Aminopeptidase dependent pore formation of *Bacillus thuringiensis* Cry1Ac toxin on *Trichoplusia ni* membranes

Argelia Lorence, Alberto Darszon, Alejandra Bravo*

Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca 62250, Mor., Mexico

Received 9 July 1997

Abstract The insecticidal *Bacillus thuringiensis* Cry1Ac δ-endotoxin specifically binds to a 120 kDa aminopeptidase N (APN) in the midgut of susceptible insects such as *Manduca sexta*, *Heliothis virescens*, *Lymantria dispar* and *Plutella xylostella*. The 120 kDa APN has a glycosylphosphatidylinositol (GPI) anchor susceptible to the action of GPI-specific phospholipase C (PIPLC). Here we show that Cry1Ac pore-forming activity depends on the amount of APN present on brush border membrane vesicles (BBMV) from *Trichoplusia ni* larvae. Inhibition of APN activity with bestatin did not affect Cry1Ac pore formation, suggesting that Cry1Ac action depends on the presence of APN, but not on its enzymatic activity. *N*-Acetyl-D-galactosamine blocks the actiono of the toxin, indicating that this sugar is also directly involved in the Cry1Ac toxin-receptor interaction. Membrane potential measurements using PIPLC treated and non-treated BBMV suggest that both APN could participate as Cry1Ac receptor. The kinetic characterization of PIPLC sensitive and resistant APN indicates that they could be different isoforms. Finally, we show that in the presence of 200 mM Cs^+ intrinsic BBMV *T. ni* channel permeability is not observed, while the toxin induced permeability is not affected, allowing an accurate analysis of the effect of the Cry1Ac toxin on *T. ni* midgut membranes.

Key words: δ-Endotoxin; Aminopeptidase N; Toxin receptor; Mode of action; Ionic channel; *Bacillus thuringiensis*

1. Introduction

The crystal proteins (Cry) from *Bacillus thuringiensis* (*Bt*) are toxic to different insect larvae. After solubilization and proteolytic activation, Cry toxins bind to specific high affinity toxin binding proteins on the surface of midgut epithelial cells [1]. It is thought that after binding, Cry toxins undergo a conformational change and insert into the membrane. Oligomerization of the toxin follows, and this oligomer forms ionic channels [2] which lyse the cell and eventually lead to the death of the insect [3,4].

Cry1Aa, Cry1Ab, Cry1Ac, and Cry1C binding proteins have been identified and purified from *Manduca sexta* [5–9], *Heliothis virescens* [10], *Lymantria dispar* [11], *Plutella xylostella* [12], and *Bombyx mori* [13]. The Cry1Ab binding protein is a cadherin-like glycoprotein [7], and Cry1Aa, Cry1Ac and Cry1C binding proteins are aminopeptidase N (APN) (EC 3.4.11.2) family members [5,6,8–13]. The 120 kDa APN has a C-terminal glycosylphosphatidylinositol (GPI) anchor [14], susceptible to endogenous GPI-specific phospholipase C (PIPLC) digestion [15]. When a mixture of 120 kDa APN and 65 kDa phosphatase was incorporated into phospholipid vesicles, the toxin concentration required to induce $^{86}Rb^+$-K^+ release from the vesicles was lowered 1000-fold, suggesting their participation as functional Cry1Ac receptors [5]. Recently, it was shown that a fraction of APN resistant to PIPLC, isolated from *M. sexta* as a complex with attached lipids, reconstituted in phosphatidylcholine:phosphatidylserine (PC:PS) liposomes and fused to phosphatidylethanolamine:phosphatidylcholine:cholesterol (PE:PC:C) planar lipid bilayers could also lower the Cry1Ac and Cry1C toxin concentrations required to observe ionic channels [16].

In this study we analyzed the pore-forming activity of the most potent *Bt* toxin against *Trichoplusia ni* larvae, the Cry1Ac protein. The results indicate that this activity is directly related to the amount of APN present in the *T. ni* brush border membrane vesicles (BBMV), but not to the APN enzymatic activity. We also analyze the different types of APN activities present in the BBMV and their relation with the toxin action.

2. Materials and methods

2.1. Materials

All materials were from Sigma (St. Louis, MO), unless otherwise stated.

2.2. Preparation of the brush border membrane vesicles

BBMV from fifth instar *T. ni* larvae were prepared and analyzed as previously reported [2] except that neomycin sulfate (2.4 μg/ml) was included in the buffer (300 mM mannitol, 20 mM 2-mercaptoethanol, 5 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, 150 μg ml^{-1} pepstatin A, 100 μg ml^{-1} leupeptin, 1 μg ml^{-1} soybean trypsin inhibitor, 10 mM HEPES-HCl pH 8.0). BBMV were dialyzed overnight against 500 volumes of 150 mM KCl, 2 mM EGTA, 0.5 mM EDTA, 10 mM HEPES-HCl pH 8.0 and stored at −70°C until used.

2.3. Preparation of Cry1Ac and Cry1C toxins

Bt var. *kurstaki* strain HD-73 encoding Cry1Ac toxin was grown for 3 days at 29°C in nutrient broth sporulation medium [17]. The spores and crystals were harvested and washed with 0.01% Triton X-100, 50 mM NaCl, 50 mM Tris-HCl pH 8.5 buffer. Crystals were

*Corresponding author. Fax: (52) (73) 17 23 88.
E-mail: bravo@ibt.unam.mx

Abbreviations: APN, aminopeptidase N; BBMV, brush border membrane vesicles; CHES, (2-[*N*-cyclohexylaminol]-ethanesulfonic acid); dis-C_3-(5), 3,3′-dipropylthiodicarbocyanine; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GalNAc, *N*-acetyl-D-galactosamine; GPI, glycosylphosphatidylinositol; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); LpNA, L-leucine-*p*-nitroanilide; MeGluCl, methylglucamine chloride; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG 8000, polyethylene glycol molecular weight 8000; PIPLC, GPI-specific phospholipase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris-HCl, tris-[hydroxymethyl]aminomethane hydrochloride

PII S0014-5793(97)01014-4

purified by sucrose gradients as in [18]. Purified crystals were solubilized and activated by trypsin 1:10 w/w for 2 h and purified by anion exchange chromatography (Q-sepharose) as described [19]. The purified toxin was concentrated in dialysis bags (Spectra/Por cutoff 12–14 kDa; Fisher Scientific, Pittsburgh, PA) covered with PEG 8000, dialyzed against 1000 volumes of 100 mM methylglucamine chloride (MeGluCl), 10 mM CHES pH 9.5 and stored at 4°C until used. Toxin purity was examined by SDS-PAGE [20] staining with Coomassie brilliant blue or silver (Bio-Rad, Richmond, CA). Western blot analyses [21] were done using a polyclonal anti-Cry1Ab [22] and a goat anti-rabbit immunoglobulin coupled to peroxidase as secondary antibody. Protein was measured by a protein dye method [23] using bovine serum albumin (New England Bio-Labs, Beverly, MA) as standard.

Escherichia coli recombinant strain containing the *cry*1C gene, derived from *Bt* var. *entomocidus* was kindly supplied by Dr. M. Peferoen. The inclusion bodies were solubilized, activated and purified as described [24].

2.4. Bioassay

Insect toxicity was assayed with first instar *T. ni* larvae by the diet surface contamination procedure as described [25]. LD_{50} value is reported as ng of protein applied to artificial diet (cm^2).

2.5. Fluorescence measurements

Membrane potential was monitored with the positively charged fluorescent dye, 3,3′-dipropylthiodicarbocyanine (dis-C_3-(5) (Molecular Probes, Eugene, OR; 1.75 μM final, 1.4 mM stock in DMSO) as previously described [2]. Ba^{2+} and Cs^+ were from Merck (Darmstadt).

2.6. Aminopeptidase assays

APN activity was assayed at 25°C in 250 mM NaCl, 200 mM Tris-HCl pH 8.0 buffer using 1 mM L-leucine-*p*-nitroanilide (LpNA) as substrate. The initial rate at 405 nm (Ultrospec II spectrophotometer; Pharmacia LKB) was used to calculate specific enzymatic activity. The absorption coefficient of *p*-nitroanilide used was 9.9×10^{-3} mol l^{-1} [26]. One unit of specific APN activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of LpNA min^{-1} mg $protein^{-1}$ at 25°C.

The initial velocity rate (v) of both APN activities (sensitive and resistant to PIPLC) was studied as a function of substrate concentration (S, 0.05–5 mM LpNA). The Michaelis constant, K_m, was calculated from the double reciprocal plot and, the maximum velocity, V_{max}, from an Eadie-Hofstee plot as described [27].

The pH dependence of APN catalysis was determined measuring the maximum velocity as a function of pH (5.5–9.5). The assay conditions were as described above, with 5 mM of LpNA.

2.7. Phosphatidylinositol specific phospholipase C digestions

T. ni brush border membrane vesicles (100 μg) were suspended in 500 μl buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.2). One unit of PIPLC from *Bacillus cereus* (Boehringer, Mannheim) was added to BBMV suspension and incubated for 90 min at 30°C. The vesicles were washed twice with PBS (14 000 rpm, 20 min) and suspended in 150 mM KCl, 2 mM EGTA, 0.5 mM EDTA, 10 mM HEPES-HCl pH 8.0.

3. Results and discussion

3.1. The resting membrane potential of T. ni BBMV

The resting membrane potential of *T. ni* BBMV loaded with 150 mM KCl, and assayed with the membrane potential sensitive fluorescent dye dis-C_3-(5), is close to the K^+ equilibrium potential ($E_{K+} \approx -92$ mV) calculated by the Nernst equation, in the presence of either 140 mM NaCl or 140 mM MeGluCl (Table 1). These data suggest that at rest, K^+ permeability contributes significantly to the membrane potential in *T. ni* midgut BBMV. K^+ addition experiments indicate that vesicles have intrinsic channels permeable to K^+ (Fig. 1A, control trace). This is shown quantitatively as the slope of the control traces (Table 2).

3.2. Effect of Cry1Ac

Toxicity assays showed that the Cry1Ac toxin preparation was highly active against *T. ni* larvae ($LD_{50} = 15$ ng/cm^2), whereas the denatured Cry1Ac form or Cry1C toxin showed no activity.

Addition of 50 nM Cry1Ac to BBMV suspended in 140 mM MeGluCl produced a fast hyperpolarization (Fig. 1A). After toxin exposure, the vesicles increased their response to KCl additions, when compared to controls to which the same amount of buffer, 200 nM of boiled Cry1Ac toxin or 200 nM of the non-toxic Cry1C protein was added (Fig. 1A, Table 2). The hyperpolarization (-14 ± 4 mV, $n=7$) and the higher sensitivity to external K^+, seen as a higher slope of the toxin trace compared to the control trace, are consistent with a toxin-induced increase in K^+ permeability probably due to ion channel opening.

3.3. Involvement of APN on the effect of Cry1Ac toxin

It has been reported that treating BBMV with PIPLC resulted in 30–40% APN release in *Bombyx mori* [28,29] and 59% in *Manduca sexta* [14]. To explore the possible involvement of APN in the pore formation of Cry1Ac toxin, *T. ni* BBMV were preincubated with PIPLC. After this treatment 25% ($n=3$) of the total APN activity was left in the BBMV. A second PIPLC treatment did not cause further APN release. When 50 nM Cry1Ac was added to PIPLC treated BBMV, a toxin effect on membrane permeability was not observed ($m_{toxin} = 0.40$, $n=3$ vs. $m_{control} = 0.40$, $n=7$, Table 2). This response suggests that in *T. ni* midgut membranes, the APN-GPI anchored could play a major role as Cry1Ac receptor. A similar observation was reported in *Lymantria dispar*, where treatment of midgut membranes with PIPLC substantially re-

Table 1
The resting membrane potential of *T. ni* BBMV

Potential	Batch			
	A	B	C	D
E_{K^+}	−94.4 mV	−92.3 mV	−84.5 mV	−92.5 mV
E_r	−87 ± 4 mV	−94 ± 5 mV	−76 ± 1 mV	−70 ± 2 mV
140 mM MeGluCl, 10 mM HEPES pH 8.0	$n=20$	$n=18$	$n=6$	$n=8$
E_r	−87 ± 3 mV	N.D.	−78 ± 2 mV	N.D.
140 mM NaCl, 10 mM HEPES pH 8.0	$n=7$		$n=10$	

The response of dis-C_3-(5) was calibrated and the resting membrane potential (E_r) calculated as described [2]. The values show the average ± S.D.; n = number of experiments. $E_{K^+} = K^+$ equilibrium potential calculated with the Nernst equation. N.D. = not determined.

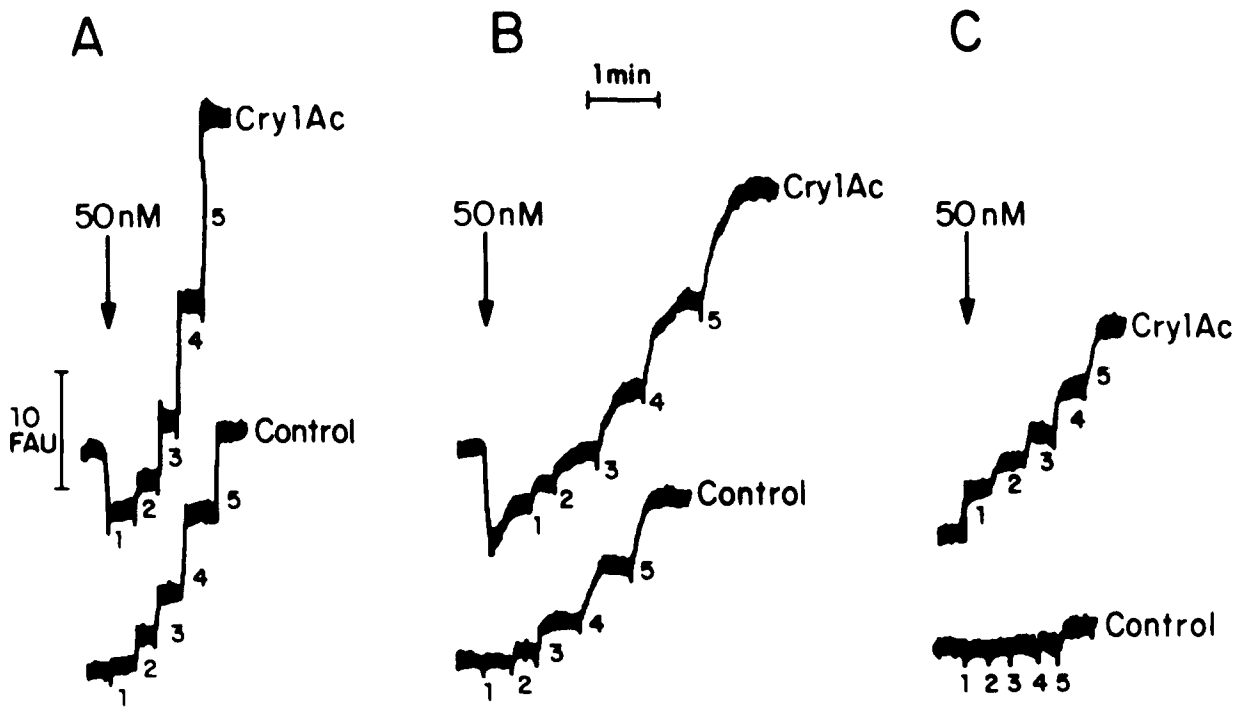

Fig. 1. Effect of Cry1Ac toxin on the membrane potential in *T. ni* midgut BBMV. Membrane potentials of BBMV (20 μg) loaded with (mM): 150 KCl, 2 EGTA, 0.5 EDTA, 10 HEPES-HCl pH 8.0 and suspended in 140 mM MeGluCl, 10 mM HEPES-HCl pH 8.0, were recorded as described [2]. A downward deflection indicates a membrane potential hyperpolarization. The arrow at the top of the traces corresponds to the time of toxin or buffer A (100 mM MeGluCl, 10 mM CHES pH 9.5) addition. Final K^+ concentrations were (mM): 1 = 4; 2 = 12; 3 = 27; 4 = 56; 5 = 114. FAU, fluorescence arbitrary units. The calibration bar is on the left of the figure and the time scale is in the middle. As previously observed for Cry1C and Cry1D toxins, Cry1Ac interacts with the dye in the absence of BBMV, producing a small fluorescence decrease (data not shown) which was determined for each ionic condition and subtracted as described [2]. A: Toxin response on *T. ni* BBMV. Control trace was obtained with addition of buffer A, 200 nM Cry1C toxin or 200 nM boiled Cry1Ac toxin. The toxin effect on BBMV preincubated with 250 μM GalNAc gave a trace similar to the control trace. B: Cry1Ac effect on BBMV preincubated with 10 mM bestatin. In the control, buffer A was added instead of toxin. C: Effect of Cry1Ac on BBMV preincubated with 200 mM Cs^+. In the control, buffer A was added instead of toxin. All experiments were repeated at least 3 times on two different vesicle batches.

duced the inhibition of short circuit current produced by Cry1Ac toxin [30].

In order to analyze if the PIPLC resistant APN could also function as receptor in *T. ni*, the Cry1Ac toxin concentration was increased in the assay with PIPLC treated BBMV. When the Cry1Ac concentration was increased four times (From 0.2 to 0.8 pmol toxin/μg BBMV protein) the pore-forming activity was recovered. This is shown quantitatively as the slope of the traces: $m_{1\times\text{toxin}} = 0.64$, $n = 3$; $m_{2\times\text{toxin}} = 0.42$, $n = 3$ and $m_{\text{control}} = 0.40$, $n = 7$. We excluded the possibility that this effect was due to a direct action of Cry1Ac on the bilayer, since in liposomes, where specific receptors are absent, 50–200-fold higher toxin concentrations are needed to see a response [5,31–33]. These data indicate that the Cry1Ac toxin induced K^+ permeability effect can be observed even in the absence of GPI anchored APN, and suggest that other proteins resistant to PIPLC treatment could function as Cry1Ac receptors. In accordance with this finding it was shown that PIPLC resistant APN from *M. sexta* function as Cry1Ac receptor when it was reconstituted and fused to planar lipid bilayers [16].

It was observed that BBMV with higher APN activity apparently display a higher Cry1Ac induced K^+ permeability

Table 2
BBMV biochemical characterization and their response to Cry1Ac toxin

	Batch		
	A	B	A-PIPLC
Neomycin sulfate	+	–	+
% BBMV facing out[a]	99	96	99
BBMV APN specific activity (U min^{-1} mg $protein^{-1}$)	51.8 ± 1.0	32.0 ± 0.4	13.0 ± 1.0
APN specific activity enrichment BBMV/homogenate	12	4	3
m_{control}[b]	0.40 ($n = 10$)	0.37 ($n = 5$)	0.40 ($n = 3$)
$m_{\text{toxin(50nM)}}$[c]	0.62 ($n = 10$)	0.46 ($n = 5$)	0.40 ($n = 3$)
% K^+ permeability increase	55	22	0

[a]Calculated from the ratio of alkaline phosphatase specific activity in the presence and absence of 0.1% Triton X-100.
[b]Slope of the traces obtained when buffer or boiled Cry1Ac was added instead of active toxin and after successive KCl additions.
[c]Slope of the traces obtained when BBMV were treated with 50 nM Cry1Ac and successive KCl additions. Values are averages; all standard deviations were less than 5%; n = number of experiments. A-PIPLC, vesicles from batch A treated with phosphatidylinositol-specific phospholipase C.

increase (Table 2). These data indicate that the toxin effect depends on the quantity of APN.

Some authors have shown that Cry1Ac toxin has no effect on the activity of purified APN from *M. sexta* [5,8]. Experiments in the fluorescence system using preincubated BBMV with the APN inhibitor, bestatin, were done in order to analyze if an active APN is necessary for Cry1Ac activity. Bestatin inhibits 100% of APN, without affecting the Cry1Ac induced K^+ permeability increase in *T. ni* BBMV (Fig. 1B). The hyperpolarization value obtained in this condition (-16 ± 2 mV, $n = 3$) is within the range of the normal response (see Section 3.2). These data indicate that the presence of APN is necessary for Cry1Ac pore formation, but not its enzymatic activity.

3.4. *N*-Acetyl-D-galactosamine inhibits Cry1Ac pore-forming activity

It has been reported that *N*-acetyl-D-galactosamine (GalNAc) blocks the binding of Cry1Ac to *M. sexta* and *P. xylostella* BBMV [34,12]. Also, it has been shown that GalNAc blocks the binding of Cry1Ac to the purified APN [6,19]. Fluorescence measurements performed preincubating the BBMV with different GalNAc concentrations showed that 250 μM GalNAc was able to inhibit the Cry1Ac pore-forming activity ($m_{toxin} = 0.44$, $n = 3$ vs. $m_{control} = 0.40$, $n = 3$). These results suggest that also in *T. ni* larvae the Cry1Ac receptor is a GalNAc bearing glycoconjugate, and that GalNAc is directly involved in the binding of Cry1Ac toxin to its receptor, as has been reported for *M. sexta* and *P. xylostella* larvae.

3.5. APN characterization

In order to analyze the possibility that both APN (sensitive and resistant to PIPLC) are different enzymes, their kinetic constants, K_m and V_{max}, were determined by performing experiments where the initial rate, v, was studied as a function of L-leucine-*p*-nitroanilide concentration. It was found that PIPLC sensitive APN displays a $K_m = 0.052 \pm 0.003$ mM and $V_{max} = 18.2 \pm 0.3$ μmol min^{-1} mg $protein^{-1}$, while the PIPLC resistant APN has a $K_m = 0.203 \pm 0.005$ mM and $V_{max} = 21.3 \pm 0.8$ μmol min^{-1} mg $protein^{-1}$.

The profile of V_{max}/pH curves showed that the optimal pH of both APN was close to 7.2. Thus, the K_m values suggest that both APN, sensitive and resistant to PIPLC, could be different isoforms. However, the V_{max} and optimal pH were similar. We cannot exclude the possibility that differences in K_m could be due to the influence of the lipid environment on GPI anchored APN activity. The existence of at least two different APN, putative receptors of Cry1Ac and Cry1C toxins, in *M. sexta* has been reported [5,6,9]. Further experiments with purified APN are needed to clarify if PIPLC sensitive and resistant APN could be different isoforms on the *T. ni* midgut.

Table 3
Relative ion permeability

Cation	BBMV intrinsic permeability (m_{X^+}/m_{K^+})	Cry1Ac induced permeability (m_{X^+}/m_{K^+})[a]
K^+	1.00	1.00
Na^+	0.97	0.99
Li^+	2.04	2.60
Cs^+	0.19	0.96
Rb^+	1.12	0.71

Ion selectivity was estimated from the ratio of the slope of the fluorescence rises which result from additions of increasing concentrations of a certain cation vs. than a K^+. m_{X^+}, slope of the fluorescence changes induced by different XCl additions to BBMV; X^+, different monovalent cations; m_{K^+}, slope for KCl additions.
[a]Relative permeability of Cry1Ac induced pores measured in the presence of 200 mM Cs^+, which blocks the intrinsic permeability. Standard deviations were less than 5%, $n = 5$ in two different vesicle batches.

3.6. Cs^+ effect and ion selectivity

Intrinsic channels of *Spodoptera frugiperda* BBMV are sensitive to Cs^+ [2]. Table 3 shows that Cs^+ permeability of the *T. ni* intrinsic channels is 5-fold lower than K^+ permeability. Fig. 1C illustrates that K^+ permeability of intrinsic *T. ni* channels is extremely low in the presence of 200 mM Cs^+, when compared to permeability of the Cry1Ac induced pores. The slope of the toxin trace in the presence of Cs^+ ($m_{toxin-Cs^+} = 0.25$, $n = 5$) is equivalent to the slope of the toxin effect without Cs^+ ($m_{toxin} - m_{control} = 0.22$, $n = 7$), indicating that this condition could be useful for analysis of the Cry1Ac pore-forming activity. The depolarization induced by Cry1Ac in this condition is due to the electrogenic uptake of Cs^+, and suggests that the toxin induced permeability pathway is not very selective among monovalent cations. Therefore the relative selectivity X^+/K^+ of the Cry1Ac pores was analyzed in the presence of 200 mM Cs^+ (Table 3). The selectivity series was: $Li^+ > K^+ \approx Na^+ \approx Cs^+ > Rb^+$. Cry1Ac induced pores are poorly selective among different monovalent cations. A similar behavior was previously reported for Cry1Aa and Cry1D induced pores in *B. mori* and *S. frugiperda* BBMV, respectively [35,2].

The results obtained in this study suggest that APN could participate as Cry1Ac receptor on *T. ni* midgut, but its enzymatic activity is not necessary to get the toxin-induced permeability. Finally, we found a condition to assay separately the Cry1Ac effect without interference of the intrinsic channels. The utilization of Cs^+ will be a very useful tool to perform more precise studies both in the fluorescence system and in the planar lipid bilayer system.

Acknowledgements: We thank E. Aranda and L. Lina for bioassays; J. Sánchez and R. Campos for technical assistance and Dr. M. Peferoen for providing *E. coli* strain. This work was supported in part by DGAPA, CONACyT and PADEP. A.L. acknowledges CONACyT for a Ph.D. fellowship.

References

[1] Hofman, C., Vanderbruggen, H., Höfte, H., Van Rie, J., Jansens, S. and Van Mellaert, H. (1988) Proc. Natl. Acad. Sci. USA 85, 7844–7848.
[2] Lorence, A., Darszon, A., Díaz, C., Liévano, A., Quintero, R. and Bravo, A. (1995) FEBS Lett. 360, 217–222.
[3] Knowles, B.H. (1994) Adv. Insect Physiol. 24, 275–308.
[4] Pietrantonio, P.V., and Gill, S.S. (1996) in: M.J. Lehane and P.F. Billingsley (Eds.), Biology of the Insect Midgut. Chapman and Hall, London, pp. 345–372.
[5] Sangadala, S., Walters, F.S., English, L.H. and Adang, M.J. (1994) J. Biol. Chem. 269, 10088–10092.
[6] Knight, P.J.K., Crickmore, N. and Ellar, D.J. (1994) Mol. Microbiol. 11, 429–436.
[7] Vadlamudi, R.K., Weber, E., Ji, I., Ji, T.H. and Bulla Jr., L.A. (1995) J. Biol. Chem. 270, 5490–5494.
[8] Knight, P.J.K., Knowles, B.H. and Ellar, D.J. (1995) J. Biol. Chem. 270, 17765–17770.
[9] Luo, K., Lu, Y.J. and Adang, M.J. (1996) Insect Biochem. Mol. Biol. 26, 783–791.

[10] Gill, S.S., Cowles, E.A. and Francis, V. (1995) J. Biol. Chem. 270, 27277–27282.
[11] Valaitis, A.P., Lee, M.K., Rajamohan, F. and Dean, D. (1995) Insect Biochem. Mol. Biol. 25, 1143–1151.
[12] Luo, K., Tabashnik, B.E. and Adang, M.J. (1997) Appl. Environ. Microbiol. 63, 1024–1027.
[13] Yaoi, K., Kadotani, T., Kuwana, H., Shinkawa, A., Takahashi, T., Iwahana, H. and Sato, R. (1997) Eur. J. Biochem. 246, 652–657.
[14] Garczynski, S.F. and Adang, M.J. (1995) Insect Biochem. Mol. Biol. 25, 409–415.
[15] Lu, Y.J. and Adang, M.J. (1996) Insect Biochem. Mol. Biol. 26, 33–40.
[16] Adang, M.J., Garczynski, S., Luo, K., Sangadala, S., Lu, Y.-J., Masson, L., Schwartz, J.L. and Tabashnik, B. (1996) Abstracts, SIP 29th Annual Meeting and IIIrd International Colloquium on *Bacillus thuringiensis*, 1–6 September, Córdoba, Spain, p. 1.
[17] Lereclus, D., Agaisse, H., Gominet, M. and Chaufaux, J. (1995) Bio/Technology 13, 67–71.
[18] Thomas, W.E. and Ellar, D.J. (1983) J. Cell Sci. 60, 181–197.
[19] Masson, L., Lu, Y.-J., Mazza, A., Brousseau, R. and Adang, M.J. (1995) J. Biol. Chem. 270, 20309–20315.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Towbin, H.T., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[22] Meza, R., Nuñez, M.A., Sánchez, J. and Bravo, A. (1996) FEMS Microbiol. 145, 333–339.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Höfte, H., Grave, H., Seurinck, J., Jansens, S., Mahillon, J., Ampe, C., Vanderckhove, J., Vanderbruggen, H., Van Montagu, M., Zabeau, M. and Vaek, M. (1986) Eur. J. Biochem. 161, 272–280.
[25] Beegle, C. (1990) in: L.A. Hickle and W.L. Fitch (Eds.), Analytical Chemistry of *Bacillus thuringiensis*. American Chemical Society, Washington, DC, pp. 14–21.
[26] Hafkenscheid, J.C.M. (1984) in: J. Bergemayer (Ed.), Methods of Enzymatic Analysis, Vol. V, Enzymes 3; Peptidases, Proteinases and their Inhibitors. Verlag Chemie, Weinheim, pp. 2–34.
[27] Hammes, G.G. (1982) Enzyme Catalysis and Regulation. Academic Press, Orlando, FL.
[28] Takesue, S., Yokota, K., Miyajima, S., Taguchi, R., Ikezawa, H. and Takesue, Y. (1992) Comp. Biochem. Physiol. 102B, 7–11.
[29] Tomita, M., Obara, H., Takesue, Y., Tamura, H.O., Miyajima, S., Taguchi, R. and Ikezawa, H. (1994) Int. J. Biochem. 26, 977–986.
[30] Lee, M.K., You, T.H., Young, B.A., Cotrill, J.A., Valaitis, A.P. and Dean, D.H. (1996) Appl. Environ. Microbiol. 62, 2845–2849.
[31] Yunovitz, H. and Yawetz, A. (1988) FEBS Lett. 230, 105–108.
[32] Haider, M.Z. and Ellar, D.J. (1989) Biochim. Biophys. Acta 978, 216–222.
[33] English, L.H., Readdy, T.L. and Bastian, A.E. (1991) Insect Biochem. 21, 177–184.
[34] Knowles, B.H., Knight, P.J.K. and Ellar, D.J. (1991) Proc. R. Soc. Lond. B 245, 31–35.
[35] Uemura, T., Ihara, H., Wadano, A. and Himeno, M. (1992) Biosci. Biotech. Biochem. 56, 1976 1979.