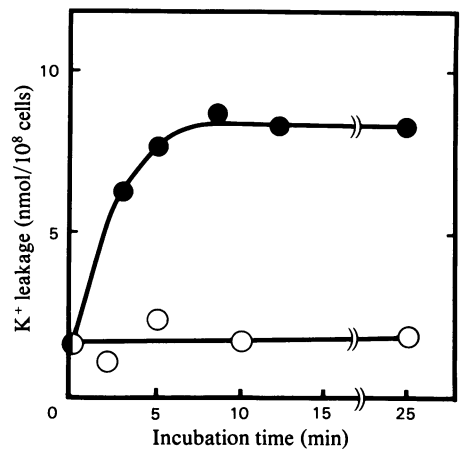

Fig. 2. *Leakage of K^+ from E. coli caused by sarcotoxin I*

E. coli K-12 594 (str^r) was suspended in 150mM-NaCl solution and preincubated for 10min at 30°C. Then sarcotoxin I was added at a final concentration of 10μg/ml, and incubation was continued. Samples (100μl) were taken at intervals and filtered, and the K^+ concentration in the filtrate was measured. The amount of K^+ extractable from the cells by 5% trichloroacetic acid was 10.6 ± 0.8nmol/10^8 cells. ○, Control cells treated with distilled water; ●, cells treated with sarcotoxin I.

Effect of sarcotoxin I on an uncA mutant

The above findings show that, when *E. coli* is treated with sarcotoxin I, the permeability of the cytoplasmic membrane changes greatly, with loss of some of its biological activity. If the uncoupling effect of sarcotoxin I is the main reason for loss of viability, an *uncA* mutant should be less sensitive than wild-type *E. coli*. Thus the *uncA* mutant is less sensitive than the wild-type strain to colicin K, which has an uncoupling effect (Kopecky *et al.*, 1975). We examined the effect of sarcotoxin I on *E. coli* K-12 AN120, an *uncA* mutant of *E. coli* K-12 AN180, under two ionic conditions. It is known that, in this *uncA* mutant, serine at residue 373 of the α-subunit of F_1-ATPase is replaced by phenylalanine, and that the ATPase activity is less than 1% that of the parent strain, but that the mutant can grow by using ATP supplied by substrate-level phosphorylation in the presence of glucose (Noumi *et al.*, 1984). When *E. coli* K-12 AN120 was treated

with sarcotoxin I in medium containing 130mM-KCl and 1mM-$MgCl_2$, it was apparently resistant to sarcotoxin I, unlike the parent strain *E. coli* K-12 AN180, and did not lose viability appreciably, as shown in Fig. 3(*a*). However, in medium containing 130mM-NaCl instead of KCl and $MgCl_2$, the *uncA* mutant was sensitive and showed more than 50% loss of viability in the presence of 2 μg of sarcotoxin I/ml, although its sensitivity was clearly less than that of the parent strain, as shown in Fig. 3(*b*). Probably, in the presence of excess K^+ and Mg^{2+}, which are essential cations for *E. coli* (Axelrod, 1967), leakage of these ions from the cells caused by sarcotoxin I was counteracted by their diffusion into the cells from the medium. Moreover, since this mutant can survive without generation of ATP by oxidative phosphorylation, sarcotoxin I may not have an effect on this mutant under these conditions. However, when KCl and $MgCl_2$ in the medium were replaced by NaCl, the mutant may become more sensitive to sarcotoxin I, because the intracellular amounts of K^+ and Mg^{2+} actually decreased on treatment with sarcotoxin I. The decreases in viability of the wild-type strain on treatment with sarcotoxin I were similar in the two environmental ionic conditions.

Next, we measured the amounts of intracellular ATP when the bacteria were treated with 0.5 μg of sarcotoxin I/ml under the two ionic conditions. As evident from Fig. 4, the amount of ATP in the wild-type strain decreased drastically on treatment with sarcotoxin I irrespective of the ionic conditions, and the extent of decrease was about the same as that on treatment with the uncoupler dinitrophenol. On the other hand, the ATP concentration of the *uncA* mutant did not change appreciably in the presence of sarcotoxin I in

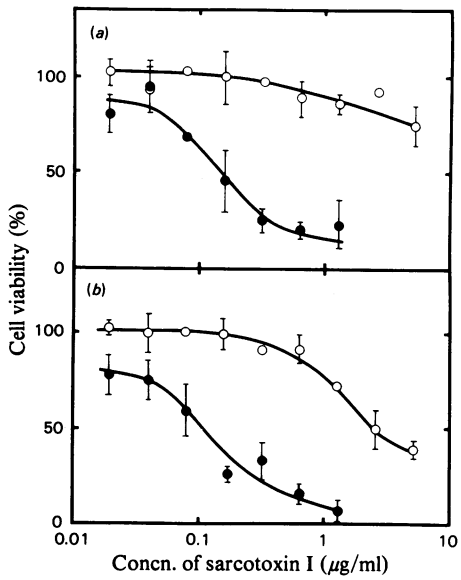

Fig. 3. *Effect of sarcotoxin I on viability of the uncA mutant of E. coli*

E. coli K-12 AN120 (*uncA* mutant) and AN180 (parent strain) were suspended in 10mM-phosphate buffer, pH6.0, containing 130mM-KCl, 1mM-$MgCl_2$ and 0.1% bovine serum albumin (*a*), or in 10mM-phosphate buffer, pH6.0, containing 130mM-NaCl and 0.1% bovine serum albumin (*b*), and incubated with various concentrations of sarcotoxin I for 10min at 30°C. Then the mixture was diluted and plated on LBK agar [for (*a*)] of LBNa agar [for (*b*)] to count viable cells. Percentage viability was plotted against the amount of sarcotoxin I. ○, *uncA* mutant; ●, parent strain.

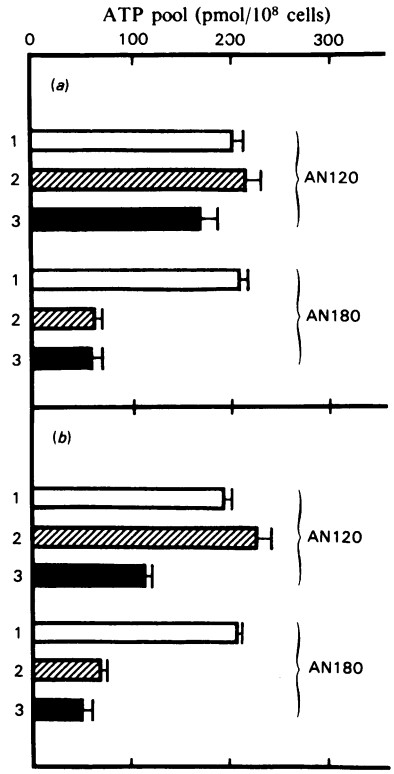

Fig. 4. *Effect of sarcotoxin I on the ATP pool of the uncA mutant of E. coli*

E. coli K-12 AN120 (*uncA* mutant) and AN180 (parent strain) were suspended in 10mM-phosphate buffer, pH6.0, containing 130mM-KCl, 1mM-$MgCl_2$, 0.1% bovine serum albumin and 0.4% glucose (*a*), or in the same buffer containing 130mM-NaCl instead of KCl and $MgCl_2$ (*b*), and preincubated for 10min at 30°C. Then sarcotoxin I (0.5 μg/ml) or dinitrophenol (5mM) was added. After incubation for 10min, the intracellular ATP pool was measured. Lane 1, control cells treated with distilled water; lane 2, cells treated with dinitrophenol; lane 3, cells treated with sarcotoxin I.

medium containing K^+ and Mg^{2+}, but decreased significantly in medium containing Na^+ instead, although the extent of the decrease was less than that in the wild-type strain. As expected, the ATP concentration of the *uncA* mutant did not change on treatment with dinitrophenol. Probably, in the wild-type strain, the uncoupling effect of sarcotoxin I, namely decrease in the ATP concentration, is essential for the bactericidal effect, because the bactericidal activity was the same irrespective of the environmental ionic conditions. In contrast, in the *uncA* mutant, leakage of essential cations induced by sarcotoxin I may cause decrease in the generation of ATP by substrate-level phosphorylation and result in loss of viability in medium lacking K^+ and Mg^{2+}.

Discussion

This paper reports the ionophore activity of sarcotoxin I, a bactericidal protein purified from the haemolymph of *Sarcophaga peregrina* larvae. In a previous paper, we suggested that the primary target of sarcotoxin I is the cytoplasmic membrane of bacteria (Okada & Natori, 1984). The present results show that the main reason for loss of viability of *E. coli* treated with sarcotoxin I is decrease in the intracellular ATP content. This decrease seems to be due to disruption of the membrane potential. As reported previously, sarcotoxin I disrupts a preformed membrane potential by changing the membrane permeability (Okada & Natori, 1984). Probably sarcotoxin I makes small channels in the membrane of bacteria and K^+ rapidly leaks out through these channels, resulting in equilibration of ionic conditions inside and outside the bacterial cells. The protons generated during respiration probably also equilibrates with protons in the medium. Thus sarcotoxin I seems to act as an ionophore and in its presence shortage of ATP seems to be the main cause of loss of viability of the wild-type strain of *E. coli*, because decrease in viability of the wild-type was not affected by the cationic conditions of the medium.

This notion was supported by studies on the *uncA* mutant, which has a defect in F_1-ATPase and as a consequence cannot synthesize ATP coupled with respiration. The *uncA* mutant was much less susceptible than the wild-type to sarcotoxin I in medium containing sufficient K^+ and Mg^{2+}, but it became susceptible in medium without these cations. We assume that sarcotoxin I has less effect on the *uncA* mutant, because the mutant generates ATP by substrate-level phosphorylation and a proton gradient is not essential for this ATP synthesis. But, probably because K^+ and/or Mg^{2+} may be essential for this reaction (Axelrod, 1967), the susceptibility of the *uncA* mutant to sarcotoxin I apparently increased in the absence of K^+ and Mg^{2+}. In parallel with decrease in viability, the ATP content of the *uncA* mutant decreased on treatment with sarcotoxin I in K^+- and Mg^{2+}-free medium. Therefore the mechanisms of the bactericidal effects of sarcotoxin I on the *uncA* mutant and the parent strain may be different: the viability of the *uncA* mutant is probably lost because K^+ and Mg^{2+} essential for substrate-level phosphorylation are lost, whereas viability of the parent strain is lost mainly because of the ionophore activity of sarcotoxin I.

Proteins or peptides with antibacterial activity have been found in various animals (Weiss *et al.*, 1978; Reddy & Bhargava, 1979; Stephen & Rafael, 1981*a*; Selsted *et al.*, 1983). These substances are supposed to participate in the non-specific defence system, together with complement and lysozyme, to prevent invasion of pathogenic bacteria (Taylor, 1983). However, the bactericidal actions of these proteins seem to be diverse, and those of two proteins, seminal plasmin in bovine seminal plasma and protein PCIII in rabbit serum, have been elucidated (Rao *et al.*, 1983; Stephen & Rafael, 1981*b*). It should be interesting to compare the mechanisms of action of the antibacterial proteins found in various organisms from the viewpoint of the evolution of non-specific defence mechanisms.

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