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DETERMINANTS FOR THE ACTIVITY OF THE NEUTRAL AMINO ACID/K^+ SYMPORT IN LEPIDOPTERAN LARVAL MIDGUT

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Summary

The columnar cells of lepidopteran larvae express, in their apical brush-border membrane, a class of symporters which *in vivo* couple the intracellularly directed amino acid and K^+ fluxes. An analysis of the functional properties of the symporter for neutral amino acids along the anterior, middle and posterior regions of the larval midgut of *Bombyx mori* demonstrated the ability of a K^+ gradient to drive leucine accumulation into brush-border membrane vesicles (BBMV) in all three preparations. However, marked differences are evident between the posterior (P) and the anterior–middle (AM) regions. In P-BBMV, much higher intravesicular accumulations were observed, V_{max} was six- to eightfold higher than in AM-BBMV, a lowering of external pH (pHe) from 8.7 to 7.2 caused a tenfold increase of K_m, and the absence of a potential difference ($\Delta\Psi$) caused a threefold decrease of V_{max}. In contrast, leucine uptake in AM-BBMV was poorly sensitive to both pH and $\Delta\Psi$. The kinetics of leucine uptake as a function of *cis* K^+ concentration were hyperbolic in P-BBMV and sigmoidal in AM-BBMV. More than 50 amino acids and analogues were used in inhibition experiments to characterize the amino acid binding site. Branched-chain amino acids modified on the carboxyl moiety were recognized only by the P-BBMV symporter. In AM-BBMV, substrate affinity was increased by the presence of a heterocyclic sidechain, even in the presence of a modified carboxyl- or α-amino group. Together, these results suggest that isoforms of the neutral amino acid/K^+ symporter are present. A natural inhibitor of amino acid symport has not yet been identified. However, several lines of evidence suggest that strong interactions exist between the amino acid/K^+ symporter and the receptor for the lepidopteran-specific *Bacillus thuringiensis* δ-endotoxins. CryIA(a) toxin, highly toxic for *B. mori* larvae, produced a dose-dependent inhibition of leucine uptake into both BBMV populations. The toxin was able to block the symporter in its ternary and leucine-only forms.

Symporter activity in the larval midgut of Lepidoptera depends on the proton-translocating V-ATPase

The identification of the primary active ion movements in an epithelium is very important, since a large number of secondary active mechanisms are expected to depend upon it. The physiology of the midgut epithelium of lepidopteran larvae is dominated by

Key words: *Bombyx mori*, neutral amino acid/K^+ symporters, *Bacillus thuringiensis* δ-endotoxin receptor.

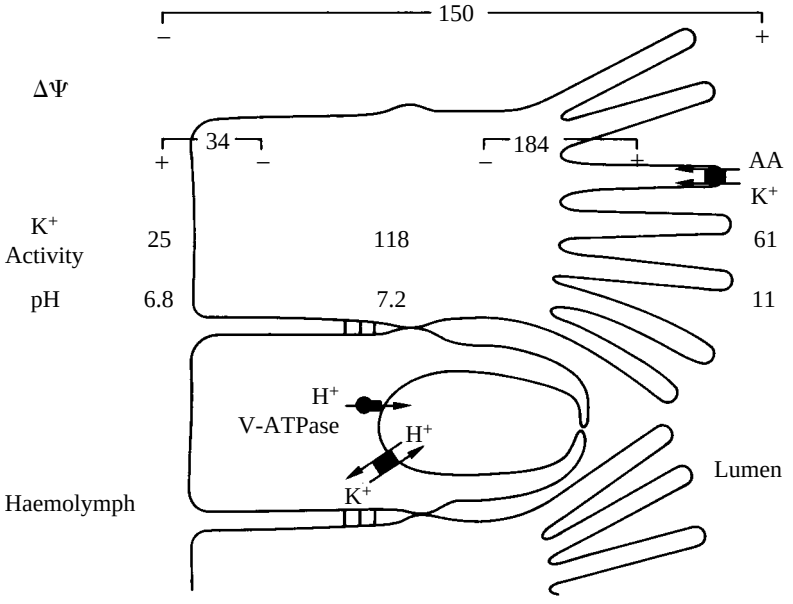

Fig. 1. Electrical potential differences ($\Delta\Psi$, mV), K^+ activities (mmol l^{-1}) and pH values in larval lepidopteran midgut. Values of electrical potential differences, potassium activities and lumen and haemolyph pH refer to *Bombyx mori in vivo*. Intracellular pH value refers to *Manduca sexta* (Chao *et al.* 1991; Dow, 1992). A goblet cell is represented between two columnar cells. AA, amino acid.

the activity of a proton pump, the H^+-translocating V-ATPase (Wieczorek *et al.* 1989) located in the luminal membrane of specialized cells, the goblet cells. The V-ATPase is associated in the goblet membrane with a K^+/nH^+ antiporter (Wieczorek *et al.* 1991; Wieczorek, 1992), thus being responsible for the ultimate active extrusion into the midgut lumen of potassium ions (Harvey and Nedergaard, 1964). The functional identification in the same epithelium of a K^+ symport, responsible for the uptake of amino acids across the brush-border membrane of midgut columnar cells, dates back to 1980 (Hanozet *et al.* 1980). Its occurrence was integrated into a model for the K^+-dependent transepithelial absorption of amino acids across the gut (Giordana *et al.* 1982). The model emphasized that it is primarily the voltage built up across the luminal membrane of columnar cells by the activity of the electrogenic proton pump, together with the ability of the symporter to bind K^+, that provides the driving force for secondary amino acid absorption. Fig. 1 gives a schematic presentation of K^+ activities, pH values and the electrical potential differences ($\Delta\Psi$) in the midgut of *B. mori* larvae. The estimated K^+ electrochemical potential across the luminal membrane of columnar cells *in vivo*, which drives amino acid uptake, is $-15.9\,kJ\,mol^{-1}$.

In the following years, with larvae of *Philosamia cynthia* as the experimental animal, the presence of at least five different K^+-dependent transport systems was established (Hanozet *et al.* 1984; Giordana *et al.* 1985, 1989). A detailed analysis of the kinetics of the K^+/symporter for neutral amino acids (Parenti *et al.* 1992) revealed the presence of

mixed-type kinetics, instead of an affinity-type mechanism that appears to be the model shared by the Na^+-dependent class of amino acid symporters. This feature might be related to the ability of the K^+ binding site to accept alkali metal cations other than K^+ (Hanozet *et al.* 1980; Giordana *et al.* 1989; Hennigan *et al.* 1993*b*; Sacchi *et al.* 1994). Moreover, both the fully loaded complex, symport/leucine/cation, and the partially loaded complex, symport/leucine, are able to perform amino acid translocation across the brush-border membrane (Giordana *et al.* 1989; Sacchi *et al.* 1990; Parenti *et al.* 1992).

The K^+/symport for neutral amino acids shares with the B^o Na^+-dependent transport system of mammalian small intestine and kidney brush-border membranes (Stevens *et al.* 1984; Lynch and McGivan, 1987; Doyle and McGivan, 1992) a broad specificity towards small, branched-chain and aromatic amino acids and it is unable to accept methylaminoisobutyric acid and cationic amino acids. The K^+-dependent broad specificity system appears to be common to most lepidopteran larvae (Hennigan *et al.* 1993*a*; Giordana *et al.* 1994).

Expression of different forms of the K^+/neutral amino acid symporter along the midgut of *Bombyx mori* larvae

The midgut of lepidopteran larvae can be divided into three different regions, identifiable by their morphological and functional differentiations (Cioffi and Harvey, 1981; Chamberlin, 1990; Azuma *et al.* 1991; Dow, 1992). The time courses of leucine uptake into brush-border membrane vesicles (BBMV) prepared by the Ca^{2+}-precipitation method (Giordana *et al.* 1982) from the anterior–middle (AM) and the posterior (P) regions of *B. mori* midgut were measured in simulated physiological conditions: i.e. an intravesicular pH of 7.2 similar to the cytoplasmic value (Chao *et al.* 1991; Dow, 1992) and an extravesicular pH of 8.7. A transmembrane electrical potential of approximately -90 mV was obtained by adding the protonophore FCCP. Fig. 2 shows that a K^+ gradient was able to drive the intravesicular accumulation of leucine in both preparations, but that in P-BBMV much higher uptake rates and accumulation were observed. Leucine uptakes into BBMV from the two separate anterior and middle tracts were almost identical (Giordana *et al.* 1994). The kinetics of leucine uptake into AM- and P-BBMV as a function of external amino acid or K^+ concentrations were then determined. The kinetic constants reported in Table 1 indicate (a) that whereas the affinity of the symporter for leucine is similar in the two midgut regions, V_{max} is markedly higher in P-BBMV, confirming that this midgut region is specifically specialized for amino acid absorption; (b) that the cation-dependent component, when both substrates are saturating, is much higher in P-BBMV than in AM-BBMV (apparently most of the amino acid taken up in the latter region is translocated under the binary form carrier/leucine); and (c) that leucine kinetics as a function of external K^+ concentration is hyperbolic in P-BBMV, but sigmoidal in AM-BBMV with a very high Hill coefficient. The Iso-Random Bi Bi system proposed for *P. cynthia* (Parenti *et al.* 1992) can account for nonhyperbolic velocity curves, assuming a steady-state model in which one route to the formation of the ternary complex is significantly favoured over the other. Alternatively, at saturating leucine concentrations, the translocation as a binary complex, kinetically less favoured, prevails when the K^+

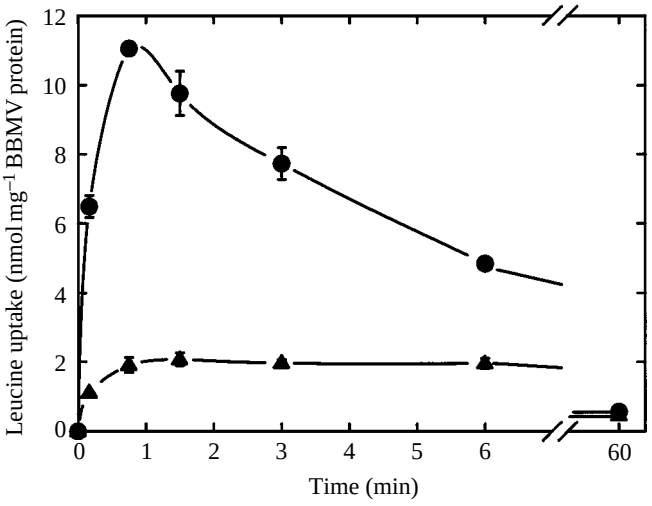

Fig. 2. Time course of leucine uptake into BBMV from the anterior–middle (triangles) and posterior (circles) midgut regions of *Bombyx mori* larvae, strain 157K. BBMV, resuspended in 90 mmol l^{-1} Hepes, 34 mmol l^{-1} Tris at pH 7.2, were diluted 1:5 to obtain the following final composition: 18 mmol l^{-1} Hepes, 55 mmol l^{-1} Tris, 0.5 mmol l^{-1} L-leucine, 1.48 MBq ml^{-1} L-[4,5-^{3}H]leucine, 50 mmol l^{-1} K_2SO_4, 0.08 mmol l^{-1} FCCP, pH 8.7. Each symbol is the mean ± S.E.M. of a typical experiment performed in triplicate.

concentration is low, whereas the more rapid translocation under the ternary form takes over as K^+ concentration increases further.

The apical membrane of the columnar cells of the larval midgut *in vivo* separates a very alkaline environment (up to a pH of 11 or more) from a nearly neutral cytoplasmic side (reviewed by Dow, 1992). When the extravesicular pH was lowered to neutrality, a drastic reduction of leucine uptake was observed only in P-BBMV. Minor effects were recorded in AM-BBMV, whereas in P-BBMV of the BC 20 *B. mori* strain the K_m value increased from 0.09±0.01 to 0.87±0.21 mmol l^{-1} (mean ± S.E.M. of three different determinations), with no modification of V_{max}. The dependence of the maximal rate of uptake of different neutral amino acids upon an extravesicular alkaline pH has been tested in AM-BBMV and P-BBMV from six different strains of *B. mori* larvae; in all cases, the effect was far more marked in P-BBMV, and it was always exerted on the affinity of the symporter for the amino acid. However, not all strains were equally sensitive, the decrease in affinity varying from five- to tenfold. Since the posterior midgut is the region where most of the amino acids are absorbed, a failure by the anterior–middle region of the midgut to alkalize the luminal contents (Dow, 1992) would severely hamper amino acid availability for the larva.

The voltage generated by V-ATPase activity is exploited mainly by symport in the posterior midgut

It had been clearly established that amino acid/K^+ symports are very sensitive to the presence of an electrical potential difference across the luminal membrane in the isolated

Table 1. *Kinetic constants of leucine uptake into BBMV from the anterior–middle and the posterior regions of the midgut of* Bombyx mori *larvae*

[Leucine] (mmol l^{-1})	[K^+] (mmol l^{-1})	V_{max} (nmol 7 s^{-1} mg^{-1})	K_m (mmol l^{-1})	*n*
Anterior–middle BBMV				
(1) 0.03–3	100	0.93±0.12	0.08±0.02	1
(2) 1	0–100	0.19±0.02	48.8±13.8	6.1±0.6
Posterior BBMV				
(3) 0.03–3	100	7.10±0.47	0.09±0.01	1
(4) 1	0–100	5.03±0.38	49.0±11.0	1

Experimental conditions for uptake were: BBMV, resuspended in 90 mmol l^{-1} Hepes, 34 mmol l^{-1} Tris at pH 7.2, were diluted 1:5 to obtain the following final composition: 18 mmol l^{-1} Hepes, 55 mmol l^{-1} Tris at pH 8.7, 0.08 mmol l^{-1} FCCP and (1, 3) 0.03–3 mmol l^{-1} L-leucine, 2.22 and 1.48 MBq ml^{-1} L-[4,5-^{3}H]leucine in AM- and P-BBMV, respectively, 50 mmol l^{-1} K_2SO_4, or (2, 4) 1 mmol l^{-1} L-leucine, 1.48 MBq ml^{-1} L-[4,5-^{3}H]leucine, 0–50 mmol l^{-1} K_2SO_4 and 50–0 mmol l^{-1} TMA sulphate. The incubations lasted 7 s.

1, the non-saturable component of leucine uptake, always present in AM-BBMV, is not reported.

2 and 4, values for the K^+-dependent component.

V_{max}, K_m, and *n* (the Hill coefficient) values are means ± S.E.M. for three different preparations.

mg, milligrams of membrane proteins.

Larvae were *B. mori* strain BC 20.

gut *in vitro* and *in vivo* (Nedergaard, 1973; Giordana *et al.* 1982) and of a transmembrane voltage in BBMV (Hanozet *et al.* 1980; Giordana *et al.* 1985; Sacchi *et al.* 1990; Hennigan *et al.* 1993*b*). Table 2 shows that, in *B. mori*, it is in P-BBMV that the $\Delta\Psi$-dependence is more marked, and that it is the translocation step that is affected, because V_{max} decreases threefold when the transmembrane potential is lowered. These results suggest that, in the absence of V-ATPase activity, the cation-dependent amino acid translocation involves the movement of a net positive charge(s), and that leucine uptake should therefore induce a depolarization of the membrane potential in BBMV. We tested this hypothesis with the fluorescent potential-sensitive dye 3,3′-diethylthiacarbocyanine iodide [DiS-C_2(5)] (Stieger *et al.* 1983; reviewed by Wright, 1984), by measuring the depolarization of an imposed inside-negative potential across the vesicle membrane, according to Cassano *et al.* (1988). The quenching of fluorescence was calibrated by generating membrane potentials of different magnitudes (Fig. 3A) and plotting the differences in fluorescence quenching *versus* the logarithmic ratio of the external and internal [K^+] (Fig. 3A, inset). The mean value of the ΔF (%) per mV, where ΔF is fluorescence quenching, was 0.41±0.01 (±S.E.M., five experiments). The ability of the amino acid symport to accept Na^+ instead of K^+ (Sacchi *et al.* 1994) was employed to evaluate the dissipation of an imposed ($[K^+]_i$=100 mmol l^{-1}, $[K^+]_o$=1 mmol l^{-1} plus valinomycin; see legend to Fig. 3) inside-negative potential by two concentrations of leucine, in the presence of a Na^+ gradient ($[Na^+]_o$>$[Na^+]_i$). Fig. 3B shows that the fluorescence quenching dissipated more rapidly with the sodium gradient and even faster when the amino acid was also present, according to its concentration.

Table 2. *Effect of the transmembrane electrical potential on the kinetic constants of leucine uptake into BBMV from the anterior–middle and the posterior regions of the midgut of* Bombyx mori *larvae*

	AM-BBMV		P-BBMV	
FCCP	V_{max} (nmol 7 s^{-1} mg^{-1})	K_m (mmol l^{-1})	V_{max} (nmol 7 s^{-1} mg^{-1})	K_m (mmol l^{-1})
+	1.14±0.25	0.09±0.01	6.02±0.76	0.12±0.01
–	0.75±0.13	0.12±0.03	1.81±0.31	0.20±0.05

Experimental conditions for uptakes were: BBMV, resuspended in 90 mmol l^{-1} Hepes, 34 mmol l^{-1} Tris at pH7.2 were diluted 1:5 to obtain the following final composition: 18 mmol l^{-1} Hepes, 55 mmol l^{-1} Tris at pH 8.7, 0.15 mmol l^{-1} L-leucine, 2.22 and 1.48 MBq ml^{-1} L-[4,5-^{3}H]leucine in AM-BBMV and P-BBMV, respectively, 50 mmol l^{-1} K_2SO_4 with (+) or without (–) 0.08 mmol l^{-1} FCCP.

The non-saturable component of leucine uptake is not reported.

V_{max} and K_m values are means ± S.E.M. of four different preparations.

mg, milligrams of membrane proteins.

Larvae of *B. mori* strain Shunrei × Shogetsu.

Thus, the functional characterization of leucine transport along the larval midgut of *B. mori* suggests the presence of two different symporters for neutral amino acids in AM and P midgut regions.

Determinants of substrate affinity of the neutral amino acid/K^+ symporters

A series of questions regarding those features of the amino acid structure that determine its affinity for the neutral amino acid/K^+ symporters of the two midgut regions was addressed. The topics investigated were: (a) the relevance of the free amino and carboxyl groups; (b) the position of the amino group; (c) the importance of the configuration and structure of the α-carbon; (d) the structure of the sidechain. Leucine uptake was measured in AM-BBMV and P-BBMV in the presence of a 20-fold excess of specific amino acids and analogues. The results (P. Parenti, M. Casartelli, M. Castagna, G. Leonardi and B. Giordana, in preparation), some of which are reported in Table 3, can be summarized as follows. Modifications to the COOH group drastically reduced the affinity for the AM cotransporter, whereas these were better tolerated by the P symporter. As expected, the free NH_2 group was also crucial for substrate recognition. However, for the K^+/symporter of the AM region, the presence in the sidechain of an imidazolic ring facilitated the interaction of the amino acid or analogue with the binding site. However, both symporters have in common a poor stereoselectivity and a relatively low selectivity for α-methyl amino acids, α-ketoacids and δ-amino acids.

These results give further support to the hypothesis that two structurally different carrier proteins for neutral amino acids are expressed along the midgut of lepidopteran larvae.

The activity of the amino acid/K^+ symporters is impaired by the lepidopteran active entomocidal *Bacillus thuringiensis* δ-endotoxins

Bacillus thuringiensis produces during sporulation a parasporal crystalline inclusion

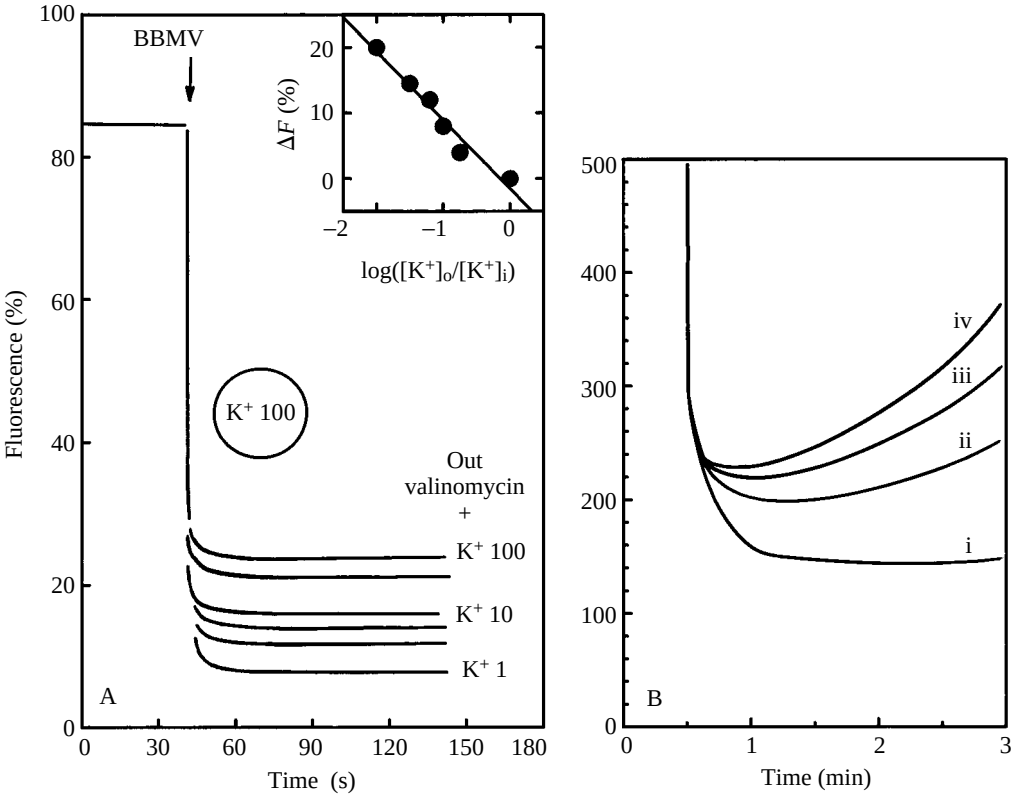

Fig. 3. Dissipation of an inside-negative membrane potential in the presence of a Na^+ gradient and of L-leucine in *Bombyx mori* P-BBMV. The fluorescence quenching was calibrated, generating membrane potentials of different magnitudes (A), by dilution of BBMV preloaded with (mmol l^{-1}) 100 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes–Tris, pH 7.2, into a buffer with a final concentration of 20 mmol l^{-1} Hepes–Tris, pH 7.2, 0.003 mmol l^{-1} cyanine dye, 0.004 mmol l^{-1} valinomycin, 100–1 mmol l^{-1} potassium gluconate and 0–99 mmol l^{-1} TMA–gluconate. Differences of recorded fluorescence quenching (ΔF) *versus* the logarithmic ratio of the external and internal K^+ concentrations are reported in the inset of A. (B) The dissipation of an imposed membrane potential (100 mmol l^{-1} $[K^+]_i$, 1 mmol l^{-1} $[K^+]_o$ and valinomycin, trace i) in the presence of 20 mmol l^{-1} sodium gluconate alone (trace ii), and after the addition of 0.1 mmol l^{-1} leucine (trace iii) or 5 mmol l^{-1} leucine (trace iv).

highly specific against several orders of insects (Höfte and Whiteley, 1989). In lepidopteran larvae, the crystal is processed by gut juice proteases to yield the entomocidal polypeptides called δ-endotoxins or Cry proteins. The molecular mechanism of action of these toxins consists of the binding to a brush-border-specific receptor, which determines the host specificity (Hofmann *et al.* 1988). Binding appears to be followed by a lytic phase with an alteration in the permeability of the apical membrane to solutes, until membrane disruption occurs (reviewed by Wolfersberger, 1992; Knowles and Dow, 1993). The activity and specificity of a given Cry protein against a lepidopteran species can be rapidly assessed by testing its ability to inhibit K^+-dependent amino acid transport into midgut BBMV (Sacchi *et al.* 1986; Wolfersberger, 1991; Parenti *et al.* 1993).

Table 3. *Specificity for different amino acid analogues of the neutral amino acid/K^+ symporters of the anterior–middle and posterior midgut regions of* Bombyx mori *larvae*

	Anterior–middle	Posterior	*P*
Leucine	100	100	–
D-Leucine	39±2	48±5	NS
α-Methyl-leucine	66±3	61±5	NS
Norleucine	98±2	96±3	NS
α-Aminobutyric acid	99±2	94±2	NS
Leucinamide	0	20±7	<0.01
Leucine methyl ester	2±1	46±5	<0.01
Isoleucine methyl ester	18±2	48±5	<0.01
Histidine methyl ester	65±5	48±4	<0.01
Histidine hydroxamate	50±3	28±4	<0.01
N-Methyl leucine	12±7	20±6	NS
N-Methylaminoisobutyric acid	4±1	5±1	NS
1-Methyl-4-imidazolacetic acid	58±5	31±3	<0.01
γ-Aminobutyryl histidine	59±5	40±6	<0.05
δ-Aminovaleric acid	50±8	40±5	NS
α-Ketoisocaproic acid	48±5	61±8	NS
α-Ketoisovaleric acid	64±5	65±1	NS

Experimental conditions as reported in the legend to Fig. 1.

Labelled leucine (0.15 mmol l^{-1}) uptake was measured in the presence of a 20-fold excess of the different substrates. To minimize interference due to dissipation of the K^+ gradient, an incubation time of 3 s was used.

Percentage inhibitions were calculated with respect to the control condition (3 mmol l^{-1} mannitol) after subtraction of the residual uptake in the presence of 3 mmol l^{-1} leucine.

Data represent mean ± S.D. of at least three different experiments.

Comparisons between AM- and P-BBMV preparations were made using Student's *t*-test. NS, not significant.

Recently, evidence has been presented that *B. thuringiensis* subsp. *aizawai* δ-endotoxin acts as a non-competitive inhibitor of amino acid/K^+ symport in *B. mori* larval midgut even in the absence of a K^+ gradient (Giordana *et al.* 1993).

The activity of three crystal proteins, CryIA(a), CryIA(b) and CryIA(c), obtained from *Escherichia coli* clones, was tested on leucine uptake into AM-BBMV and P-BBMV at a pHe of 8.7 (P. Parenti *et al.* 1994). Fig. 4 shows that the activated CryIA(a) toxin produced a dose-dependent inhibition of amino acid uptake in both midgut regions. The effect is not due to leakage of the substrate from the vesicles and it is specific, since the two related toxins CryIA(b) and CryIA(c) did not cause significant inhibition in the same assay.

The ability of the active toxin to inhibit amino acid uptake can be observed (a) in the presence of a 100 mmol l^{-1} K^+ gradient (not shown); (b) with 100 mmol l^{-1} K^+ inside and outside the vesicle (Fig. 4: IC_{50} 6.9±1.3 μg mg^{-1} BBMV protein for AM-BBMV and 7.0±0.3 μg mg^{-1} BBMV protein for P-BBMV); (c) in the complete absence of K^+ (Fig. 4: IC_{50} 3.5±0.3 μg mg^{-1} BBMV protein for AM-BBMV and 2.5±0.6 μg mg^{-1}

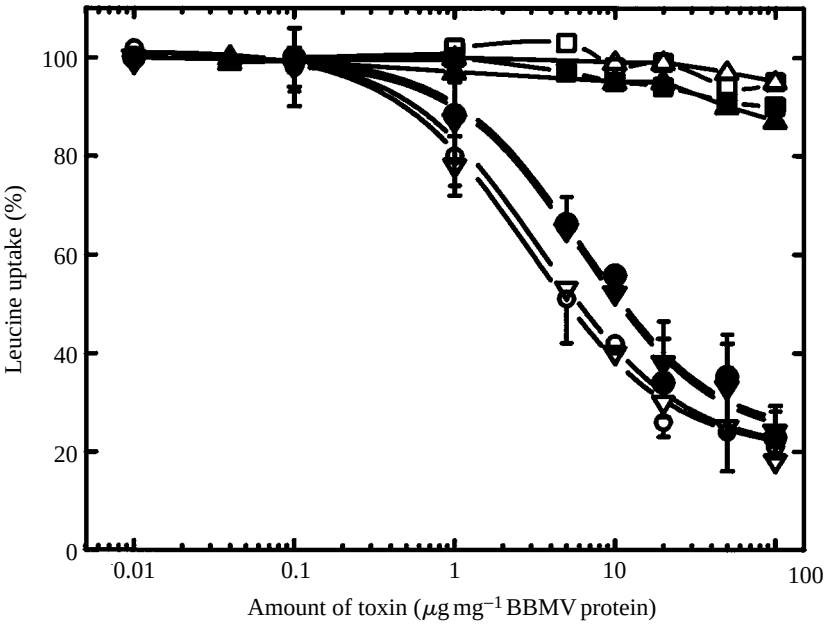

Fig. 4. Effect of CryIA(a), CryIA(b) and CryIA(c) δ-endotoxins on leucine uptake into midgut AM-BBMV (▼, ▽, □, △) and P-BBMV (●, ○, ■, ▲) from *Bombyx mori* larvae. BBMV, resuspended in the usual buffer at pH 7.2 with (▼, ●) or without (▽, ○) 50 mmol l^{-1} K_2SO_4, were preincubated for 30 min with CryIA(a) toxin concentrations from 0.01–100 μg mg^{-1} BBMV protein, then diluted 1:5 into the buffer at pH 8.7 reported in the legend of Fig. 2, with (filled symbols) or without (open symbols) 50 mmol l^{-1} K_2SO_4. For CryIA(b) (□, ■) and CryIA(c) (△, ▲) only conditions with intra- and extravesicular K^+ are reported. Leucine (0.15 mmol l^{-1}) uptake was terminated after 3 min. Data, referred to a control in the absence of toxin, represent the mean ± S.E.M. of a typical experiment performed in triplicate.

BBMV protein for P-BBMV). Very similar IC_{50} values, though with different Hill coefficients (discussed in P. Parenti *et al*. 1994), were also obtained in the absence of a pH gradient (pHi=pHe=7.2). Therefore, inhibition of amino acid transport in larval midgut BBMV is not secondary to the well-documented pore formation, since the toxin inhibited leucine uptake irrespective of the presence of K^+ and in the absence of any other ion gradient.

To explain the inhibition of the amino acid/K^+ symporters by *B. thuringiensis* subsp. *aizawai* toxin, we suggested (Giordana *et al.* 1993) that the complex mechanism of action of *B. thuringiensis* toxins could be mediated by the binding to a membrane protein represented by the K^+/amino acid symporters or a strictly associated protein. The receptor for CryIA(c) toxin active on *Manduca sexta* larvae has now been identified with the midgut brush-border membrane ectoenzyme aminopeptidase N (Knight *et al.* 1994; Sangadala *et al.* 1994). McGivan and coauthors have shown that the integrity of this enzyme is essential for an efficient amino acid transport by the Na^+-dependent B^o system in bovine renal brush-border membranes (Plakidou-Dymock *et al.* 1993).

Our present working hypothesis is that one action – but not the only one (Knowles and

Dow, 1993) – of lepidopteran entomocidal *B. thuringiensis* δ-endotoxins is to impair the activity of the amino acid/K^+ symporters either directly or through their interaction with the functionally associated aminopeptidase N.

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