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Biochimica et Biophysica Acta 1561 (2002) 171–179

BIOCHIMICA ET BIOPHYSICA ACTA
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Amino acid and divalent ion permeability of the pores formed by the *Bacillus thuringiensis* toxins Cry1Aa and Cry1Ac in insect midgut brush border membrane vesicles

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Received 6 September 2001; received in revised form 5 December 2001; accepted 13 December 2001

Abstract

The pores formed by *Bacillus thuringiensis* insecticidal toxins have been shown to allow the diffusion of a variety of monovalent cations and anions and neutral solutes. To further characterize their ion selectivity, membrane permeability induced by Cry1Aa and Cry1Ac to amino acids (Asp, Glu, Ser, Leu, His, Lys and Arg) and to divalent cations (Mg^{2+}, Ca^{2+} and Ba^{2+}) and anions (SO_4^{2-} and phosphate) was analyzed at pH 7.5 and 10.5 with midgut brush border membrane vesicles isolated from *Manduca sexta* and an osmotic swelling assay. Shifting pH from 7.5 to 10.5 increases the proportion of the more negatively charged species of amino acids and phosphate ions. All amino acids diffused well across the toxin-induced pores, but, except for aspartate and glutamate, amino acid permeability was lower at the higher pH. In the presence of either toxin, membrane permeability was higher for the chloride salts of divalent cations than for the potassium salts of divalent anions. These results clearly indicate that the pores are cation-selective.

Keywords: Insecticidal toxin; Ion selectivity; Membrane permeability; Brush border membrane vesicle; *Bacillus thuringiensis*; *Manduca sexta*

1. Introduction

Bacillus thuringiensis insecticidal toxins act specifically at the level of the luminal brush border membrane of midgut epithelial cells where they bind to specific receptors and form lethal transmembrane permeability channels [1,2]. Several putative toxin receptors have been identified from a variety of lepidopteran species. Among these, various toxin-binding glycosylphosphatidylinositol-anchored aminopeptidase N isoforms from *Manduca sexta* [3–8], *Heliothis virescens* [9,10], *Lymantria dispar* [11,12], *Bombyx mori* [13–15] and *Plutella xylostella* [7,16] and cadherin-like proteins from *M. sexta* [17,18] and *B. mori* [19] have been purified, cloned and sequenced. More recently, an anionic glycoconjugate that binds Cry1Aa, Cry1Ab and Cry1Ac was isolated from the brush border membrane of *L. dispar* [20].

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0005-2736/02/$ – see front matter
PII: S0005-2736(02)00342-5

Further evidence that some of these proteins act as toxin receptors was provided by experiments in which the 120 kDa aminopeptidase from *M. sexta* [4] and the 170 kDa aminopeptidase from *H. virescens* [10] were shown to enhance Cry1Ac-induced $^{86}Rb^{+}$ efflux from liposomes. The former protein was also shown to stimulate ion channel formation by this toxin when reconstituted in planar lipid bilayers [21]. In addition, Sf9 cells expressing the 175 kDa cadherin-like protein from *B. mori* acquired sensitivity to Cry1Aa [22]. Finally, detailed analyses of toxin-mediated inhibition of amino acid transport into insect midgut brush border membrane vesicles has provided evidence suggesting that certain potassium-dependent amino acid symporters could also act as *B. thuringiensis* toxin receptors [23–26].

The pores formed by *B. thuringiensis* toxins in the plasma membrane of cultured insect cells [27–29] and in brush border membrane vesicles [30–34] allow the diffusion of a variety of monovalent cations and anions and relatively large neutral solutes such as sucrose, raffinose, alanine and polyethylene glycols. On the other hand, the formation of cation-selective pores has been clearly demonstrated in planar lipid bilayer experiments, both in the presence [21,35] and absence [21,36–38] of reconstituted membrane receptors, although ion selectivity of the pores can be modified by the incorporation of brush border membranes in the lipid bilayer [39]. The fact that anion-selective pores have also been observed with Cry1C at pH 6.0 [37] has suggested that the apparent lack of selectivity in cultured cells and brush border membrane vesicles could have resulted from the use of a near neutral pH [1,2], appropriate for the culture of insect cells, but much lower than what is normally found in the lepidopteran midgut lumen [40,41]. Nevertheless, experiments conducted at pH 7.5 with a membrane potential-sensitive fluorescent probe also suggested the formation of cation-selective pores in brush border membrane vesicles [35,42]. In addition, the pore-forming properties of Cry1Aa and Cry1Ac, in contrast with those of Cry1C, were recently found to be remarkably similar at pH 7.5 and 10.5 [33,34].

In the present study, we took advantage of the possibility of modifying the charge of amino acids, without significantly altering their size nor the properties of the toxin channels, by changes in pH to investigate the ion selectivity of the pores formed by Cry1Aa and Cry1Ac in brush border membrane vesicles isolated from the midgut of *M. sexta*. Toxin-induced permeability to salts of divalent cations and anions was also examined at pH 7.5 and 10.5. The results demonstrate that the pores are cation-selective.

2. Materials and methods

2.1. Preparation of toxins and brush border membrane vesicles

Cry1Aa and Cry1Ac toxins were produced as insoluble inclusions in *Escherichia coli*, solubilized, trypsin-activated and purified by fast protein liquid chromatography as described earlier [43]. Whole midguts from 5th-instar *M. sexta* larvae (Carolina Biological Supply Co., Burlington, NC) were freed of attached Malpighian tubules, cut longitudinally to remove the peritrophic membrane and gut contents and rinsed thoroughly with ice-cold 300 mM sucrose, 17 mM Tris–HCl (pH 7.5) and 5 mM EGTA. Brush border membrane vesicles were prepared with a magnesium precipitation and differential centrifugation technique [44].

2.2. Light-scattering assay

Membrane permeability was analyzed using an osmotic swelling technique based on light-scattering measurements [31]. In preparation for the experiments, vesicles were resuspended to about 90% of the desired final volume in 10 mM HEPES–KOH (pH 7.5) or CAPS–KOH (pH 10.5) and allowed to equilibrate overnight at 4°C. At least 1 h before the start of the experiments, they were diluted to a final concentration of 0.4 mg of membrane protein/ml with the appropriate buffer and enough bovine serum albumin to achieve a final concentration of 1 mg/ml. Vesicles were then incubated with the specified concentration of toxin for 60 min at 23°C and rapidly mixed with an equal volume of a solution composed of 10 mM HEPES–KOH (pH 7.5) or CAPS–KOH (pH 10.5), 1 mg/ml bovine serum albumin and 100 mM L-aspartic acid, L-glutamic acid, L-serine, L-leucine, L-histidine, L-lysine, L-arginine,

$MgCl_2$, $CaCl_2$, $BaCl_2$, K_2SO_4 or potassium phosphate using a stopped-flow apparatus (Hi-Tech Scientific Co., Salisbury, UK). Because Mg^{2+} and Ba^{2+} form insoluble hydroxides at high pH, $MgCl_2$ and $BaCl_2$ were only tested at pH 7.5. Scattered light intensity was monitored at a wavelength of 450 nm and a frequency of 10 Hz in a PTI spectrofluorometer (Photon Technology International, South Brunswick, NJ) with a photomultiplier tube located at 90° from the incident light beam. Percent volume recovery was defined as $100(1-I_t)$, where I_t is the scattered light intensity measured at a given time t relative to the maximum attained in the absence of toxin. Unless specified otherwise, data are mean ± S.E.M. of three experiments, each performed in quintuplicate with a different vesicle preparation. The proportion of the different amino acid and phosphate species at pH 7.5 and 10.5 were calculated from published pK_a values with the Henderson–Hasselbalch equation [45].

3. Results and discussion

3.1. Membrane permeability to amino acids

Vesicles preincubated with Cry1Ac for 60 min were subjected to a hypertonic shock by rapid mixing with an equal volume of 100 mM aspartate or leucine at pH 7.5 (Fig. 1). During the first second, water exit from the vesicles and vesicle shrinking were evidenced by a rapid increase in scattered light intensity. Subsequently, the amino acids, along with water, diffused through the membrane and caused the vesicles to swell as indicated by a decrease in scattered light intensity. The rate and magnitude of vesicle swelling, which depend on substrate permeability, increased with toxin concentration. The initial swelling rates were considerably higher for leucine than for aspartate. In fact, leucine diffused so quickly through the pores that vesicles never shrank to the same extent as in control experiments done without toxin. Permeability to aspartate was nevertheless substantial since scattered light intensity reached comparable levels for both amino acids after approximately 30 s.

Membrane permeability, measured as percent volume recovery after 3 s, to aspartate, glutamate, serine, leucine, histidine, lysine and arginine was ana-

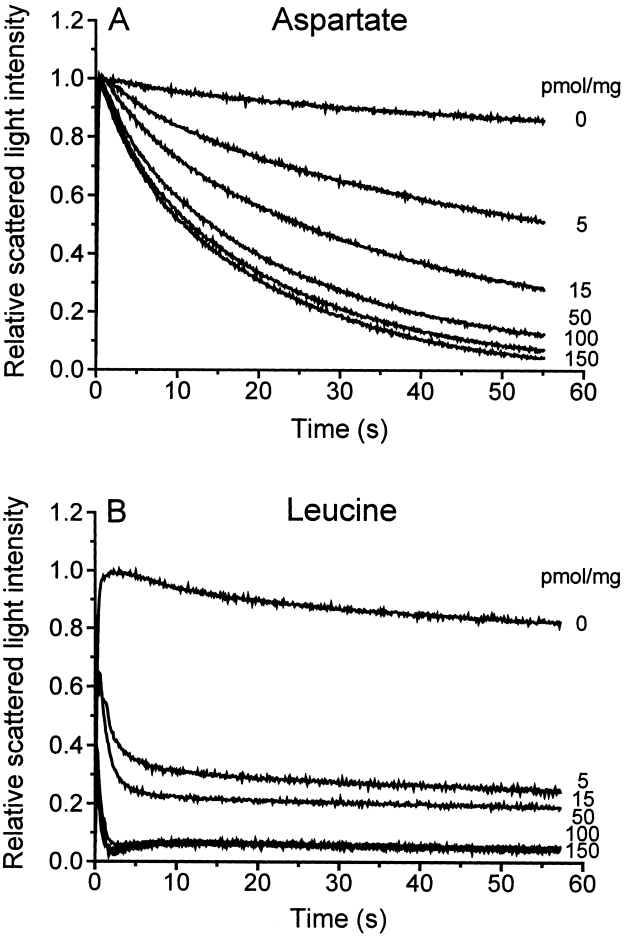

Fig. 1. Osmotic swelling of *M. sexta* midgut brush border membrane vesicles induced by Cry1Ac in the presence of aspartate and leucine. Vesicles preincubated for 60 min with the indicated concentrations of toxin (in pmol toxin/mg membrane protein) in 10 mM HEPES–KOH (pH 7.5) and 1 mg/ml bovine serum albumin were rapidly mixed, using a stopped-flow apparatus, with an equal volume of 100 mM L-aspartic acid (A) or L-leucine (B), 10 mM HEPES–KOH (pH 7.5) and 1 mg/ml bovine serum albumin. Scattered light intensity was monitored at an angle of 90° in a PTI spectrofluorometer. Each trace represents the average of five experiments.

lyzed at pH 7.5 and 10.5 as a function of toxin concentration (Fig. 2). All tested amino acids diffused readily across the pores formed by Cry1Aa and Cry1Ac at both pH values. Toxin-induced permeability was nevertheless much smaller for aspartate and glutamate than for the other amino acids. Osmotic swelling rates were significantly smaller for glutamate (Fig. 2B) than for aspartate (Fig. 2A), in agreement with the presence of an extra methylene group on the side group of glutamate. Nevertheless,

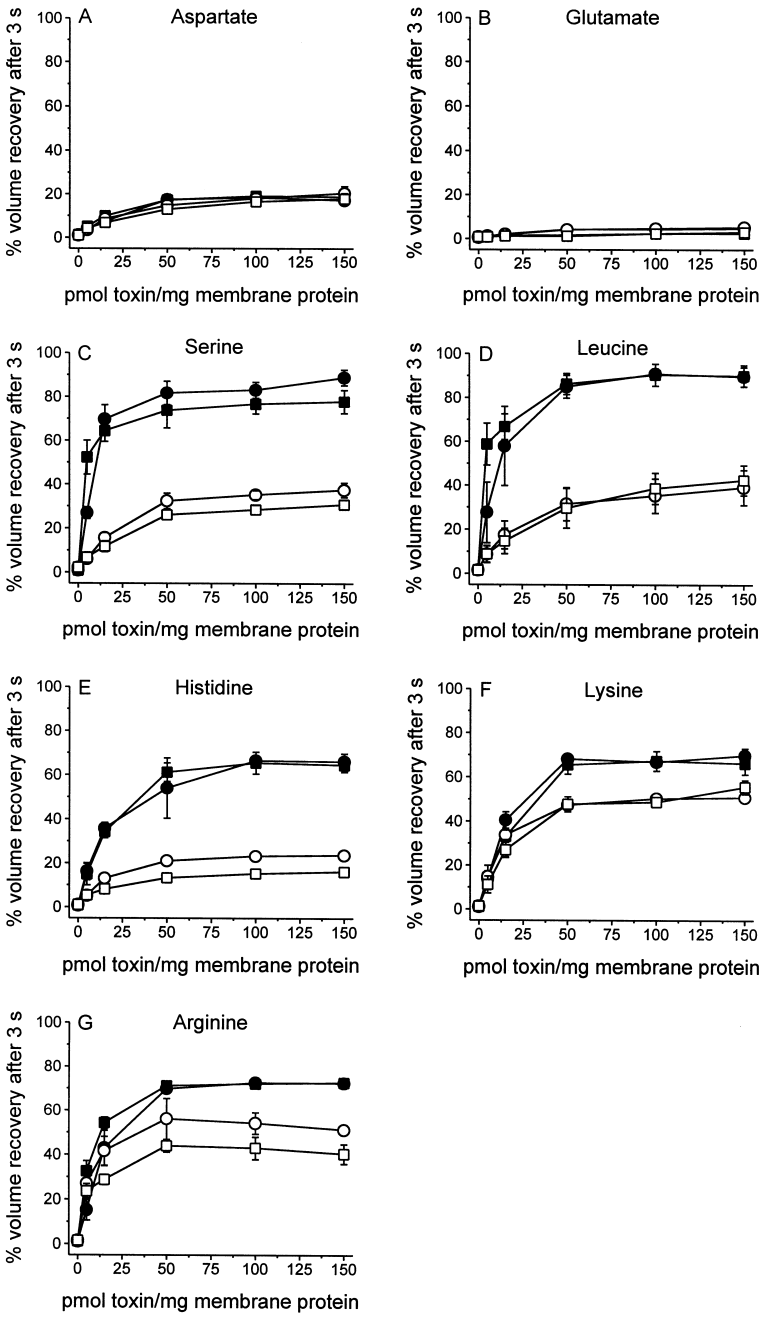

Fig. 2. Permeability of the pores formed by Cry1Aa and Cry1Ac to amino acids. Vesicles were preincubated for 60 min with the indicated concentrations of Cry1Aa (○, ●) or Cry1Ac (□, ■) in 10 mM HEPES–KOH (pH 7.5) (●, ■) or CAPS–KOH (pH 10.5) (○, □) and 1 mg/ml bovine serum albumin and mixed with a solution containing the same buffer supplemented with 100 mM L-aspartic acid (A), L-glutamic acid (B), L-serine (C), L-leucine (D), L-histidine (E), L-lysine (F) or L-arginine (G). Scattered light intensity was monitored as illustrated in Fig. 1 and percent volume recovery after 3 s was calculated as described in Section 2.

both amino acids diffused well across the toxin channels, percent volume recovery reaching values of about 73–84% for aspartate and 27–48% for glutamate after 30 s, at the higher toxin concentrations (100–150 pmol toxin/mg membrane protein), for both toxins and pH values. Also in contrast with aspartate and glutamate, the other five amino acids diffused significantly less rapidly at pH 10.5 than at

pH 7.5. Among those tested, aspartate and glutamate are the only amino acids which bear a net negative charge at pH 7.5. The second net negative charge carried by about 76% of aspartate molecules and 78% of glutamate molecules at pH 10.5 did not further decrease their rate of diffusion (Fig. 2A,B). Serine, leucine and histidine are neutral at pH 7.5, but at least 85% of their molecules bear a net negative charge at pH 10.5. The introduction of this negative charge caused a strong decrease in the rate of diffusion of these amino acids (Fig. 2C–E). Lysine and arginine bear a net positive charge at pH 7.5. At the higher pH, lysine become either neutral or negatively charged, in the proportion of about 2/3 and 1/3, and arginine becomes neutral. Thus, removal of a net positive charge also decreased the rate of diffusion of these amino acids (Fig. 2F,G), but its effect was less pronounced than that of adding a negative charge on a neutral molecule such as the previous three amino acids. The above-mentioned light-scattering results thus correlate well with the charge carried by the different amino acids at each pH value and are therefore consistent with the pores formed by the toxins being cation-selective.

Earlier studies dealing with the amino acid permeability of the pores formed by *B. thuringiensis* toxins have yielded somewhat conflicting results. While alanine was concluded to be permeant [30,46], diffusion of histidine [24] and leucine [25] across toxin pores could not be demonstrated. Our finding that the latter two amino acids diffuse readily across the membrane in the presence of Cry1Aa and Cry1Ac was probably facilitated by the fact that much stronger amino acid gradients were used in the light-scattering experiments than in previous measurements of radiolabeled amino acid efflux from vesicles or liposomes.

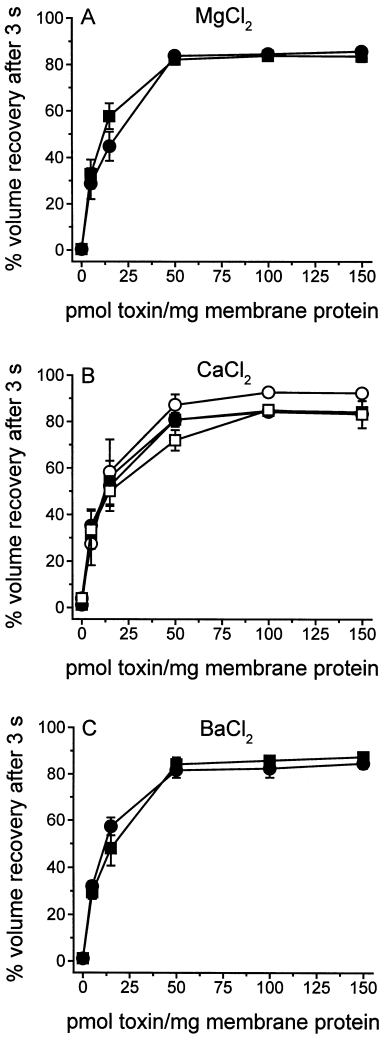

Fig. 3. Permeability of the pores formed by Cry1Aa and Cry1Ac to divalent cations. Vesicles were preincubated for 60 min with the indicated concentrations of Cry1Aa (●, ○) or Cry1Ac (■, □) in 10 mM HEPES–KOH (pH 7.5) (●, ■) or CAPS–KOH (pH 10.5) (○, □) and 1 mg/ml bovine serum albumin and mixed with a solution containing the same buffer supplemented with 100 mM $MgCl_2$ (A), $CaCl_2$ (B) or $BaCl_2$ (C). Scattered light intensity was monitored as illustrated in Fig. 1 and percent volume recovery after 3 s was calculated as described in Section 2.

3.2. Are amino acid symporters toxin receptors?

Amino acid symporters, in particular those for leucine [23–26] and histidine [24], have been suggested as possible receptors for Cry1Aa [24–26] and Cry1Ac [23] in the midgut brush border membrane. This hypothesis was first put forward by Reuveni and Dunn [23] to explain why Cry1Ac inhibits more efficiently the uptake of leucine into brush border membrane vesicles than that of aspartic acid. Further evidence in favor of this hypothesis was provided by experiments demonstrating an inhibitory effect of Cry1Aa on amino acid transport measured both in the presence and in the absence of a potassium transmembrane gradient [24–26]. This observation was taken as evidence that the toxins could not inhibit amino acid transport by simply abolishing the potassium gradient and therefore suggested a direct interaction of the toxins with amino acid transport-

ers. Our finding that leucine diffuses more rapidly than aspartate across the pores formed by Cry1Aa and Cry1Ac and that these pores are permeable to a variety of amino acids, however, suggests that earlier results could be explained by a significant amino acid efflux from the vesicles during the filtration and washing steps of the transport experiments. Although our results cannot exclude the possibility that *B. thuringiensis* toxins may interact with amino acid transporters, they suggest a different interpretation of the results on which this hypothesis is based.

3.3. Membrane permeability to divalent cations and anions

The properties of the pores formed by Cry1Aa and Cry1Ac were further analyzed by comparing their effects on membrane permeability to the chloride salts of divalent cations (Fig. 3) and the potassium salts of divalent anions (Fig. 4). $MgCl_2$, $CaCl_2$ and $BaCl_2$ diffused readily across the pores formed by both toxins (Fig. 3), at rates comparable to those previously reported for the chloride salts of the small monovalent cations K^+, Na^+, tetramethylammonium and tetraethylammonium [31,33,34]. Osmotic swelling caused by a dissociated salt depends on the movement of both ion species and is limited by the rate of diffusion of the least permeable ion [31,34]. In the presence of these chloride salts, vesicle swelling rates thus appear to be limited by the rate of diffusion of chloride ion. In agreement with the anion being the rate limiting species, much slower diffusion rates were observed for K_2SO_4 and potassium phosphate at both pH values (Fig. 4) than for the chloride salts of divalent cations (Fig. 3) and the potassium salts of the small monovalent anions Cl^- and SCN^- [31,33,34]. The slow rates of diffusion of potassium phosphate and the fact that they were not influenced by pH (Fig. 4B) are probably mostly due to the predominance of the divalent phosphate species at both pH values since $H_2PO_4^-$ accounts for only about 1/3 of the phosphate ions at pH 7.5 and is absent at pH 10.5. These results indicate that the pores are much more permeable to small monovalent anions than to divalent anions and, in agreement with the results on amino acid permeability, they provide further evidence that the pores formed by Cry1Aa and Cry1Ac are cation-selective.

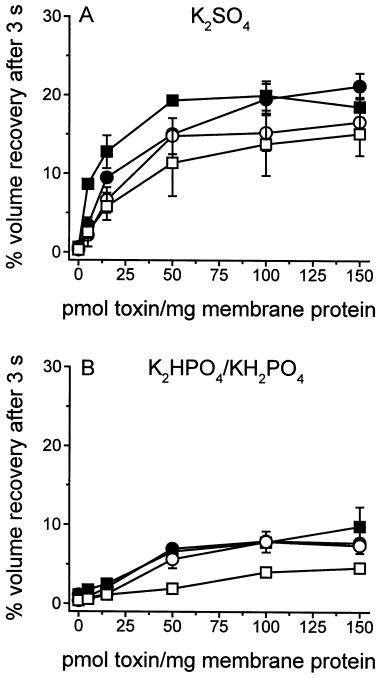

Fig. 4. Permeability of the pores formed by Cry1Aa and Cry1Ac to divalent anions. Vesicles were preincubated for 60 min with the indicated concentrations of Cry1Aa (●, ○) or Cry1Ac (■, □) in 10 mM HEPES–KOH (pH 7.5) (●, ■) or CAPS–KOH (pH 10.5) (○, □) and 1 mg/ml bovine serum albumin and mixed with a solution containing the same buffer supplemented with 100 mM K_2SO_4 (A) or potassium phosphate (B). Scattered light intensity was monitored as illustrated in Fig. 1 and percent volume recovery after 3 s was calculated as described in Section 2.

In the presence of either toxin, pH had little effect on membrane permeability to $CaCl_2$ (Fig. 3B) and K_2SO_4 (Fig. 4A). Because these two salts are always completely dissociated, regardless of pH, these results confirm the validity of our assumption, based on earlier studies [33,34], that the properties of the pores formed by Cry1Aa and Cry1Ac are not influenced to any great extent by changes in pH, at least between pH 7.5 and 10.5. Both Cry1Aa [33] and Cry1Ac [34] nevertheless allowed sucrose and raffinose to diffuse faster across the brush border membrane at pH 10.5 than at pH 7.5 in agreement with a slightly larger pore diameter at the higher pH [32]. This increased pore size cannot account for the pH effects observed in the present study. On the contrary, a larger pore size should have allowed the tested solutes to diffuse more readily across the membrane at the higher pH and, in this way, attenuated

the differences in swelling rates observed at both pH values.

In contrast with earlier suggestions [35,47] and in agreement with other experiments using brush border membrane vesicles [30,48], calcium (Fig. 3B) and barium (Fig. 3C) diffused very efficiently across the membrane in the presence of Cry1Aa and Cry1Ac and therefore had no detectable inhibiting effect on the pores formed by these toxins. This is in agreement with previous results that have documented calcium permeability of the pores formed by Cry1Ac in cultured insect cells and in planar lipid bilayers [49].

3.4. Ion selectivity of the pores

The results of the present study all concur to indicate that *B. thuringiensis* toxins form cation-selective pores that allow the passage of a variety of charged and uncharged solutes. This conclusion is consistent with the findings of practically all studies dealing with the properties of the pores formed by these toxins. Several planar lipid bilayer studies have reported reversal potential estimates indicative of cation selectivity [35–38]. None of these estimates reached the Nernst potential for the cation, however, a strong indication that the anion permeability of the pores is not negligible as demonstrated by a variety of osmotic swelling experiments [27,29,31,33,34]. Cry1C appears to be somewhat exceptional in this respect in being the only toxin for which anion-selective pores have been described, in addition to cation-selective pores [37], and being very active at acidic pH, at least in comparison with Cry1Ac [34]. The pores it forms in Sf9 cells were also found to have a very poor ion selectivity [29].

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Fonds pour la formation de chercheurs et l'aide à la recherche of Quebec.

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