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Leucine Transport Is Affected by *Bacillus thuringiensis* Cry1 Toxins in Brush Border Membrane Vesicles from *Ostrinia nubilalis* Hb (Lepidoptera: Pyralidae) and *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae) Midgut

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Abstract. The pore-forming activity of Cry1Ab, Cry1Fa and Cry1Ca toxins and their interaction with leucine transport mediated by the K^+/leucine co-transporter were studied in brush border membrane vesicles (BBMVs) isolated from the midgut of *Ostrinia nubilalis* and *Sesamia nonagrioides*. In both species, as in other Lepidoptera, leucine uptake by BBMVs can take place in the absence of cations, but it can also be driven by a K^+ gradient. Experiments with the voltage-sensitive fluorescent dye 3,3′-diethylthiacarbocyanine iodide proved that Cry1Ab, a *Bacillus thuringiensis* toxin active *in vivo*, enhanced the membrane permeability to potassium in *O. nubilalis* BBMVs. This result is in agreement with similar effects observed in *S. nonagrioides* BBMV incubated with various Cry1 toxins active *in vivo*. The effect of the above toxins was tested on the initial rate of 0.1 mM leucine influx. Instead of an increase in leucine influx, a reduction was observed with the Cry1 toxins active *in vivo*. Cry1Ab and Cry1Fa, but not the inactive toxin Cry1Da, inhibited in a dose-dependent manner leucine uptake both in the absence and in the presence of a K^+ gradient, a clear indication that their effect is independent of the channel formed by the toxins and that this effect is exerted directly on the amino acid transport system.

Key words: *Bacillus thuringiensis* mode of action — Corn borer — Brush border membrane vesicle — Pore formation — K^+/leucine cotransport

Introduction

Bacillus thuringiensis (Bt) Cry toxins are biopesticides widely used as an alternative to chemical insecticides, sprayed as formulations or expressed in transgenic plants (Schnepf et al., 1998; de Maagd & Stiekema, 1999; Whalon & Wingerd, 2003). However, a successful long-term use of Bt toxins for pest control depends on an adequate management of the development of insect resistance, which might arise at any step of the toxic mechanism (Herrero, Oppert & Ferré, 2001; Ferré & Van Rie, 2002). The complete elucidation of the interactions of Bt toxins with their cell membrane target sites is important for resistance management strategies, especially because most of the data indicate that resistance is frequently associated to an alteration of the toxin binding sites (Ferré & Van Rie, 2002; Jurat-Fuentes & Adang, 2006).

In lepidopteran larvae, it is long accepted that the specificity and toxicity of Cry1 toxins involve their binding to receptor(s) located in the apical membrane of midgut columnar cells and their insertion into the membrane to form pores which will lead to cell death (Knowles, 1994; Schnepf et al., 1998). A recent mechanism has been proposed in which binding of the toxin to the membrane receptor triggers a signaling pathway which leads to the death of the cell by a complex cellular response (Zhang et al., 2006). Given the different responses obtained upon laboratory selection for resistance to Bt toxins, it is likely that a number of mechanisms may be involved in the final toxin effect (Ferré & Van Rie, 2002).

The absorption of amino acids by the midgut epithelium of lepidopteran larvae is largely dependent on the functional properties of goblet cells, a specialized cell type only present in Lepidoptera. The vacuolar-type proton-ATPase (V-ATPase) in their apical membrane actively pumps H^+ in the goblet cavity, and a $K^+/2H^+$ antiporter exploits the created proton gradient to perform the extrusion of potassium into the midgut lumen (Harvey et al., 1998). The V-ATPase is

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also responsible for the high lumen-positive transepithelial electrical potential difference (Moffett & Koch, 1992). The K^+ electrochemical gradient thus generated across the apical membrane of the adjacent columnar cells favors the passive influx of the cation, and this driving force is exploited by K^+/amino acid cotransporters located in the brush border membrane to concentrate the amino acid severalfold into brush border membrane vesicles (BBMVs) or midgut cells (Giordana et al., 1998; Casartelli et al., 2001). Any lesion of the apical membrane forming a pore permeable to K^+ would provide a dissipative pathway for the cation, decreasing the driving force available for the amino acid accumulation. In addition to this, it has been shown that the cotransporter responsible for leucine uptake can also bind and transport the amino acid in the absence of K^+ (Parenti, Villa & Hanozet, 1992): in this case, leucine concentration inside the vesicle will only equilibrate with the external concentration and no accumulation will take place.

BBMVs isolated from midgut epithelial cells of target larvae are a basic tool for the evaluation of Bt toxin activity and specificity and to elucidate the types of interactions of Bt toxins with their membrane receptors (Sacchi et al., 1986; Wolfersberger, 1989, 1991; Gill, Cowles & Pietrantonio, 1992; Giordana et al., 1993; Parenti et al., 1995; Soberón et al., 2000; Estela, Escriche & Ferré, 2004). Previous studies from our laboratories have shown that Bt toxins can interact with leucine transport *in vitro*. In BBMVs of the susceptible insect *Bombyx mori*, the Bt toxins active *in vivo* inhibited leucine uptake regardless of the presence of the cation (Giordana et al., 1993; Parenti et al., 1995; Leonardi et al., 1997). It is likely that the interaction of Bt toxins with the K^+/leucine cotransporter contributes to the toxicity of these toxins; therefore, it was desirable to explore whether it also occurred in other insect species.

The European corn borer *Ostrinia nubilalis* and the Mediterranean corn borer *Sesamia nonagrioides* are two of the most harmful insect pests of maize. Since 1996, the adoption of genetically modified Bt maize producing the Bt toxin Cry1Ab has proved an effective way to control these pests. We present here a study performed with midgut BBMVs from these insects on the ability of Cry1Ab and other Cry1 toxins active *in vivo* (Gonzáles-Cabrera et al., 2006) to form pores and to interact with the K^+/leucine cotransporter.

Materials and Methods

Materials

L-[4,5-^{3}H]Leucine and L-[2,3,4,5-^{3}H]arginine were purchased from Amersham Biosciences (Cologno Monzese, Italy). 3,3′-Diethylthiacarbocyanine iodide ($DiSC_3[5]$) was purchased from Molecular Probes; Invitrogen (San Giuliano Milanese, Italy). Tetramethylammonium (TMA) and all other analytical-grade reagents were obtained from Sigma-Aldrich (Milan, Italy).

Insects

O. nubilalis larvae, kindly provided by Prof. C. Lozzia (Istituto di Entomologia Agraria, University of Milan, Milan, Italy), and *S. nonagrioides* eggs, kindly provided by Prof. P. Castañera (Departamento de Biologia de Plantas, Consejo Superior do Investigaciones Cientifics, Centro do Investigaciones Biológicas, Madrid, Spain), were reared on an artificial diet at 25 ± 1°C, 65–70% relative humidity and an 18:6 h light:dark photoperiod. Actively feeding fifth-instar *O. nubilalis* and sixth-instar *S. nonagrioides* larvae were used.

BBMV Preparation

Larval midguts were dissected, rinsed in a medium containing a mix of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μM pepstatin A, 2 μg/ml aprotinin, 0.1 mM leupeptin) and preserved in liquid nitrogen for no more than 6 months. BBMVs prepared from lepidopteran midguts preserved in liquid nitrogen largely retain their ability to transport amino acids (Giordana et al., 1992) and respond to Bt toxins, in both pore formation and inhibition of amino acid transport, qualitatively in the same manner as BBMVs prepared from fresh midguts, although uptake values and fluorescence signals were slightly lower (*data not shown*).

BBMVs were prepared from rapidly thawed midguts by divalent cation precipitation and differential centrifugation. Ca^{2+} and Mg^{2+} precipitations were performed according to Giordana, Sacchi & Hanozet (1982) and Wolfersberger et al. (1987), respectively. A shorter homogenization step (six instead of nine strokes at 1,500 rpm in a glass-Teflon potter) was necessary to obtain brush border fragments that effectively formed closed and tight vesicles. Protein concentration was assessed with the Coomassie Brilliant Blue G-250 Protein Assay (Pierce, Rockford, IL), using bovine serum albumin as standard.

Enzymes Assays

Aminopeptidase N (E.C. 3.4.11.2) and alkaline phosphatase (E.C. 3.1.3.1) activities were determined at 25°C by measuring (spectrophotometer Ultrospec 3000; Pharmacia Biotech, Cambridge, UK) the release of *p*-nitroaniline from L-leucine-*p*-nitroanilide in 40 mM Tris-HCl at pH 7.5 or of *p*-nitrophenol from *p*-nitrophenylphosphate in 1 M Tris-HCl at pH 8, respectively.

Toxin Preparation

The following Cry proteins were produced in *B. thuringiensis* strains expressing just one type of Cry protein: EG7077 (Cry1Ab), EG1081 (Cry1Ca), EG7300 (Cry1Da) and EG11096 (Cry1Fa) (obtained from Ecogen, Langhorne, PA). Each *B. thuringiensis* strain was grown at 29°C for 48 h in CCY medium (Stewart et al., 1981) supplemented with the suitable antibiotic. Spores and crystals were collected by centrifugation at 9,700 × *g* at 4°C for 10 min. Pellets were washed four times with 1 M NaCl/10 mM ethylenediaminetetraacetic acid (EDTA) and suspended in 10 mM KCl. Crystals were solubilized in 50 mM sodium carbonate buffer at pH 10.5 containing 10 mM dithiothreitol. Protoxins were trypsin-activated at 37°C for 2 h (1 mg trypsin/10 mg protoxin). Finally, activated toxins were purified by anion-exchange chromatography as described by Estela et al. (2004). Protein concentration in the preparations of Cry proteins was measured by the method of Bradford (1976) using the Bio-Rad (Munich, Germany) Protein Assay with bovine serum albumin as standard.

To remove Na^+ from the buffer, toxin preparations were dialyzed overnight against 20 mM Tris-HCl, 150 mM CsCl at pH 8.6 with regenerated cellulose SPECTRA/POR membranes (cut-off 12,000–14,000 daltons) (Spectrum, Los Angeles, CA).

Fluorescence Measurements

BBMVs were preloaded with 1 mM KCl by resuspension in 300 mM mannitol, 1 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-Tris at pH 7.2 before the last centrifugation. BBMVs were preincubated at 4°C for 30 min with the toxin concentrations reported in the legends of the figures, and control vesicles were preincubated with the toxin buffer.

The transmembrane electrical potential difference generated by subsequent extravesicular additions of KCl 20 mM was measured by means of the voltage-sensitive fluorescent dye $DiSC_3(5)$ in control and Bt-incubated BBMVs. Fluorescence measurements were performed in a polyacril cuvette thermostatted at 25°C (spectrofluorophotometer Jasco FP-777; Japan Spectroscopic, Tokyo Japan). Excitation and emission wavelengths were 645 and 665 nm, respectively. Fluorescence values were expressed in arbitrary units.

Transport Experiments

Transport experiments were performed in triplicate or quadruplicate at room temperature by rapid filtration under vacuum (Giordana et al., 1982). To measure leucine and arginine uptakes, one volume of the vesicle suspension was mixed with four volumes of the radiolabeled incubation medium, whose final composition is reported in the legends of the figures. BBMVs were preincubated at 4°C for 30 min with the toxin concentrations reported in the legends to the figures, and control vesicles were preincubated with the toxin buffer. Uptakes were terminated by a 50-fold dilution of the incubation mixture with an ice-cold stop solution (150 mM NaCl, 10 mM HEPES-Tris at pH 7.2) followed by rapid filtration, a procedure that lasted less than 2 s.

To determine the amount of leucine or arginine uptake due to unspecific binding of the labeled substrate and/or to a noncarrier-mediated uptake, 0.1 mM labeled leucine or 0.05 mM arginine uptake into *S. nonagrioides* BBMVs was measured at 1 and 3 min, respectively, in the presence of a 100-fold excess of the corresponding cold amino acid, in the presence or in the absence of a potassium gradient. The excess of substrate suppresses exclusively the carrier-mediated component, and the residual uptake can only be due to unspecific binding or noncarrier-mediated transport of the amino acid. The residual uptake was 26% and 39% of total uptake for leucine and 19% and 21% for arginine, with or without a potassium gradient, respectively. The unspecific uptake was routinely subtracted from total uptakes at 1 and 3 min in both species.

Leucine effluxes were measured in *O. nubilalis* BBMVs preincubated for 30 min with either 15 μg Cry1Ab/mg BBMVs protein or the toxin buffer (controls), then equilibrated for 10 min with 0.1 mM ^{3}H-L-leucine. Effluxes were measured at time zero by directly filtering the BBMVs suspension and at the different time intervals by leaving the BBMVs in the stop solution (the same one used for the uptake experiments) for the indicated time intervals.

Uptakes and efflux reaction mixtures were filtered through prewetted mixed cellulose ester filters (0.45 μm pore size; Micro Filtration Systems, Dublin, CA) and the filters counted for radioactivity in a liquid scintillation spectrometer (Tri-Carb 1600; Packard, Warrenvill, IL).

Calculations

Leucine uptake values as a function of Bt toxin concentration, expressed as percent of the control value, were fitted to the following equation:

$$v = V \times \frac{IC_{50}{}^n}{IC_{50}{}^n + [I]^n} + k$$

where v represents the leucine uptake value in the presence of the toxin, V represents leucine uptake in the absence of the toxin (control), I represents the toxin concentration, IC_{50} represents the half-maximal inhibition constant, n is the Hill coefficient and k is the uninhibited leucine uptake. Parameters were calculated by a multiparameter, iterative regression program (Sigma Plot; Jandel, Corte Madera, CA). Differences between mean values were tested by Student's *t*-test.

Results

BBMVs Preparation and Leucine Transport

For studies on membrane permeability and amino acid transport, it is essential that the brush border membrane fragments are adequately purified and sufficiently closed to form osmotically active spaces. We tested, therefore, if a better final membrane suspension could be obtained by using either the Ca^{2+} or the Mg^{2+} precipitation procedure (Giordana et al., 1982; Wolfersberger et al., 1987) to prepare BBMVs from *O. nubilalis* isolated midguts. BBMVs purity was evaluated from the enrichment of the specific activities of the two brush border membrane marker enzymes alkaline phosphatase (AP) and aminopeptidase N (APN), measured in the initial homogenates and in the final suspensions. Both enzymes were enriched to a similar extent with the two methods (about threefold AP and ninefold APN), and no significant difference in protein yield was observed. To test if the two preparations were equally composed of tight vesicles, we measured 0.1 mM leucine uptake after 60 min of incubation, i.e., at equilibrium (*see* Fig. 1 and related comment below), as a function of the intravesicular volume, experimentally modified by increasing the external medium osmolarity with the addition of sucrose. In both preparations, leucine uptake decreased linearly with the increase of the extravesicular osmolarity, namely with the decrease of the intravesicular volume (*data not shown*), so leucine was effectively transported into an osmotically active space in both cases. Since no significant differences were observed, BBMVs from *O. nubilalis* and *S. nonagrioides* isolated midguts were routinely prepared by Ca^{2+} precipitation.

A limited characterization of leucine transport in *O. nubilalis* and *S. nonagrioides* BBMVs was performed in the presence of a high external potassium concentration (100 mM) and a pH gradient (pH_{in} 7.2, pH_{out} 8.6). These experimental conditions ensure intravesicular accumulation of the amino acids transported by K^+ cotransporters in midgut BBMVs of many lepidopteran species

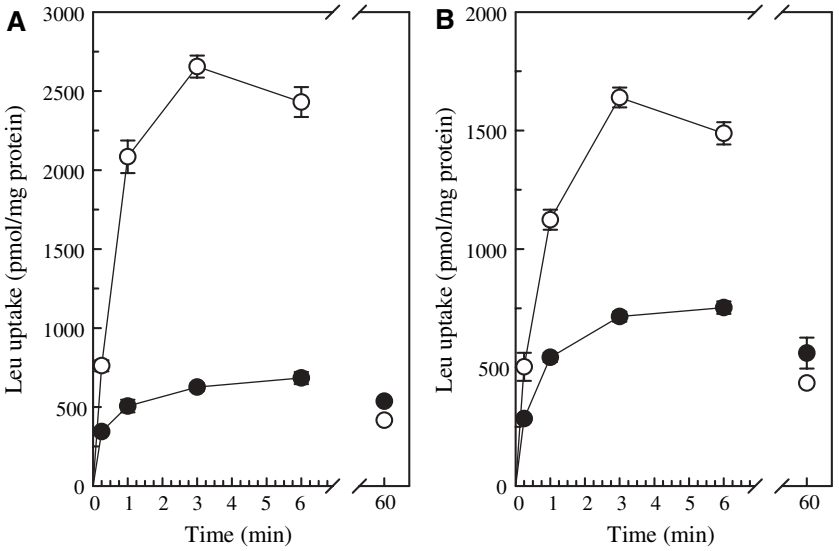

Fig. 1. Time course of leucine uptake into *O. nubilalis* (*A*) and *S. nonagrioides* (*B*) BBMVs. BBMVs, resuspended in 100 mM mannitol, 10 mM HEPES-Tris at pH 7.2, were diluted 1:5 to obtain the following final composition: 2 mM HEPES, 22 mM Tris at pH 8.6, 0.1 mM ^{3}H-L-leucine 30 μCi/ml, 50 mM K_2SO_4 (*open symbols*) or $(TMA)_2SO_4$ (*closed symbols*). Values are means ± standard error of typical experiments performed in triplicate.

(Giordana et al., 1982, 1998; Wolfersberger, 1989, 1991). In the presence of a K^+ gradient, 0.1 mM leucine was transiently accumulated within BBMVs (Fig. 1). The accumulation value is the ratio between the maximal and the equilibrium uptake values, reached after 60 min: equilibrium is attained when leucine net efflux along its concentration gradient reduces the intravesicular amino acid concentration to that of the external solution. In *O. nubilalis* BBMVs, leucine was accumulated sixfold (Fig. 1A) and in *S. nonagrioides* BBMVs, fourfold (Fig. 1B). If the cation was omitted, leucine was not accumulated and its internal concentration equaled the external one, i.e., reached the equilibrium value, within 3 min.

Pore Formation by Cry1 Toxins

The pore formation activity of Cry1Ab was tested in *O. nubilalis* BBMVs by means of the fluorescent voltage-sensitive dye $DisC_3(5)$. Increasing inwardly directed K^+ gradients were generated across control and toxin-treated vesicles by successive additions of KCl to the extravesicular buffer, to obtain progressive final concentrations of 20, 40, 60 and 80 mM. The inside-positive potentials of different magnitude thus generated caused a progressive increase of the fluorescence signal in control vesicles (Fig. 2). An increase of the membrane permeability to K^+ will produce larger variations of fluorescence, due to the higher diffusion potentials generated. Cry1Ab caused indeed a significant increase of the signal, even if considerably lower than that induced by the K^+ ionophore valinomycin (Fig. 2). Similar variations of the signal were induced in *S. nonagrioides* BBMVs by the Cry1 toxins active *in vivo* (González-Cabrera et al., 2006).

Inhibition of Amino Acid Transport by Bt Toxins

The amino acid initial uptake rate was measured in the absence of potassium: if the increase of the membrane passive permeability induced by the active Bt toxins appreciably affected the diffusional component of leucine transport, an enhanced amino acid uptake should be recorded. Instead, leucine uptake rate was significantly reduced in *O. nubilalis* BBMVs preincubated with Cry1Ab (Fig. 3A) as well as in *S. nonagrioides* BBMVs preincubated with Cry1Ab, Cry1Fa and Cry1Ca, three toxins active *in vivo* (González-Cabrera et al., 2006), while no inhibition was seen with the inactive toxin Cry1Da (Fig. 3B).

To rule out the possibility that the reduced amount of leucine inside the toxin-treated vesicles could be due to an increased efflux of the labeled amino acid into the diluting solution during the filtration step, we measured the efflux of 0.1 mM leucine in control and Cry1Ab-treated *O. nubilalis* BBMVs. The vesicles were incubated with the toxin buffer or with Cy1Ab for 30 min, then the labeled amino acid was added and the vesicles were left to stand for a further 10 min, to obtain the equilibration of labeled leucine within the vesicles. Then aliquots of the suspension were withdrawn and either immediately filtered (time zero) or diluted in the stop solution for the selected time intervals prior to filtration. As shown in Figure 4, the amount of leucine inside toxin-treated vesicles was always lower than that in controls, an indication that the inhibition of leucine uptake into the vesicles had prevented the attainment of equilibrium within 10 min.

The effect of toxins on leucine uptake was further investigated by adding increasing amounts of Cry1Ab (0–2 μg/mg BBMV proteins) in *O. nubilalis* BBMVs (Fig. 5A) or of Cry1Ab, Cry1Fa or Cry1Da (0–15 μg/mg BBMV proteins) in *S. nonagrioides* BBMVs

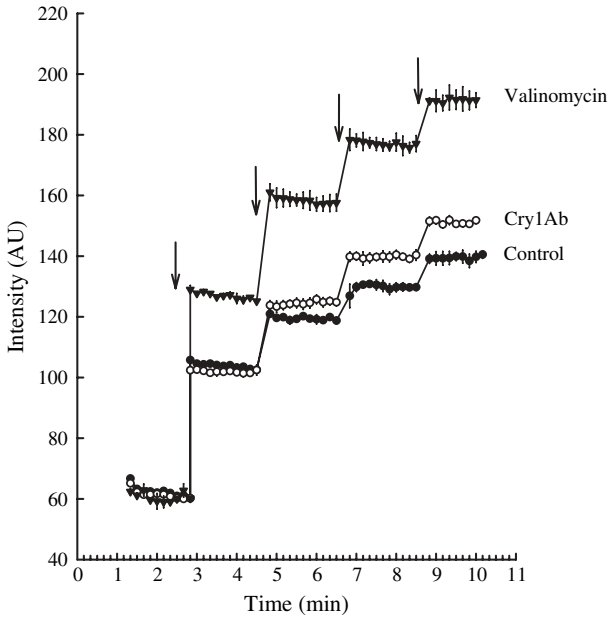

Fig. 2. Effect of Cry1Ab on K^+ permeability in *O. nubilalis* BBMVs. BBMVs, resuspended in 300 mM mannitol, 1 mM KCl, 10 mM HEPES-Tris at pH 7.2 and preincubated for 30 min with the toxin buffer (control) or 20 μg/mg of membrane proteins of Cry1Ab, were diluted in a cuvette in a buffer of the following final composition: 300 mM mannitol, 1 mM KCl, 10 mM HEPES-Tris at pH 7.2, 6 μM $DiSC_3(5)$. When present, 23 μM valinomycin was added to the diluting buffer. KCl to a progressive final concentration of 20, 40, 60 and 80 mM was added at the time indicated by the *arrows*. Fluorescence signals were normalized to the mean basal value, recorded in the absence of external K^+. Each trace is the mean ± standard error of a typical experiment performed in quadruplicate.

(Fig. 5B, C). The results were expressed as percent of the control value. With no K^+ (Fig. 5A, B), Cry1Ab reduced in a dose-dependent manner the amino acid uptake in both *O. nubilalis* and *S. nonagrioides* BBMVs. Cry1Fa, but not the nontoxic Cry1Da, also inhibited uptake in *S. nonagrioides* BBMVs. The dose-dependent inhibition of uptake by the active toxins also occurred in the presence of a K^+ gradient (Fig. 5A, C), while, again, Cry1Da was not effective (Fig. 5C).

The IC_{50} values, expressed as μg toxin/mg of membrane proteins, were 0.34 ± 0.05 and 0.32 ± 0.02 for Cry1Ab in *O. nubilalis* BBMVs in the absence and in the presence of potassium, respectively. In *S. nonagrioides* BBMVs, the values were 1.42 ± 0.22 and 1.21 ± 0.09 for Cry1Ab and 0.86 ± 0.11 and 1.10 ± 0.51 for Cry1Fa in the absence and in the presence of potassium, respectively. The IC_{50} values for Cry1Ab were significantly different ($P < 0.001$) between the two insect species in both conditions. The susceptibility of *S. nonagrioides* to Cry1Fa does not seem to be appreciably different from that to Cry1Ab.

To assess if the inhibition induced by Cry1 toxins was restricted to the specific cotransporter for leucine, we tested the effect of the toxins on arginine transport, which is mediated by a different transport system (Giordana et al., 1989; Liu & Harvey, 1996; Casartelli et al., 2001). The uptake of 0.05 mM arginine, with or without K^+, in *O. nubilalis* and *S. nonagrioides* BBMVs preincubated with the different toxins was significantly inhibited by Cry1Ab, Cry1Fa and Cry1Ca, while Cry1Da was ineffective (Fig. 6A, B).

Discussion

BBMVs isolated from *O. nubilalis* and *S. nonagrioides* larval midgut by Ca^{2+} precipitation were enriched in apical membranes, were satisfactorily tight and retained the ability to transport leucine and accumulate this amino acid by exploiting an inwardly directed potassium gradient (Fig. 1). Carrier-mediated leucine transport in *O. nubilalis* and *S. nonagrioides* midgut displayed the basic functional properties common to lepidopteran larvae so far studied, i.e., the ability to cross the brush border membrane alone or cotransported with K^+.

Cry1Ab, a toxin active *in vivo* on *O. nubilalis*, significantly increased the permeability to K^+ in *O. nubilalis* BBMVs (Fig. 2), a result consistent with those obtained in *S. nonagrioides* BBMVs with Cry1Ab and Cry1Fa (González-Cabrera et al., 2006), two toxins to which *S. nonagrioides* larvae are susceptible. These findings are in line with the well-documented ion channel activity of Bt toxins (Carroll & Ellar, 1993; Schwartz et al., 1993; Soberón et al., 2000; Bravo et al., 2002).

However, the pores formed by the toxins did not cause increased passive diffusion into the vesicles of 0.1 mM leucine. In fact, instead of higher leucine uptakes, significant inhibition of the amino acid initial uptake rates was detected in *O. nubilalis* and *S. nonagrioides* BBMVs preincubated with the three active toxins (Fig. 3). We had previously shown that in *Bombyx mori* BBMVs, Bt toxins active on larvae *in vivo* interacted with the cotransporter responsible for the amino acid transport (Giordana et al., 1993; Parenti et al., 1995; Leonardi et al., 1997), so the inhibition of leucine uptake recorded in *O. nubilalis* and *S. nonagrioides* BBMVs was not unexpected.

Kirouac et al. (2002) have shown that Cry1Ac and Cry1Aa insertion in *Manduca sexta* BBMVs produced a lesion that increased the passive influx of several amino acids, present outside the vesicles at a concentration of 100 mM. With such a high external concentration, amino acid transport into the vesicles would effectively be due mainly to diffusion and would be therefore very sensitive to a variation of the membrane permeability, i.e., to the pores formed by the toxins. Since we were interested in studying the

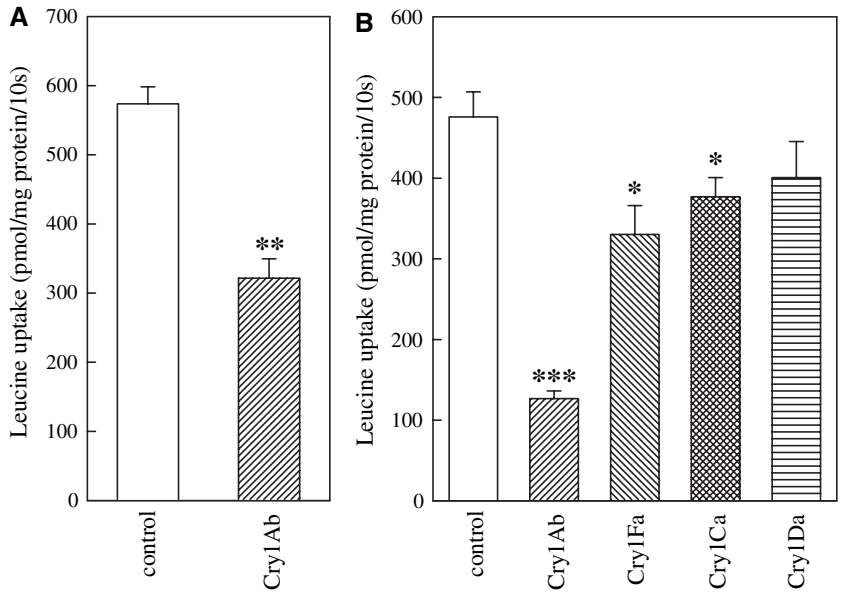

Fig. 3. Effect of different Bt toxins on leucine initial uptake rate in *O. nubilalis* (*A*) and *S. nonagrioides* (*B*) BBMVs. BBMVs resuspended in 100 mM mannitol, 10 mM HEPES-Tris at pH 7.2 were preincubated for 30 min without (control) or with 11.5 μg/mg of membrane proteins of Cry1Ab (*A*) or 16.5 μg/mg of membrane proteins of Cry1Ab, 7.3 μg/mg of membrane proteins of Cry1Fa, 15 μg/mg of membrane proteins of Cry1Ca and 15 μg/mg of membrane proteins of Cry1D (*B*). BBMVs were diluted 1:5 to obtain the following final composition: 2 mM HEPES, 22 mM Tris at pH 8.6, 68 mM mannitol, 0.1 mM ^{3}H-L-leucine 30 μCi/ml. Values are means ± standard error of a typical experiment performed in triplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

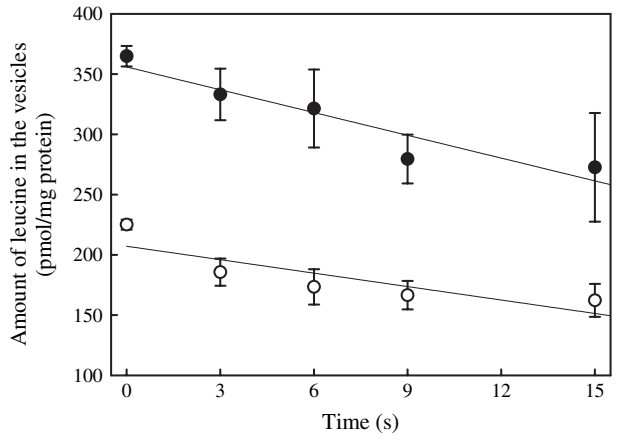

Fig. 4. Effect of Cry1Ab on leucine efflux in *O. nubilalis* BBMVs. BBMVs, resuspended in 100 mM mannitol, 10 mM HEPES-Tris pH 7.2 were preincubated for 30 min with the toxin buffer (*closed symbols*) or with 15 μg/mg of membrane proteins of Cry1Ab (*open symbols*), then incubated for 10 min in 5 mM HEPES, 25 mM Tris at pH 8.6, 100 mM mannitol, 0.1 mM ^{3}H-L-leucine 30 μCi/ml. At the end of incubation, the reaction mixture was diluted 50-fold in the stop solution and filtered after different time intervals. Time zero values were obtained by delivering the suspension directly on the filter. Values are means ± standard error of a typical experiment performed in quadruplicate.

interaction of Bt toxins with the K^+/amino acid co-transporter, we measured in *O. nubilalis* and *S. nonagrioides* BBMVs uptake of the amino acid leucine when its concentration was 0.1 mM, a condition that completely minimizes amino acid diffusion and amplifies carrier-mediated transport (Giordana et al., 1998). Kirouac et al. (2002) also suggested that the diminished amount of leucine found in *Bombyx mori* toxin-treated vesicles (Giordana et al., 1993; Parenti et al., 1995; Leonardi et al., 1997) could be due to enhanced outflux of the amino acid through the pores into the stop solution during the filtration step of the transport experiment. However, Figure 5 shows that the amount of leucine present inside toxin-treated *O. nubilalis* BBMVs at time zero, when no dilution of the incubated BBMVs had occurred, was lower than that of control. Moreover, toxin-treated vesicles should deplete more rapidly than in the control if effluxes had increased: instead, a small reduction, albeit not significant, of leucine efflux was observed in toxin-treated BBMVs. Therefore, the pores formed by the toxin (Fig. 2) do not represent a significant pathway for the passive diffusion of the amino acid because at the low concentration of leucine used (which corresponds to the K_m of the cotransporter Giordana et al., 1998), carrier-mediated transport is the major component of the amino acid influx. Only with a low amino acid concentration is it possible to reveal the Bt toxin interaction with the transporter.

In *O. nubilalis* and *S. nonagrioides* BBMVs, leucine uptake was inhibited in a dose-dependent manner by Cry1Ab and Cry1Fa both in the absence and in the presence of K^+ ions, and the inhibition was strictly related to the toxic effect *in vivo* because Cry1Da, a nontoxic Cry protein, did not impair amino acid uptake (Fig. 5). According to IC_{50} values, the leucine transport system was more sensitive to Cry1Ab in *O. nubilalis* than in *S. nonagrioides*.

The present results, in full agreement with those previously reported in *Bombyx mori* (Parenti et al., 1995; Leonardi et al., 1997), provide further evidence that Bt toxins, in addition to their binding to the identified receptors (cadherin, APN and AP), can interact with different amino acid transport systems (Figs. 5 and 6) and hinder amino acid translocation. We have shown that, at least in *Bombyx mori*, the inhibition exerted on amino acid transport in BBMVs

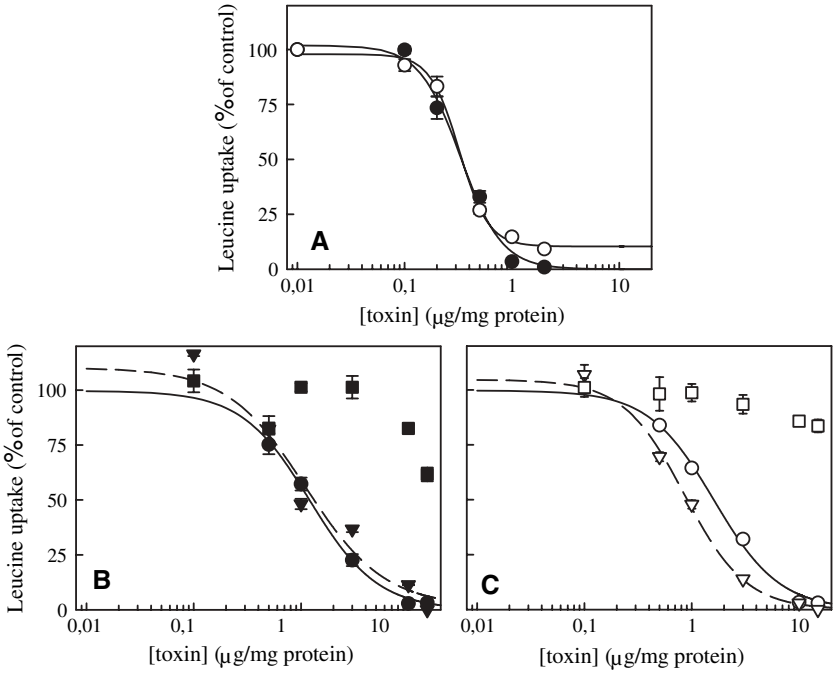

Fig. 5. Dose-response relationship of different Bt toxins on leucine uptake in *O. nubilalis* (*A*) and *S. nonagrioides* (*B, C*) BBMVs. BBMVs, resuspended in 100 mM mannitol, 10 mM HEPES-Tris pH 7.2 and preincubated for 30 min with increasing amounts of Cry1Ab (*circle*), Cry1Fa (*triangle*) and Cry1Da (*square*), were diluted 1:5 to obtain the following final composition: 2 mM HEPES, 22 mM Tris at pH 8.6, 0.1 mM ^{3}H-L-leucine 30 μCi/ml, 50 mM $(TMA)_2SO_4$ (*closed symbols*) or K_2SO_4 (*open symbols*). Data are expressed as percent of control values. Values are means ± standard error of typical experiments performed in triplicate.

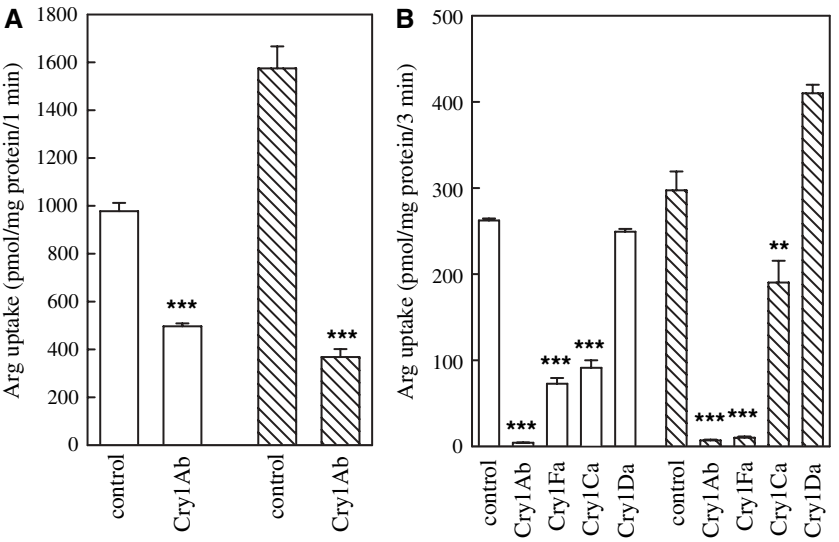

Fig. 6. Effect of different Bt toxins on arginine uptake in *O. nubilalis* (*A*) and *S. nonagrioides* (*B*) BBMVs. BBMVs, resuspended in 100 mM mannitol, 10 mM HEPES-Tris at pH 7.2 and preincubated for 30 min with 11.5 (*A*) or 15.8 μg/mg of membrane proteins of Cry1Ab, 10.1 μg/mg of membrane proteins of Cry1Fa and 16.3 μg/mg of membrane proteins of Cry1Da (*B*), were diluted 1:5 to obtain the following final composition: 2 mM HEPES, 22 mM Tris at pH 8.6, 0.05 mM ^{3}H-L-arginine 30 μCi/ml, 50 mM K_2SO_4 (*open bars*) or $(TMA)_2SO_4$ (*hatched bars*). Values are means ± standard error of typical experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$.

by different Bt toxins was always noncompetitive (Giordana et al., 1993; Leonardi et al., 1997); but it is not yet clear how this inhibition takes place. The interaction between Bt toxins and the transporter might consist of direct binding of the toxin to the transporter, possibly to the glycan moieties shown to be present in the first K^+/amino acid cotransporter cloned from a lepidopteran larval midgut (Castagna et al., 1998). Alternatively, the toxins may interact with a membrane protein functionally or spatially related to the transporter.

Whichever the molecular mechanism, the active toxins are able to impair the intestinal absorption of a number of essential amino acids, critical for lepidopteran larval growth and development. While the pore formation ability of Bt toxins and the deriving osmotic imbalance may be the principal reasons for the rapid death of susceptible larvae, the inhibition of nutrient absorption could be responsible for the decreased growth rate and fitness observed in larvae supplemented with low toxin concentrations, i.e., in those cases in which acute toxicity and mortality do not occur.

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