Bacillus thuringiensis toxin inhibits K$^+$-gradient-dependent amino acid transport across the brush border membrane of *Pieris brassicae* midgut cells

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The luminal membrane of larval midgut cells is the site of action of insecticidal delta-endotoxin from *Bacillus thuringiensis*. At concentrations that correspond to normal effective doses in vivo, the toxin inhibits the uptake of amino acids by brush border membrane vesicles prepared from midguts of *Pieris brassicae* larvae. The toxin does not interact with the K$^+$-amino acid symport but rather increases the K$^+$ permeability of the membrane. The toxin does not increase the permeability of lepidopteran midgut brush border membrane to either Na$^+$ or H$^+$ nor does it increase the K$^+$ permeability of brush border membrane vesicles prepared from mammalian small intestine.

Endotoxin (Bacillus thuringiensis, *Lepidopteran midgut*) *Amino acid transport* *Membrane permeability* *Brush border membrane vesicle*

1. INTRODUCTION

The parasporal protein crystal, delta-endotoxin, from many subspecies of *Bacillus thuringiensis* is toxic to the larvae of numerous species of lepidopteran insects. The delta-endotoxin crystals from especially subspecies *kurstaki* and *thuringiensis* have been used as commercial insecticides for more than 20 years. Despite extensive field use and laboratory investigation, the molecular basis for the insecticidal action of the lepidopteran-specific *B. thuringiensis* delta-endotoxin is not known. The delta-endotoxin crystals dissolve readily in the alkaline environment of the lepidopteran larva's midgut. The dissolved protein (130 kDa) is cleaved enzymatically into a stable toxic protein of approx. 55 kDa [1], which passes readily through the peritrophic membrane and gains access to the midgut epithelial cells.

Numerous histopathological studies have identified the luminal plasma membrane of larval midgut epithelial cells as the primary target of the toxin [2]. The first step in the interaction of the toxin with the membrane is presumed to be its binding to a specific receptor [3,4], whose identity is still unknown. The details of what transpires immediately after the toxin binds to the susceptible cell membrane are also unknown. The longer range effects of the toxin are swelling of the cells and eventually disruption of the midgut epithelium [2].

The uptake of amino acids by the columnar epithelial cells from the lumen of lepidopteran midgut has been shown to occur by a cotransport process which is dependent upon the electrochemical gradient of K$^+$ across the epithelium

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[5–8]. Here, we describe primarily the effects of *B. thuringiensis* toxin on histidine transport using BBMV prepared from *Pieris brassicae* larval midguts [8].

2. MATERIALS AND METHODS

2.1. Toxin preparation

B. thuringiensis ssp. *kurstaki* (ETH culture 4432) and *thuringiensis* (ETH culture 4412) were grown as described [1]. Parasporal crystals were purified from sporulated cultures by the method of Delafield et al. [9]. They were solubilized by incubation at 3 mg/ml in 10 mM dithiothreitol, 50 mM sodium carbonate buffer (pH 9.5) for 30 min at 37°C. Undissolved material was removed by centrifugation. SDS-polyacrylamide gel electrophoresis [10] of the supernatants revealed them to contain only polypeptides of approx. 130 kDa. The solubilized crystal proteins were hydrolyzed with trypsin and the activated toxins were isolated by size-exclusion chromatography according to Huber-Lukač et al. [11]. SDS-polyacrylamide gel electrophoresis of the isolated activated toxins revealed each of them to be composed of two polypeptides of approx. 55 and 70 kDa.

2.2. BBMV preparation and transport experiments

BBMV were prepared from rabbit small intestine according to Kessler et al. [12] and from midguts isolated from last instar *P. brassicae* larvae by a modification of the method of Biber et al. [13], as recently described [8]. BBMV were routinely resuspended in 100 mM mannitol, 10 mM Hepes-Tris, pH 7.4 (buffer A). Transport experiments were performed by a rapid filtration technique [8]. Toxin, when present, was added to the vesicle suspension from concentrated stock solutions, so as to not change significantly either the concentration or pH of the BBMV suspension. The toxin-vesicle mixtures were routinely preincubated for 12 min at 25°C. The protein concentration of toxin preparations was determined by the method of Lowry et al. [14]. The method of Bradford [15], using a Bio-Rad kit, was used to determine the protein concentration of BBMV suspensions. Each value in table 1 and the figures

represents the mean ± SE of a typical experiment carried out in triplicate. When not given, SE bars in the figures were smaller than the symbol used.

2.3. Chemicals

D-[U-^{1C}]Glucose (257 mCi/mmol), L-[2,5-^{3}H]histidine (60 Ci/mmol) and L-phenyl[2,3-^{3}H]alanine (50 Ci/mmol) were purchased from Amersham International (England), valinomycin from Boehringer (Mannheim), FCCP from Sigma (St. Louis, MO) and trypsin (from bovine pancreas, diphenyl carbamyl chloride treated) from Serva (Heidelberg). All other reagents were analytical grade products from either Merck (Darmstadt) or Fluka (Buchs, Switzerland).

3. RESULTS AND DISCUSSION

At 0.8 μg toxin per mg BBMV protein, *B. thuringiensis* toxins depressed by 50% or more both the initial uptake rate and the transient accumulation of histidine by *P. brassicae* BBMV induced by a KSCN gradient. The toxins did not affect the equilibrium distribution of histidine between the intravesicular space and the external solution (fig.1). The effect of the toxins on 1 min histidine uptake by the BBMV was dose-dependent (fig.2A).
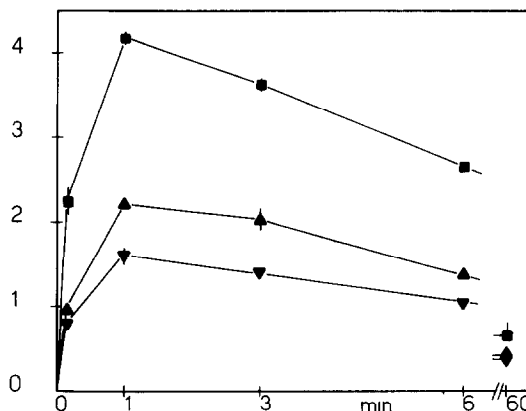

Fig.1. Effect of *B. thuringiensis* toxin on K$^+$-driven histidine accumulation in BBMV from *P. brassicae* midgut. BBMV resuspended in buffer A were preincubated for 12 min in the absence (■) or presence of 0.8 μg/mg BBMV protein of toxin from *B. t.* ssp. *thuringiensis* (▲) or *kurstaki* (▼) and incubated in buffer A containing 100 mM KSCN, 0.5 mM L-[^{3}H]histidine (final concentrations).
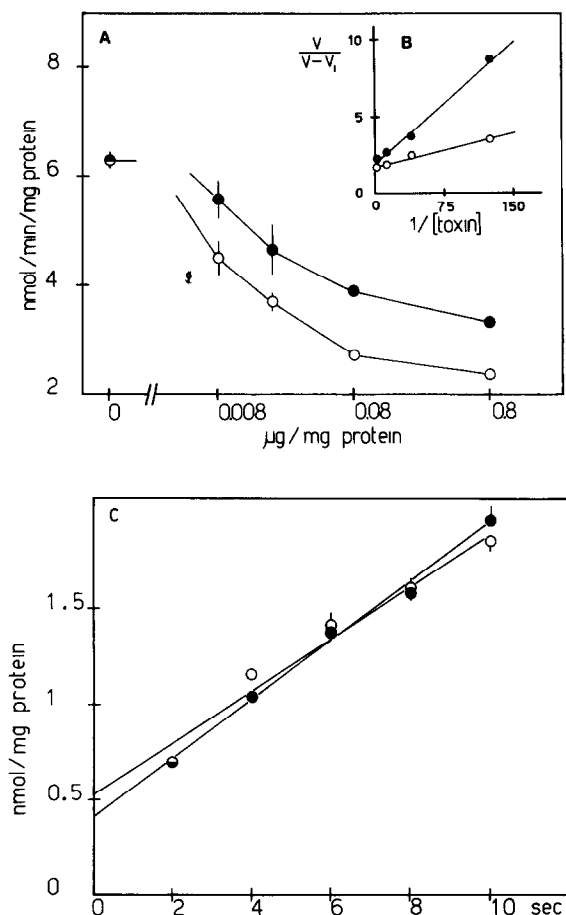
Fig.2. Effect of *B. thuringiensis* toxin on histidine uptake in BBMV from *P. brassicae* midgut. (A) Effect of the toxin concentration. BBMV resuspended in buffer A were preincubated for 12 min in the presence of increasing concentrations of *B. t.* ssp. *thuringiensis* (●) or *kurstaki* (○) toxin and incubated as in fig.1. The uptake was determined at 1 min incubation. Toxin concentration is expressed as μg/mg BBMV protein. In the inset (B) the reciprocal of inhibition is plotted vs the reciprocal of the toxin concentration. v, uptake in the absence of toxin; v_i, uptake in the presence of toxin. (C) Effect of the absence of the K^+ gradient. BBMV resuspended in buffer A containing 100 mM KSCN were preincubated for 12 min in the absence (○) or presence (●) of 0.1 μg/mg BBMV protein of *B. t.* ssp. *kurstaki* toxin and incubated in buffer A containing 100 mM KSCN and 0.5 mM L-[³H]histidine.

Both the *B. t.* ssp. *thuringiensis* and especially the *B. t.* ssp. *kurstaki* toxin caused a clearly discernable depression of transient histidine accumula-

tion at a concentration of less than 10 ng toxin per mg BBMV protein. The depression of the uptake overshoot increased almost linearly with the log of increasing toxin concentrations up to approx. 100 ng toxin per mg BBMV protein, then increased only slightly with another 10-fold increase in toxin concentration. The curves reported in fig.2 showed saturation kinetics, but the apparent limiting value of the uptake rate was not zero. This limiting value has been calculated by plotting the reciprocal of inhibition vs the reciprocal of toxin concentration (fig.2B). The maximal inhibition calculated is 61 ± 3 and 52 ± 4% for *B. t.* ssp. *kurstaki* and *thuringiensis* toxins, respectively. These values are not statistically different, as evaluated by *t*-test. From the same plot K_i values can be calculated, i.e. the toxin concentration that, in the experimental conditions adopted, causes half-maximal effect. These values are 9.1 ± 0.9 and 26 ± 2 ng/mg BBMV protein for *B. t.* ssp. *kurstaki* and *thuringiensis* toxins, respectively. These values are of the same order of magnitude as the amount of toxin required in vivo for a 50% reduction in weight gain by fifth instar *P. brassicae* larvae after a 24 h ad libitum feeding period (ED_{50}). For instance, the ED_{50} for *B. t.* ssp. *kurstaki* toxin is approx. 16 ng/larva [3]. Our method for preparing BBMV from *P. brassicae* has a yield of 26% and we obtain approx. 100 μg BBMV from each larva [8]. The effect of *B. t.* ssp. *kurstaki* toxin on histidine uptake by *P. brassicae* BBMV is half maximal at a toxin concentration of 9.1 ng per mg BBMV. Simple arithmetic reveals that this concentration corresponds to a dose of 3.5 ng toxin per larva.

Two ways in which the toxin could depress the K^+-dependent transient accumulation of histidine by *P. brassicae* BBMV are either by providing routes for K^+ to enter the vesicles other than along with histidine through the cotransport carrier (thus increasing the velocity of dissipation of the initial $\Delta\mu K^+$) or by interacting directly with the cotransport carrier. One means of distinguishing between these possibilities is by studying the initial rate of histidine uptake in the absence of a K^+ gradient. Under these conditions histidine uptake is purely an equilibrium process which should be unaffected by changing the K^+ permeability of the BBMV. On the other hand, the main path for histidine entry into the vesicle is through the carrier, so that any effect of the toxin on the carrier

should be reflected in the rate at which histidine enters the vesicles. When the K^+ concentration was equal on both sides of the membrane, *B. t.* ssp. *kurstaki* toxin had no effect on the initial rate of histidine uptake by the BBMV (fig.2C). This result indicates that the effect of the toxin is on the K^+ permeability of the vesicle rather than on the amino acid-K^+ cotransporter.

Several types of experiments were carried out in order to determine the specificity of the effect of the toxin on the ion permeability of BBMV. Na^+ can substitute, in part, for K^+ in supporting histidine uptake by midgut BBMV [7]. Therefore, we studied the effect of *B. t.* ssp. *kurstaki* toxin on histidine uptake driven by an Na^+ gradient. As seen in table 1, the toxin caused a striking dose-dependent inhibition of K^+-driven histidine uptake but had only a small and not dose-dependent effect on Na^+-driven histidine uptake. These results already rule out the possibility of *B. t.* ssp. *kurstaki* toxin having a nonspecific detergent-like effect on the permeability of target cell membranes, as has been proposed for the *B. t.* ssp. *israelensis* toxin [16].

In the presence of a transmembrane electrical potential difference generated by proton diffusion, it is possible to accumulate histidine into lepidopteran BBMV with either K^+ or Na^+ at the same concentration on both sides of the membrane [7]. Vesicles with an acidic internal medium are suspended in a medium of neutral pH and the driving force is switched on by the addition of the proton ionophore FCCP. In principle, an influence of

the different charge carried by histidine at the two membrane sides, under these conditions, should be expected. However, this influence should be the same whether the toxin is present or not. The results of such an experiment with *P. brassicae* BBMV are shown in fig.3. In the presence of K^+ and a proton gradient (fig.3A) and in the absence of toxin and FCCP, histidine uptake was little
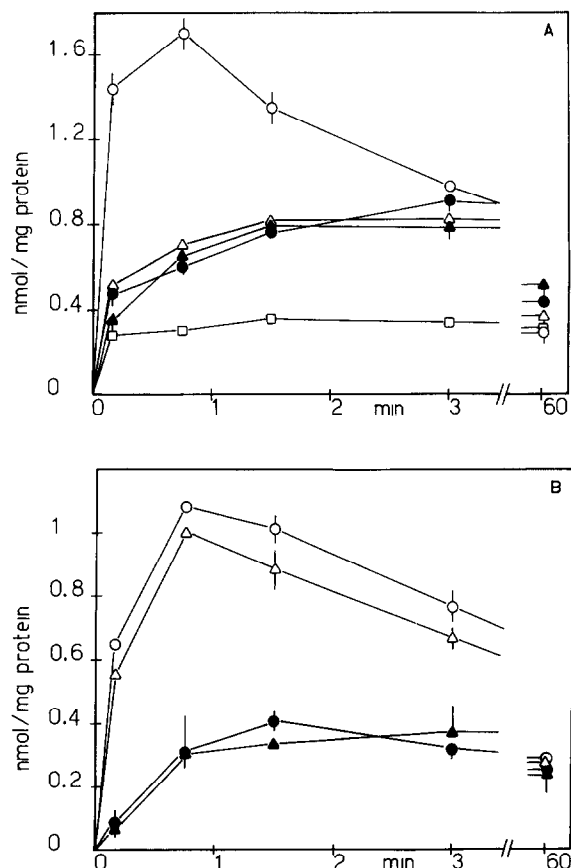

Fig.3. Effect of proton gradient, FCCP and *B. t.* ssp. *kurstaki* toxin on the histidine uptake in BBMV from *P. brassicae* midgut in the presence of K^+ (A) or Na^+ (B). BBMV resuspended in 193 mM mannitol, 90 mM Mes, 17 mM Tris, pH 5.5 and 50 mM K_2SO_4 (A) or 50 mM Na_2SO_4 (B) were preincubated for 12 min in the absence (○,●) or presence of 0.1 μg toxin/mg BBMV protein (△, ▲) or 7 μg valinomycin/mg BBMV protein (□) and incubated in a medium of the following final composition: 166 mM mannitol, 18 mM Mes, 72 mM Hepes, 39 mM Tris (pH 7.21), 50 mM K_2SO_4 (A) or 50 mM Na_2SO_4 (B), 0.5 mM L-[^{3}H]histidine in the absence (●, ▲) or presence of 80 μM FCCP (○, △, □).

Table 1

Effect of *B. t.* ssp. *kurstaki* toxin on K^+- or Na^+-driven histidine uptake in BBMV from *P. brassicae* midgut

Toxin concentration (ng/mg protein)	Histidine uptake (nmol/min per mg protein)	
	+ KSCN	+ NaSCN
–	6.30 ± 0.10	3.00 ± 0.02
8	4.50 ± 0.34	2.54 ± 0.02
80	2.73 ± 0.12	2.68 ± 0.05

Membrane vesicles resuspended in buffer A were preincubated for 12 min with the indicated toxin concentrations and incubated in buffer A containing 100 mM KSCN or NaSCN, and 0.5 mM L-[^{3}H]histidine

more than equilibrative (●). However, in the presence of FCCP, a transient overshoot of histidine uptake resulted (○). The addition of the toxin alone to the vesicles did not mimic the FCCP effect (▲), indicating that the toxin does not affect their proton permeability. By contrast no accumulation of histidine took place in the presence of both toxin and FCCP (△), which indicates that the toxin allows K^+ to enter the vesicles by a path of lower resistance than through the cotransport carrier with the histidine, thus dissipating the transmembrane electrical potential difference generated by the proton diffusion. This condition resembles the classic voltage clamp obtained with valinomycin (□). Similar experiments in which Na^+ was substituted for K^+ are shown in fig.3B. In these experiments we obtained essentially the same results that we obtained in the experiments with K^+, provided that FCCP was not present (solid symbols). However, FCCP caused an accumulation of histidine in both the presence and absence of toxin (open symbols). These results confirm the lack of an effect of the toxin on both the Na^+ and H^+ permeability of *P. brassicae* BBMV.

The above results demonstrate clearly that very small amounts of toxin from *B. thuringiensis* profoundly increase the K^+, but not the Na^+ or H^+, conductance of brush border membranes from the

tissue that is the target of the toxin in vivo, the midgut of lepidopteran larvae. However, they provide no evidence that this is a cell-specific effect. Therefore, it was of great interest to determine whether this toxin would have an effect on an intestinal cell plasma membrane transport system of non-lepidopteran origin. Fig.4 shows the Na^+-gradient-dependent uptakes of D-glucose and L-phenylalanine by BBMV prepared from rabbit small intestine. The toxin, at a dose that would inhibit greatly amino acid uptake by *P. brassicae* midgut BBMV, did not affect the uptake of either solute, indicating that also in this tissue it modifies neither Na^+ permeability nor the activities of the cotransporters examined. The effect of the toxin on the K^+ permeability of the rabbit membranes was studied by measuring Na^+-dependent glucose uptake driven by an outwardly directed K^+ gradient (fig.5). Since the K^+ ionophore valinomycin but not the toxin was able to stimulate D-glucose uptake, we conclude that the toxin is unable to increase the K^+ permeability of these mammalian BBMV.

In the light of the conclusion drawn, the experimental design used in this paper to assess the possible mode of action of *B. thuringiensis* delta-endotoxin on intestinal cell membrane of lepidopteran larvae is indeed an indirect approach.
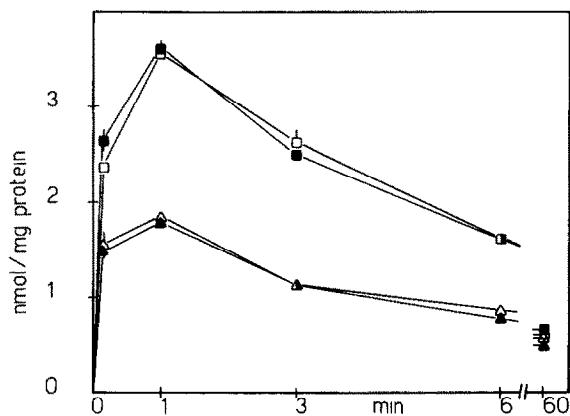

Fig.4. Effect of *B. t.* ssp. *kurstaki* toxin on the Na^+-driven D-glucose and L-phenylalanine uptake in BBMV from rabbit jejunum. BBMV resuspended in buffer A were preincubated for 12 min in the absence (□, △) or presence of 0.1 μg toxin/mg BBMV protein (■, ▲) and incubated in buffer A containing 100 mM NaSCN and 0.2 mM D-[^{14}C]glucose (□, ■) or 0.5 mM L-[^{3}H]phenylalanine (△, ▲) (final concentrations).
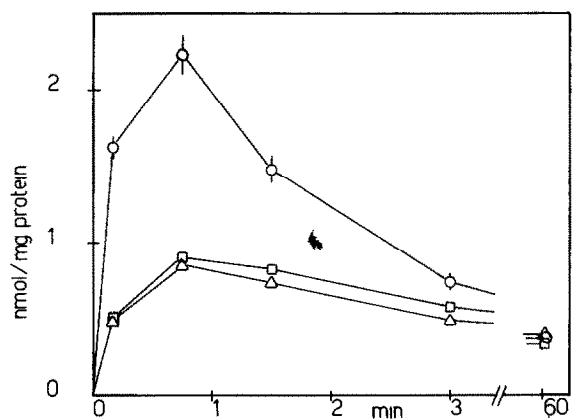
Fig.5. Effect of *B. t.* ssp. *kurstaki* toxin and valinomycin on D-glucose uptake in BBMV from rabbit jejunum. BBMV resuspended in buffer A containing 50 mM K_2SO_4 were preincubated for 12 min in the absence (□) or presence of 0.1 μg toxin/mg BBMV protein (△) or 2 μg valinomycin/mg protein (○) and incubated in buffer A containing 5 mM K_2SO_4, 50 mM Na_2SO_4, 0.5 mM D-[^{14}C]glucose (final concentrations).

Therefore, less than 100% inhibition of histidine uptake by infinite toxin concentration (see fig.2) cannot be surprising. It has been shown [7] that part of histidine transport across BBMV from larval intestinal cells does not depend on K^+ and is driven by electrical potential difference (a factor presumably operating in the experimental conditions of fig.2). This implies that, at present, we cannot ascribe entirely this incomplete effect to an interaction of the toxin molecules with a limiting concentration of a receptor on the membrane surface. Nonetheless, the experimental approach presented here, which allows the control of the environment both inside and outside the membrane vesicle, seems particularly suitable for elucidating the first events in the mode of action of the entomocidal delta-endotoxins of *B. thuringiensis*.

ACKNOWLEDGEMENTS

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