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Bacillus thuringiensis CrylAa δ-Endotoxin Affects the K^+/Amino Acid Symport in *Bombyx mori* Larval Midgut

M.G. Leonardi[1], **P. Parenti**[2], **M. Casartelli**[1], **B. Giordana**[1]

[1]Department of Biology and [2]Department of General Physiology and Biochemistry, University of Milan, via Celoria 26, 20133 Milano, Italy

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Abstract. We have examined the type of inhibition exerted by an activated preparation of the *Bacillus thuringiensis* δ-endotoxin CrylAa on K^+-dependent leucine transport into midgut brush border membrane vesicles or epithelial cells of the isolated midgut from *Bombyx mori* to study its possible interaction with the amino acid symporter.

K^+ permeability and the cation-dependent amino acid translocation into brush border membrane vesicles were evaluated by monitoring the fluorescence of the voltage-sensitive cyanine dye 3,3′-dipropylthiadicarbocyanine iodide. The symporter ability to accept Na^+ instead of K^+ was exploited and the dissipation of an imposed inside-negative potential (K^+ gradient in>out and valinomycin) was registered in the presence of a Na^+ gradient (out>in) and of the amino acid. The fluorescence quenching dissipated more rapidly when the amino acid was present. Preincubation of brush border membrane vesicles with CrylAa caused a significant decrease of the amino acid-dependent recovery of fluorescence, whereas K^+ permeability was sparely affected.

In the isolated midgut, CrylAa inhibits leucine uptake as well as the transepithelial electrical potential difference. The strong inhibition exerted by the δ-endotoxin was observed also in the absence of potassium and the transepithelial electrical potential difference. The results obtained strongly suggest a direct interaction of CrylAa δ-endotoxin with the K^+/amino acid symporter.

Key words: CrylAa δ-endotoxin — *Bacillus thuringiensis* — *Bombyx mori* larval midgut — K^+/amino acid symporters — Brush border membrane vesicles — Isolated midgut

Correspondence to: M.G. Leonardi

Introduction

The soil bacterium *Bacillus thuringiensis* (Bt) produces parasporal crystalline inclusions during sporulation which exhibit highly specific entomocidal activity against several order of insects (Höfte & Whitely, 1989). The crystal is composed of a number of inactive polypeptides, the protoxins, which in lepidopteran larvae are solubilized in the alkaline fluids of the midgut and cleaved to active proteins by endogenous proteases. The δ-endotoxins present a two-step mechanism of action: a binding phase to a specific receptor located on the brush border membrane of midgut columnar cells, which determines the host specificity, and a lytic phase with an increased permeability to several solutes until membrane disruption (Knowles & Dow, 1993). The toxin is supposed to insert into the membrane forming a pore whose size is still not unequivocally determined.

In lepidopteran larval midgut the absorption of neutral amino acids occurs by means of a K^+-dependent broad specificity transport system (Hanozet, Giordana & Sacchi, 1980; Giordana, Sacchi & Hanozet, 1982; Wolfersberger, 1989; Reuveni & Dunn, 1991; Parenti, Villa & Hanozet, 1992; Giordana et al., 1994) similar to the B^0 Na^+-dependent transport system of small intestine and kidney (Lynch & McGivan, 1987; Doyle & McGivan, 1992). In brush border membrane vesicles (BBMV) of *Bombyx mori* larval midgut, K^+/leucine cotransport was inhibited by Bt. subsp. *aizawai* even in the presence of equal concentrations of K^+ at the two sides of the vesicles, suggesting a direct interaction with the transporter (Giordana et al., 1993). More recently it has been shown (Parenti et al., 1995) that the activated Bt δ-endotoxin CrylAa, prepared from an *Escherichia coli*-cloned gene product, was also able to cause a specific dose-dependent inhibition of leucine uptake into *B. mori* BBMV, both in the presence and in the absence of a K^+-gradient. Further, the inhibition was exerted also in

the absence of K^+, i.e., the toxin affected the translocation of the carrier in its binary form (Parenti, Villa & Hanozet, 1992). CrylAa is by far the most active CrylAa δ-endotoxin in *B. mori* in vivo (Ge, Shivarova & Dean, 1989; Van Frankenhuyzen et al., 1991) as in vitro (Parenti et al., 1995).

In this paper the interaction between the CrylAa toxin and the K^+/leucine symport has been examined both in BBMV and in the isolated middle midgut from *B. mori* larvae.

Materials and Methods

Materials

L-[4,5-^{3}H]Leucine (2.04 TBq/mmol) and [U-^{14}C]Sucrose (23.2 GBq/mmol) were purchased from Amersham International plc, UK. The fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide ($DisC_3(5)$), the ionophore valinomycin, the protonophore carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine (bestatin) and all other reagents were supplied by Sigma Chemical (St. Louis, MO).

Experimental Animals

Five-day-old fifth instar larvae of *Bombyx mori* fed on mulberry leaves or artificial diet (Yakult, Japan or ENEA-Ratti Research Laboratory, Italy) were used.

Bt Toxin and its Activation and Purification

Overexpressing *Escherichia coli* strain ECE52 containing the CrylAa gene was kindly provided by Professor Donald H. Dean, The Ohio State University, Columbus, Ohio. The partially purified crystal protein was obtained from fresh cultures as described by Lee et al. (1992). Crystals were solubilized in 50 mM $NaHCO_3$, 10 mM dithiothreitol, pH 9.5, for 2 hr at 37°C. After centrifugation, the supernatant, adjusted to pH 8 with 6 N HCl, was incubated with trypsin 1:4 (w/w) at 37°C for 60 min. The proteolysis produced a polypeptide of about 60 kDa, as revealed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Laemmli, 1970). Trypsin and the small fragments of low molecular weight were removed by gel filtration in a Sephacryl S-200 column equilibrated in 50 mM $NaHCO_3$, 150 mM NaCl, pH 9.5 (bicarbonate buffer). A 2.5-ml sample (1.5 mg/ml of protein) was applied to the top of a 1.6 × 35-cm column and was eluted with bicarbonate buffer at 25 ml/hr. The fraction containing the proteins of 60 kDa was then dialyzed overnight against 5 mM $KHCO_3$, pH 9.5, at 4°C, lyophilized (Edwards Instrument), and stored at −20°C. The purity of the δ-endotoxin was evaluated by SDS-PAGE electrophoresis (Parenti et al., 1995).

Isolation and Preservation of the Midgut for BBMV Preparation

Midguts were isolated and preserved in liquid nitrogen as previously reported (Giordana et al., 1992). BBMV were prepared by Ca^{++}-precipitation and differential centrifugation as reported by Giordana et al. (1982). The final pellets were resuspended by 10 passes through a 22-gauge needle in the appropriate buffer as successively indicated. The protein concentration was assessed with the Coomassie Brilliant Blue G-250 protein assay (Pierce, IL), using bovine serum albumin as standard, and adjusted to a final concentration of 5 mg/ml.

Aminopeptidase N Assay

Aminopeptidase N (EC 3.4.11.2) activity in BBMV was determined by measuring the release of *p*-nitroaniline from L-leucine-p-nitroanilide in 50 mM TrisHCl at pH 8. Incubations were carried out in a spectrophotometer (Ultrospec 3000 Pharmacia Biotech, Cambridge UK) with a thermostatized (30°C) cuvette holder, under such conditions that activity was proportional to protein concentrations and time. One unit of enzyme is defined as the amount that hydrolyzes 1 μmol of substrate/min.

Transport Experiments

Transport experiments were performed in quadruplicate at room temperature by rapid filtration of the vesicle suspension through a prewetted cellulose nitrate filter (Hanozet, Giordana & Sacchi, 1980) with a pore size of 0.45 μm (Micro Filtration System, Dublin, CA). Incubations were performed with an automated device at 7 sec by mixing 10 μl of the BBMV resuspended in 90 mM Hepes, 34 mM Tris, pH 7.2 to 40 μl of the incubation medium to obtain the following final composition: 18 mM Hepes, 55 mM Tris, 0.1 mM L-leucine, 1.48 MBq/ml ^{3}H leucine, 50 mM K_2SO_4, 0.1 mM FCCP, pH 8.7. FCCP was added in order to generate an inside negative membrane potential of about 90 mV; the impermeant anion SO_4^{2-} was used as potassium counterion to avoid interference in the generation of the membrane potential. The presence of the pH gradient and the membrane potential simulated the physiological conditions (Dow, 1992). Samples were counted for radioactivity in a scintillation spectrometer Tri-Carb, Packard, model 300 C.

Fluorescence Measurements

The electrical potential difference generated across the BBMV was measured, into a polyacril cuvette, by means of the voltage-sensitive dye $DisC_3(5)$. The measurements were done in a spectrofluorophotometer (Jasco FP-777) with a thermostatized (25°C) cuvette holder. Excitation and emission wavelengths were 645 and 665 nm (Cassano et al., 1988; Uemura et al., 1992). Different extravesicular and intravesicular conditions were tested as indicated in the legends to the figures. Fluorescence values were expressed in arbitrary units.

Preincubation of BBMV and Incubation of the Isolated Midgut with δ-Endotoxin

The toxin was resuspended immediately before the experiments at about 1 mg/ml in H_2O; the protein concentration was measured with the Coomassie Brilliant Blue G-250 protein assay (Pierce, IL). BBMV were preincubated with the indicated CrylAa concentration at 25°C for 30 min, a time interval that provides the maximal inhibition of K^+-dependent amino acid uptake (Giordana et al., 1993).

In the isolated midgut the indicated CrylAa concentrations were injected in the lumenal compartment 15 min before the addition of the labeled leucine.

Transepithelial Electrical Potential Difference (TEP) Measurements and Leucine Uptakes in the Isolated Midgut

The middle region of the midgut, separated from silkworms kept in crushed ice for 15–20 min and deprived of the peritrophic membrane, was mounted as a tube on a suitable apparatus (Sacchi et al.,

1981). The buffer used had the following composition (in mM): 20 K_2SO_4, 5 $KHCO_3$, 44 $MgSO_4$, 9 $CaCl_2$, 1 L-leucine, 124 sucrose, 5 TrisHCl, at pH 7.2 for the hemolymph compartment or at pH 8.6 for the lumenal compartment. The saline in the lumenal and hemolymph compartments was aerated and stirred by bubbling 100% O_2. To measure leucine uptake in the absence of K^+ the following buffer was used (in mM): 44 $MgSO_4$, 9 $CaCl_2$, 1 L-leucine, 194 sucrose, 5 TrisHCl at pH 7.2. Leucine uptake was measured by adding 148 kBq/ml ^{3}H-leucine in the lumenal compartment. After an incubation of 15–20 min, the exposed tissue was severed, introduced in an eppendorf vial after removal of excess fluid and weighed to obtain fresh weight; 200 μl of distilled water was added and the midgut was then frozen and thawed twice. After centrifugation at 15.000 rpm (Force 16 centrifuge, Techne Ltd, Cambridge, UK) for 10 min, the supernatant was counted for radioactivity in the scintillation counter. Intracellular uptake values were calculated after subtraction of the radioactivity in the lumenal extracellular space (ECS). ECS was determined according to Giordana & Sacchi (1977) by incubating the midgut for 40 min with 18.5 kBq/ml ^{14}C-sucrose in the lumenal compartment. The calculation of ECS values, leucine uptakes and intracellular pools was performed as reported in Sacchi et al. (1981). On the basis of more than 30 determinations, dry weight was calculated as 19.4% ± 0.39 of the measured fresh weight. TEP was recorded throughout each experiment with a Keithley 176 microvoltmeter (Keithley, Taunton, MA), by means of calomel electrodes connected via agar-KCl (3 M) bridges to the solutions bathing both sides of the isolated midgut.

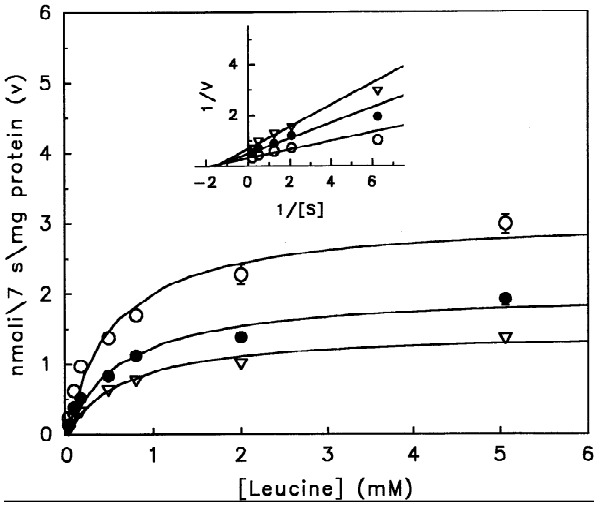

Fig. 1. Effect of CrylAa δ-endotoxin on leucine kinetics. BBMV were preincubated with 0 (○), 5 (●) or 50 (▽) μ*g* CrylAa toxin/mg BBMV protein. The initial rate of leucine uptake (*v*) was measured as a function of external amino acid concentration. Each point represents the mean ± SE of a typical experiment performed in quadruplicate. If absent, error bars were smaller than the symbols. The inset reports the reciprocal plot of the curves.

Calculations

Kinetic analysis was performed using a nonlinear regression, iterative computer program based on Marquardt-Levenberg algorithm (Sigma Plot, Jandel Scientific, Erkarath, Germany). The statistical analysis of the data was performed using the Student *t*-test.

Results

To assess the type of inhibition exerted by CrylAa toxin on the activity of the K^+/leucine cotransporter, we measured leucine uptake as a function of leucine concentration in the presence of two concentrations of the toxin (Fig. 1). The inhibition was noncompetitive, suggesting that CrylAa did not interact at the amino acid binding site.

Bestatin, a well-known inhibitor of aminopeptidase N activity in mammals and in lepidopteran larvae, behaves as competitive inhibitor of the symporter (Parenti et al., 1997). To confirm that the toxin does not interact with the amino acid binding site, we measured the inhibition of leucine transport into BBMV induced by a mixture of bestatin and CrylAa. The initial rate of 0.1 mM leucine uptake was measured in the presence of three different toxin concentrations as a function of bestatin concentration. The plot of the reciprocal of the transport rate (1/uptake) at a fixed concentration of the substrate (leucine), against the concentrations of the first inhibitor (bestatin) at each concentration of the second inhibitor (CrylAa), is linear. If the two inhibitors are mutually exclusive, the slope of each line is independent of the concentration of the second inhibitor, and the straight lines are parallel. On the contrary, when the two inhibitors do not compete for the same binding site, the slope depends on the concentration of the second inhibitor and the lines intersect. The experiment with bestatin and CrylAa produced a convergent Dixon plot (Fig. 2), which again indicated that CrylAa did not interact with the amino acid binding site on the carrier protein.

Then we addressed the question whether the inhibition of the K^+/amino acid symport could be an indirect effect of the interaction of the toxin with a functionally associated protein. As a matter of fact the brush-border membrane enzyme aminopeptidase N has been identified as a receptor for CrylAa toxin in the larva of *Manduca sexta* (Knight, Crickmore & Ellar, 1994; Knight, Knowles & Ellar, 1995; Sangadala et al., 1994), and the functional integrity of this enzyme has been shown to be essential for an efficient transport of several neutral amino acids through the Na^+-dependent B^0 system in bovine renal brush border membranes (Plakidou-Dymock, Tanner & McGivan, 1993).

Therefore we evaluated the effect of the toxin on the hydrolytic activity of aminopeptidase N. CrylAa was unable to affect the enzyme activity either directly (Fig. 3) or indirectly, since the δ-endotoxin did not influence the action of bestatin, a competitive inhibitor of aminopeptidase N activity (Fig. 4).

We then tested the possibility that the toxic lesion, which is compatible with a well documented pore formation (Hendrickx, De Loof & Van Mellaert, 1990; Li, Carroll & Ellar, 1991; Schwartz et al., 1993; Carroll &

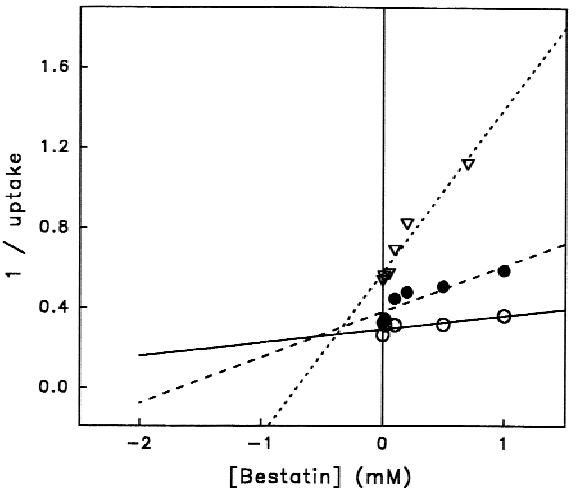

Fig. 2. Effect of bestatin and CrylAa δ-endotoxin on the uptake of 0.1 mM leucine. BBMV were preincubated with 0 (○), 11 (●) and 40 (▽) μg CrylAa toxin/mg BBMV protein. The initial rate of 0.1 mM leucine uptake (nmol/7 s/mg protein) was measured as a function of different bestatin concentrations (0–1 mM). Each point represents the mean ± SE of a typical experiment performed in quadruplicate. If absent, error bars were smaller than the symbols.

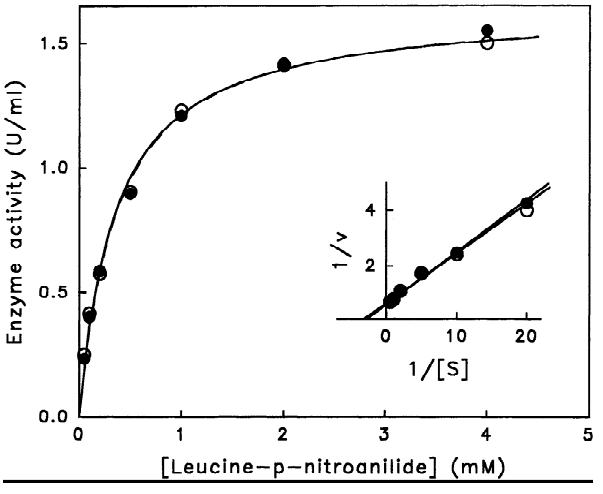

Fig. 3. Effect of CrylAa δ-endotoxin on the kinetics of aminopeptidase N activity. BBMV were preincubated with 0 (○) or 20 (●) μg CrylAa toxin/mg BBMV protein. Each point represents the mean ± SE of a typical experiment performed in quadruplicate. If absent, error bars were smaller than the symbols. The inset reports the reciprocal plot of the curves.

Ellar, 1993), might provide a pathway for the passive leakage of the amino acid.

The cation-dependent amino acid translocation involves the movement of a net charge, inducing a depolarization of the membrane potential in vertebrate (Wright, 1984; Cassano et al., 1988) and in lepidopteran brush border membranes (Giordana et al., 1989). This depolarization can be monitored in BBMV by recording

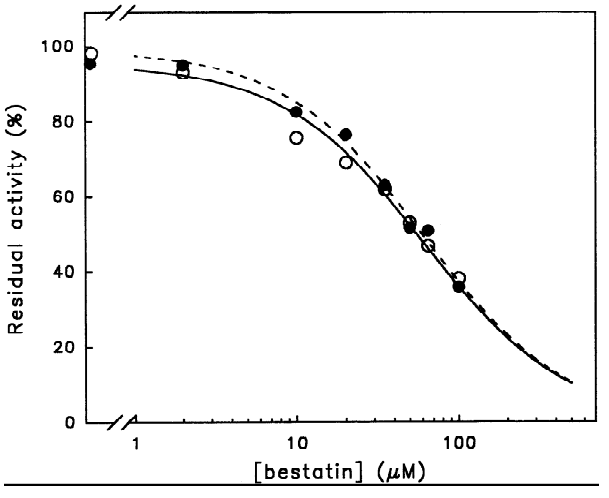

Fig. 4. Effect of CrylAa δ-endotoxin on the inhibition of aminopeptidase N activity by bestatin. BBMV were preincubated with 0 (○) or 20 (●) μg CrylAa toxin/mg BBMV protein. Each point represents the mean ± SE of a typical experiment performed in quadruplicate. If absent, error bars were smaller than the symbols.

the fluorescence quenching of the voltage-sensitive dye $DiSC_3(5)$ (Wright, 1984; Cassano et al., 1988). We used this approach, according to Cassano et al. (1988), with BBMV prepared from the posterior region of *B. mori* midgut, since leucine translocation in this region is strongly dependent on transmembrane potential difference (Giordana et al., 1994).

The fluorescence quenching was routinely calibrated by generating inside-negative potentials of different magnitudes by different outwardly directed K^+ gradients in the presence of valinomicyn (Fig. 5). The inset shows that the differences in fluorescence quenching (ΔF) plotted against the logarithmic ratio of external and internal K^+ concentration were linear. The mean value of the ΔF (%) per mV was 0.30 ± 0.05 (mean ± SE, five experiments) in agreement with previous results (Giordana & Parenti, 1994).

We employed the ability of the symporter to accept Na^+ instead of K^+ (Sacchi et al., 1994) to evaluate the dissipation of the imposed inside-negative potential caused by an inwardly directed Na^+ or Na^+ and leucine gradient (Fig. 6). As expected, in control BBMV the fluorescence quenching dissipated more rapidly when the amino acid was present (Panel A) while preincubation with CrylAa significantly decreased the amino acid-dependent recovery of fluorescence (Panel B), indicating a drastic reduction of leucine uptake.

K^+ permeability appeared to be little affected by the toxin (Fig. 7) when compared to the large quenching of fluorescence induced by the addition of 10 μM valinomycin.

The ability of the toxin to increase K^+ permeability was further investigated by measuring the fluorescence

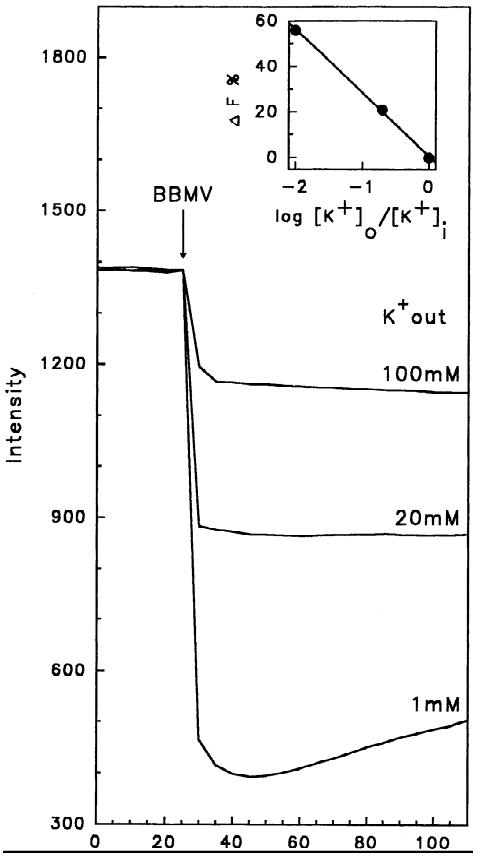

Fig. 5. Calibration of fluorescence quenching. Inside negative membrane potentials of different magnitudes were generated by dilution of BBMV resuspended (in mM): 100 Kgluconate, 50 TMAgluconate, 20 HepesTris, pH 7.2 into a buffer with the final composition (in mM): 100-20-1 Kgluconate, 50-130-149 TMAgluconate, 0.0015 $DisC_3(5)$, 0.01 valinomycin, 20 HepesTris, pH 7.2. The figure reports a typical experiment. Differences of recorded fluorescence quenching (ΔF%) *vs.* the logarithmic ratio of external and internal K^+ concentration are reported in the inset.

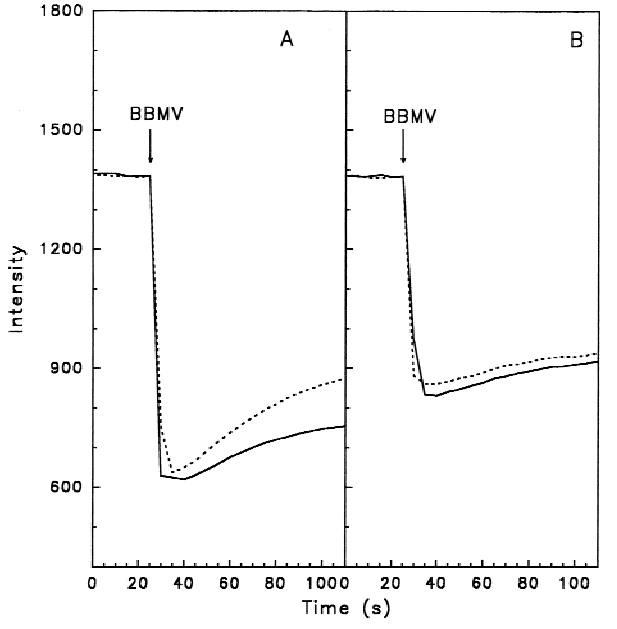

Fig. 6. Dissipation of an inside-negative potential in the presence of an inwardly directed Na^+ (solid line) or Na^+ and leucine (dotted line) gradient. BBMV, resuspended (in mM): 100 Kgluconate, 50 TMAgluconate, 20 HepesTris, pH 7.2 and preincubated with 0 (Panel *A*) or 40 (Panel *B*) μg CrylAa toxin/mg BBMV protein, were diluted into a buffer with the final composition (in mM): 1 Kgluconate, 134 TMAgluconate, 15 Nagluconate, 0.0015 $DisC_3(5)$, 0.01 valinomycin, 20 HepesTris, pH 7.2, with or without 5 leucine. The figure reports a typical experiment.

quenching in the presence of an inwardly directed K^+ gradient (Fig. 8). The addition to the extravesicular buffer of 15 mM KCl (arrow) generates a transmembrane electrical potential difference with the positive pole inside the vesicle, monitored by an increase of the fluorescence. An increase of K^+ permeability, causing a higher diffusion potential, would produce an increase of fluorescence. In the presence of CrylAa, an appreciable increase of the signal was observed, considerably enhanced by the subsequent addition of 10 μM valinomycin.

To compare the effects of CrylAa in BBMV to an experimental condition more similar to the in vivo situation, we have measured the transepithelial electrical potential difference (TEP) and the intracellular leucine uptake in the isolated midgut of *Bombyx mori*.

To mimic the physiological conditions, the experiments were performed in the presence of a lumenal pH of 8.6 and of an hemolymphatic pH of 7.2. Under these conditions, a TEP of 90–100 mV, lumen side positive, was recorded, which slowly decayed with time (Fig. 9, panel *A*).

The injection into the lumenal side of CrylAa drastically reduced the transepithelial voltage. Both the lag time i.e., the time between the application of the toxin and the start of the drop of voltage, and the slope of the response curves varied according to the dose (Fig. 9, panel B), in agreement with previous results by Liebig, Stetson & Dean (1995).

The uptake into midgut cells of 1 mM leucine was simultaneously measured by adding labeled leucine in the lumenal compartment 15 min after the toxin application (indicated by the arrows in Fig. 9). As reported in Table 1, the addition of CrylAa significantly decreased both amino acid uptake and its intracellular accumulation in a dose-dependent manner. Leucine uptake values were corrected for the lumenal extracellular space volume (ECS), which was determined by a 40-min incubation with labeled sucrose, a compound that does not cross the lumenal membrane (Giordana & Sacchi, 1977). As shown in the table, the ECS was not significantly different in control and treated midguts, suggesting that the toxic lesion does not cause an increase of sucrose permeability in the midgut in vitro.

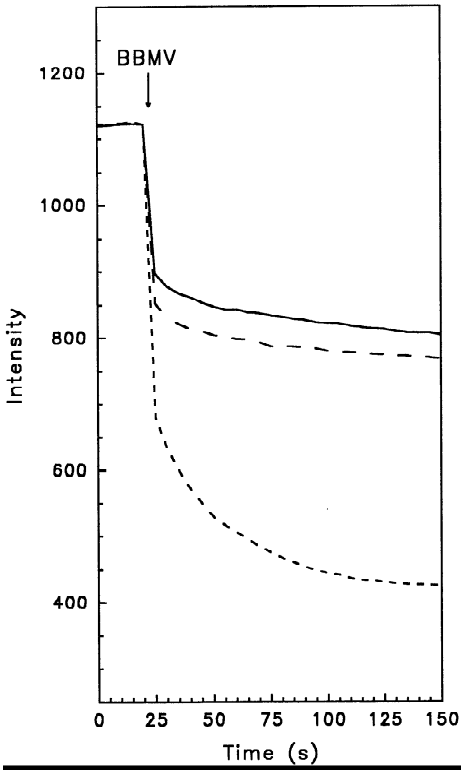

Fig. 7. Effect of CrylAa δ-endotoxin on the K^+ permeability in the BBMV, resuspended (in mM): 100 Kgluconate, 50 TMAgluconate, 20 HepesTris, pH 7.2 and preincubated with 0 (solid line) or 40 (long dashes) μg CrylAa toxin/mg BBMV protein, were diluted into a buffer with the final composition (in mM) of: 1 Kgluconate, 149 TMAgluconate, 0.0015 $DisC_3(5)$, 20 HepesTris, pH 7.2. The curve with small dashes was obtained with 0.01 mM valinomycin. The figure reports a typical experiment.

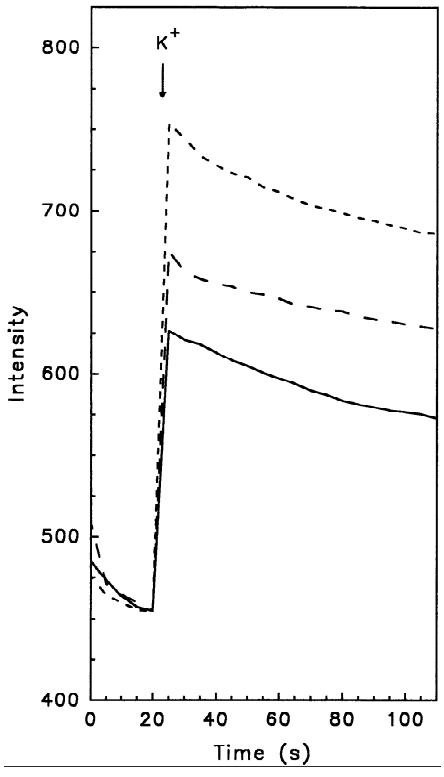

Fig. 8. Effect of CrylAa δ-endotoxin on the K^+ permeability in the presence of an inwardly directed K^+ gradient. BBMV, resuspended (in mM): 300 mannitol, 10 HepesTris, pH 7.2 and preincubated with 0 (solid line) or 40 (long dashes) μg CrylAa toxin/mg BBMV protein, were diluted into a buffer with the final composition (in mM): 300 mannitol, 0.0015 $DisC_3(5)$, 10 HepesTris, pH 7.2. The curve with small dashes was obtained with 0.01 mM valinomycin. 15 mM KCl (final concentration) was added at the time indicated by the arrow. The figure reports a typical experiment.

Amino acid translocation across the midgut is strongly dependent on TEP (Nedergaard, 1973) as expected according to the cellular model for K^+-dependent amino acid absorption (Giordana et al., 1982). So the reduced uptake of leucine with the δ-toxin could be due to the drop in TEP. However, the amino acid can cross the brush border membrane also in the absence of lumenal K^+, due to the ability of the symporter to translocate in the binary form (Giordana et al., 1989; Parenti et al., 1992). So we have measured leucine uptake in the absence of K^+ in the bathing solutions. The TEP in the isolated midgut is entirely dependent on hemolymph K^+ (Harvey & Nedergaard, 1964), so that in the absence of K^+ its value approached to zero within 20 min from the start of the incubation (Fig. 10, panel *B*), whereas in the presence of K^+ TEP values were much higher and only a 20% decline occurred in the same time lapse (panel *A*). Although the addition of CrylAa toxin to the lumenal fluid caused a lag in the drop of the TEP, its value was approaching zero when the labeled leucine was added (arrow) to start the amino acid uptake measurements (Fig. 10, panel *C*). Table 2 shows that, in the absence of K^+ and of the TEP, a 30% reduction of leucine uptake was observed, but a further significant inhibition of the amino acid uptake was caused by the addition of CrylAa.

Discussion

The inhibition of K^+-dependent amino acid transport into BBMV from the midgut of susceptible lepidopteran larvae provides a reliable test for the evaluation of Bt δ-endotoxin activity and specificity (Sacchi et al., 1986; Wolfersberger, 1989; 1991; Ge, Shivarova & Dean, 1989; Reuveni & Dunn, 1991; Parenti et al., 1993; Giordana et al., 1993).

We have shown that a mixture of endotoxins obtained from the dissolution of the native crystal of Bt subsp *aizaway* (Giordana et al., 1993) as well as the cloned product in *E. coli* CrylAa (Parenti et al., 1995)

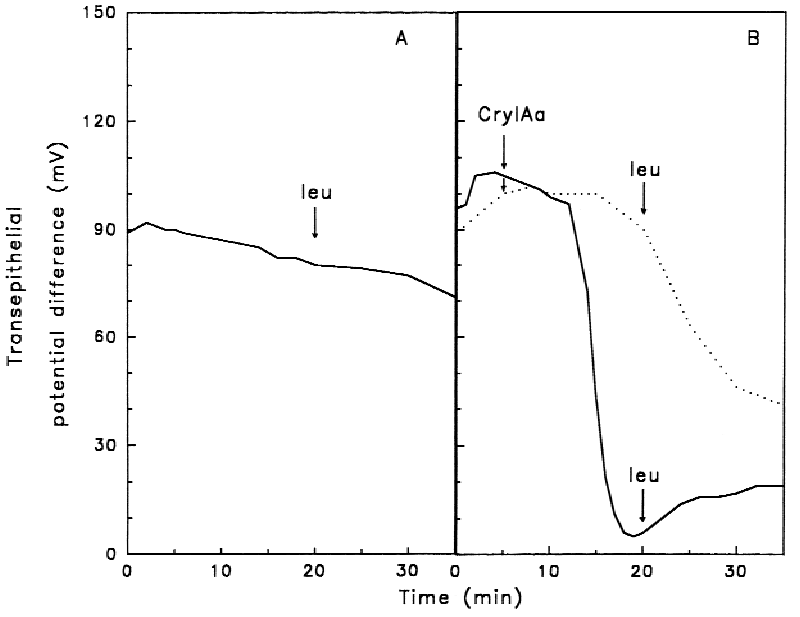

Fig. 9. Effect of CrylAa δ-endotoxin on the transepithelial potential difference across the isolated midgut. The midgut was perfused with a buffer of the following composition (in mM): 20 K_2SO_2, 5 $KHCO_3$, 44 $MgSO_4$, 9 $CaCl_2$, 1 L-leucine, 124 sucrose, 5 TrisHCl at pH 8.6 in the lumen side or pH 7.2 in the haemolymph side, bubbled with 100% O_2. Panel *A:* control condition; panel *B:* toxin effect. The arrows indicate the addition to the lumenal side of 2 (dotted line) or 20 (solid line) nM CrylAa and of 148kBq/ml ^{3}H-leucine.

Table 1. Effect of CrylAa δ-endotoxin on lumenal extracellular space (ECS) and leucine uptake in isolated *B. mori* larval midgut

	Lumenal ECS % tissue water	Leu Uptake nmol/g dry weight/min	Leu Intracellular pool mmol/l cell water
Control	16.6 ± 1.5 (5)	684.1 ± 85.1 (4)	2.93 ± 0.36 (4)
CrylaAa 2nM	17.6 ± 2.8 (7)	238.9 ± 51.3[a] (5)	0.80 ± 0.24[a] (5)
20nM	18.0 ± 1.9 (7)	107.0 ± 22.1[b] (3)	0.47 ± 0.10[b] (3)

The midgut was perfused as reported in the legend to Fig. 9. The uptake of 1 mM leucine was measured for 15 min by adding labeled leucine in the lumenal compartment 15 min after the toxin application.
[a] $P < 0.01$; [b] $P < 0.001$

produced a specific dose-dependent inhibition of leucine uptake into *B. mori* midgut BBMV also in the absence of a K^+-gradient or of K^+. These findings strongly suggested a toxin-symporter interaction. We have now studied in detail the type of interaction between the CrylAa δ-endotoxin and the K^+/leucine symporter.

The kinetic analysis performed in BBMV indicated that CrylAa toxin does not interact with the binding site for the amino acid (Figs. 1, 2). This finding did not discriminate if the toxin interacted directly with the carrier at a different site or through the inactivation of a protein functionally associated to the transporter such as the brush border membrane enzyme aminopeptidase N. This peptidase, which is a receptor for CrylAa toxin in *Manduca sexta* (Knight, Crickmore & Ellar, 1994; Knight, Knowles & Ellar, 1995; Sangadala, et al., 1994), appears to be functionally linked to the B^0 system, the Na^+-dependent neutral amino acid transporter, in bovine kidney (Plakidou-Dymock, Tonner & McGivan, 1993). Our observation that CrylAa toxin has no effect on aminopeptidase N hydrolytic activity (Figs. 3 and 4) does not support the hypothesis of a functional link in *B. mori* between the activity of the peptidase and that of the symporter. It is possible, however, that the aminopeptidase N domain involved in the toxin-binding was located at the stalk of the phosphatidilinositol anchor of the protease (Parenti et al., 1997). In such a case, the binding of the toxin could provide a steric hindrance for the symporter activity.

Besides, the diminished amount of leucine into BBMV in the presence of the toxin (Parenti et al., 1995) could be due to a leakage of the amino acid through the pores and/or channels formed by the toxin (Hendrickx, De Loof & van Mellaert, 1990; Schwartz et al., 1993; Knowles & Ellar, 1987; Knowles & Dow, 1993). However, we observed that CrylAa affected leucine transport (Fig. 6) even if the increase of K^+-permeability in our preparation of BBMV was rather modest (Figs. 7 and 8), so the inhibition of leucine uptake does not seem secondary to the presence of the toxic pore.

Although BBMV are a valid tool for the analysis of several membrane phenomena, the isolation and purification of this subcellular fraction may introduce artefacts; for that reason we evaluated the effect of CrylAa toxin on the isolated midgut. In an experimental condition that mimicked the physiological one, CrylAa toxin inhibited leucine uptake into midgut epithelial cells and affected the TEP across the isolated midgut. Moreover, in the absence of K^+ and therefore in the absence of TEP, the toxin was still able to inhibit the amino acid transport.

The lumenal extracellular space was not signifi-

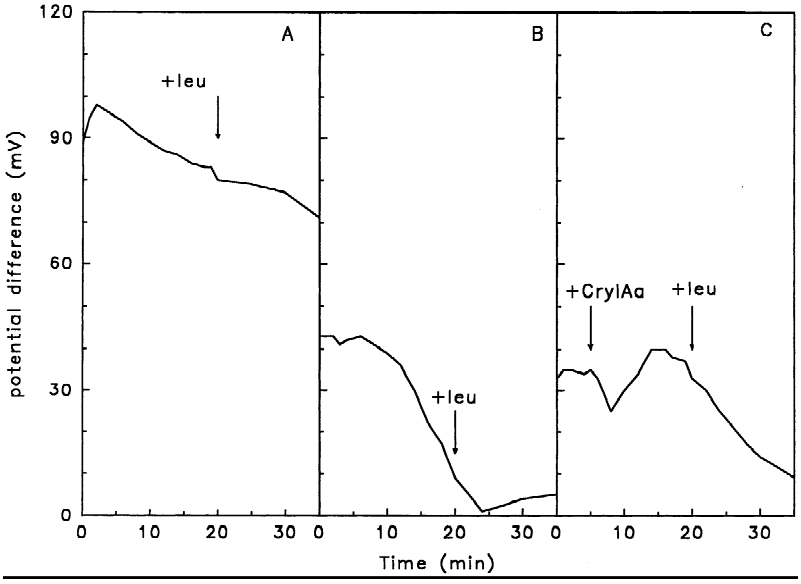

Fig. 10. Effect of CrylAa δ-endotoxin on the transepithelial potential difference across the isolated midgut in the absence of K^+. The midgut, bubbled with 100% O_2, was perfused both in the lumenal and hemolymph compartments with buffers of the following composition (in mM): 20 K_2SO_4, 5 $KHCO_3$, 44 $MgSO_4$, 9 $CaCl_2$, 1 L-leucine, 124 sucrose, 5 TrisHCl at pH 7.2 (panel *A*) or 44 $MgSO_4$, 9 $CaCl_2$, 1 L-leucine, 194 sucrose, 5 Tris-Cl pH 7.2 (panels *B* and *C*). The arrows indicate the addition to the lumen side of 20 nM CrylAa and of 148kBq/ml ^{3}H-leucine.

Table 2. Effect of the absence of K^+ and of CrylAa δ-endotoxin on leucine uptake in isolated *B. mori* larval midgut

	K^+	Leu uptake nmoli/g dry weight/min	Leu intracellular pool mmol/l cell water
Control	+	287.4 ± 50.6 (3)	1.19 ± 0.25 (4)
Control	−	178.9 ± 37.3 (3)	0.95 ± 0.22 (3)
CrylAa	−	64.8 ± 7.9[a] (3)	0.37 ± 0.05[a] (3)

The midgut was perfused as reported in the legend of Fig. 10. The uptake of 1 mM leucine was measured for 20 min by adding labeled leucine in the lumenal compartment 15 min after the toxin application. [a] $P < 0.05$.

cantly different between control and treated midguts. Therefore the toxin, also at the highest dose, was unable to produce a large increase of permeability across the lumenal membrane.

Our observation that CrylAa δ-endotoxin impairs the K^+/amino acid symporter, independently of the formation of a leakage pore in the membrane, and the nonunivocal relationship between binding and biological activity of *Bacillus thuringiensis* toxins (Garczynski, Crim & Adang, 1991; Wolfesberger, 1991), strongly suggest a multicomponent mode of action of Bt δ-endotoxin.

Therefore the ionophoric action does not seem to be the only activity exerted by the Bt toxins that has a biological significance.

Our data suggest a new case for an amino acid transport protein functioning as receptor for a toxin, analogously to system y^+, receptor for the ecotropic murine retroviruses (Kim et al., 1991; Wang et al., 1991).

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