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Video imaging analysis of the plasma membrane permeabilizing effects of *Bacillus thuringiensis* insecticidal toxins in Sf9 cells

Mario Villalon [a], Vincent Vachon [a], Roland Brousseau [b], Jean-Louis Schwartz [a,b], Raynald Laprade [a,*]

[a] *Groupe de recherche en transport membranaire, Université de Montréal, P.O. Box 6128, Centre Ville Station, Montreal, Quebec, Canada H3C 3J7*

[b] *Biotechnology Research Institute, National Research Council, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2*

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Abstract

The size and ionic selectivity of the pores formed by the insecticidal crystal protein Cry1C from *Bacillus thuringiensis* in the plasma membrane of Sf9 cells, an established cell line derived from the fall armyworm *Spodoptera frugiperda*, were analyzed with a video imaging technique. Changes in the permeability of the membrane were estimated from the rate of osmotic swelling of the cells. In the presence of Cry1C, which is toxic to Sf9 cells, the permeability of the cell membrane to KCl and glucose increased in a dose-dependent manner. In contrast, Cry1Aa, Cry1Ab and Cry1Ac, toxins to which Sf9 cells are not susceptible, had no detectable effect. Pores formed by Cry1C allowed the diffusion of sucrose, but were impermeable to the trisaccharide raffinose. On the basis of the hydrodynamic radii of these substances, the diameter of the pores was estimated to be 1.0–1.2 nm. In the presence of salts, the rate of swelling of cells exposed to Cry1C was about equally influenced by the size of the anion as by that of the cation, indicating that the ionic selectivity of the pores is low.

Keywords: Insecticidal toxin; Sf9 cell; Membrane permeability; Cell volume; Video imaging technique; *(Bacillus thuringiensis)*

1. Introduction

Bacillus thuringiensis is the most widely used microorganism, among the available alternatives to synthetic insecticides, for the biological control of insect pests in forestry and agriculture [1,2] and in public health [3]. Various strains of this spore-forming gram-positive bacterium are specifically pathogenic for different species of lepidopteran, dipteran and coleopteran insects [4], nematodes and protozoans [5]. Pathogenesis and its remarkable specificity are largely attributable to the synthesis of crystal proteins, also called δ-endotoxins, which accumulate in parasporal inclusion bodies during sporulation.

The structure, genetics and mode of action of these *B. thuringiensis* toxins have received considerable attention in recent years [6–10]. Numerous toxin genes have been sequenced, and the three-dimensional crystal structures of Cry3A, a coleopteran-specific toxin [11], Cry1Aa, a lepidopteran-specific toxin [12], and CytB, a toxin which is specifically

* Corresponding author. Fax: +1 (514) 343-7146; E-mail: laprader@ere.umontreal.ca

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active against dipteran insects in vivo, but has a broad-spectrum cytolytic activity in vitro [13], have been elucidated. Once ingested by susceptible insect larvae, crystal proteins of the Cry1 family are solubilized in the midgut and activated by intestinal proteases. The activated Cry1 toxins bind to specific receptors located on the surface of the apical membrane of columnar cells of the midgut epithelium and insert into the membrane. Pore formation causes the transmembrane electrical potential and ionic gradients to collapse. Killing of the cells disrupts the epithelium and allows the intestinal content to come in contact with the hemolymph. This eventually leads to the death of the larva, by starvation or septicemia [6–10].

In lepidopteran larvae, the midgut epithelium actively transports K^+ ions from hemolymph to the lumen and the electrical component of the K^+ electrochemical gradient thus generated serves as the main driving force for the active transport of amino acids into columnar cells. Inhibition of the transepithelial K^+ current [14–18] or K^+ gradient-dependent amino acid transport into brush border membrane vesicles [19–23] has provided highly sensitive, albeit indirect, means of assaying for toxin activity. The properties of the pores formed by Cry [124–27] and Cyt [28,29] toxins were examined more directly in planar lipid bilayer membranes. In these receptor-free artificial membranes, the pores are generally cation-selective although their ionic selectivity may depend on the pH of the bathing solution [25]. Cation-selective channels were also demonstrated in midgut brush border membrane vesicles which were either fused to planar lipid bilayer membranes [30,31] or analyzed in suspension with a membrane potential-sensitive probe [31,32]. Although, in these experiments, small inorganic cations diffused faster than chloride ions, the channels did not appear to discriminate strongly among different cations [31,32]. Moreover, a number of studies have demonstrated that the channels formed by different *B. thuringiensis* toxins in liposomes [33,34], brush border membrane vesicles [21,35] and cultured insect cells [36,37] allow the diffusion of a variety of cations, anions and neutral solutes including relatively large molecules such as alanine, uracil and oligosaccharides.

In the present study, the properties of the pores formed by Cry1C in Sf9 cells were examined with a new video imaging technique. The pores were sufficiently large to allow the diffusion of sucrose and showed little ionic selectivity.

2. Materials and methods

2.1. Cell cultures

Sf9 cells (ATCC CRL 1711) were grown at 27°C in Grace's insect cell culture medium (Gibco, Gaithersburg, MD) supplemented with 350 mg/l sodium bicarbonate, 3.33 g/l yeastolate (Difco, Detroit, MI), 3.33 g/l lactalbumin hydrolysate (Difco), 50 mg/l sodium ampicillin (Gibco) and 10% (v/v) heat-inactivated fetal bovine serum (Gibco). Cultures (100–150 ml) were carried out in spinner flasks inoculated with 1.0×10^5 cells/ml with constant stirring at 50–60 revolutions per min. Before use, cells were allowed to settle onto collagen-coated circular glass coverslips for at least 30 min.

2.2. Solutions

All organic chemicals were obtained from Sigma (St. Louis, MO), unless noted otherwise. Experiments were carried out in 50 mM KCl, 21 mM NaCl, 14 mM $MgCl_2$, 11 mM $MgSO_4$, 6.8 mM $CaCl_2$, 3.9 mM glucose, 2.2 mM fructose, 120 mM sucrose, and 10 mM 1,4-piperazinediethanesulfonic acid (Pipes)/Tris, pH 6.5 (G^* medium) [38]. The composition of this medium was modified as required for individual experiments. Hypotonic and hypertonic solutions were made by changing the sucrose concentration. For isotonic solutions, solutes were added in replacement of sucrose. The osmolarity of all solutions was measured with a DigiMatic Model 3D2 osmometer (Advanced Instruments Needham Heights, MA) and adjusted with sucrose. Recombinant Cry1Aa, Cry1Ab, Cry1Ac and Cry1C toxins were trypsin-activated and HPLC-purified as described previously [39,40].

2.3. Bath perfusion

Each coverslip was placed at the bottom of a custom-made bath composed of two communicating chambers. The cells were observed in the first cham-

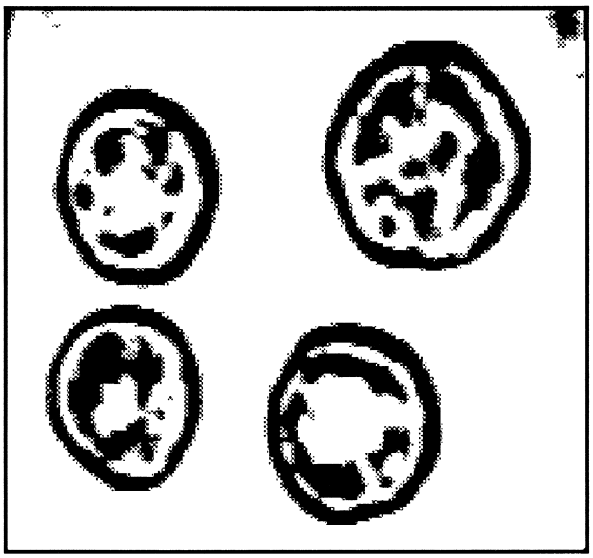

Fig. 1. Image of 4 Sf9 cells as seen during data acquisition. Cells were observed under 150× magnification with an Olympus IMT-2 inverted microscope. Images were recorded every 1 s with a monochrome CCD-72 video camera connected to a PC equipped with an IP8/AT videographics system board. The surface area occupied by each cell at its equatorial plane was measured with a custom-made program.

ber, near the end of a perfusion tube connected to pressurized reservoirs. The flow of bathing solutions was controlled electronically with magnetic valves. Excess liquid was aspirated from the second chamber to ensure a constant level of liquid in the bath and a laminar flow of solution over the cells.

2.4. Volume measurements

Cells were observed under 150× magnification with an Olympus IMT-2 inverted microscope. Images (Fig. 1) were recorded every 1 s with a monochrome CCD-72 camera (DAGE-MTI, Michigan, IN) connected to a 486 DX2 PC equipped with a IP8/AT videographics system board (Matrox, Dorval, Que.). The surface area occupied by each cell at its equatorial plane was measured. Since Sf9 cells are nearly spherical, their cellular volume was estimated as that of a sphere with the same projected surface area at the equatorial plane. Data are means ± SEM. For clarity, error bars in the figures are only shown for every tenth experimental point. Initial swelling rates were estimated by linear regression with FigP software (Biosoft, Ferguson, MO).

3. Results

To test whether changes in the volume of Sf9 cells could be used to estimate the permeability of their plasma membranes, cells were first submitted to hypotonic and hypertonic shocks by suddenly changing the sucrose concentration in the extracellular medium (Fig. 2). Decreasing the extracellular sucrose concentration caused the cells to swell until their volume reached a plateau at a level which was proportional to the change in the osmolarity of the medium. In contrast, increasing the extracellular concentration of sucrose caused the cells to shrink, but their volume stabilized at a similar level after addition of 50 or 100 mM sucrose. The cells thus share some of the properties expected from an osmometer, albeit non-ideal. A minimum volume is rapidly attained following relatively modest increases in the osmolarity of the medium. This may be due to packing constraints imposed by the cell structure. More importantly, however, Sf9 cells showed no evidence of compensatory volume regulation, at least within the timeframe of the experiments reported here.

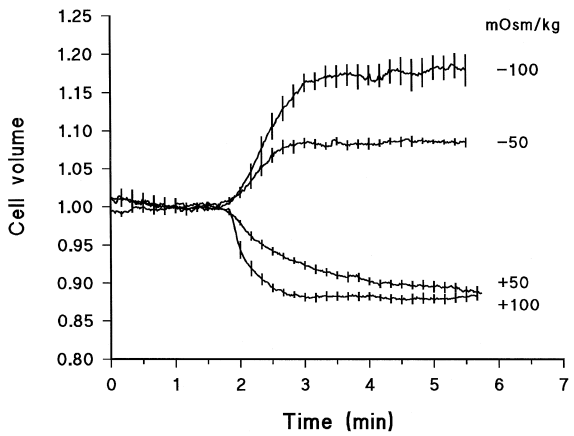

Fig. 2. Effect of hypertonic and hypotonic shocks on the volume of Sf9 cells. Cells incubated in G* medium were submitted to hypertonic and hypotonic shocks of 50 and 100 mosM/kg by replacing the G* medium from the bath by a solution of similar composition in which the sucrose concentration was either increased or decreased. Values are derived from 4 to 7 experiments.

In the absence of toxin, equivalent increases in the extracellular osmolarity with KCl, glucose, sucrose or raffinose caused the cells to shrink to a similar extent (Fig. 3(a)). The absence of volume recovery following such hypertonic shocks indicates that these solutes cannot diffuse readily across the intact cell membrane. In the presence of Cry1C, however, the cell membrane became permeable to KCl, glucose and sucrose, but remained impermeable to raffinose (Fig. 3(b)). The rate of cell volume recovery decreased as the size of the solute being tested was increased. From these results, it can be deduced that

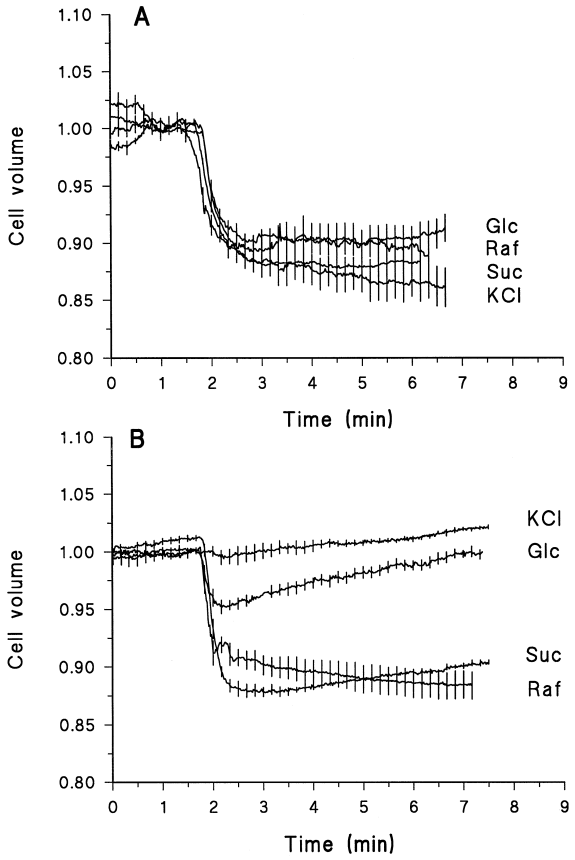

Fig. 3. Estimation of the size of the pores formed by Cry1C. Cells incubated in G* medium were submitted to a hypertonic shock by replacing the bathing solution by G* medium in which either the KCl concentration was raised from 50 to 100 mM, the glucose (Glc) concentration was raised from 3.9 to 103.9 mM, or the sucrose (Suc) concentration was raised from 120 to 220 mM, or by G* medium supplemented with 100 mM raffinose (Raf) in the absence of toxin (A) or after 15 min of preincubation with 10 μg/ml Cry1C (B). Values are derived from 4 to 6 experiments.

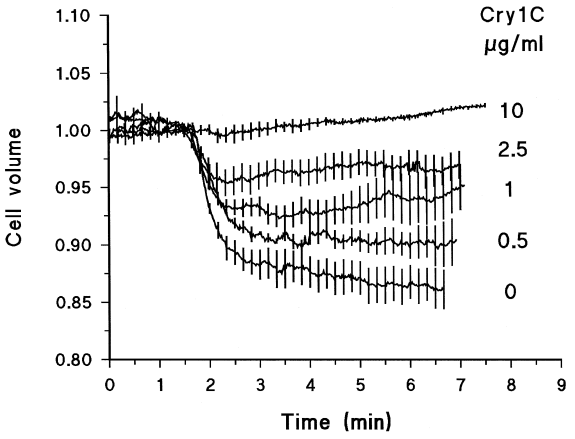

Fig. 4. Dose-dependent effect of Cry1C on the KCl permeability of the plasma membrane of Sf9 cells. Cells were preincubated 15 min in G* medium with the indicated concentrations of Cry1C. The extracellular medium was suddenly replaced by G* medium in which the KCl concentration was raised from 50 to 100 mM. Values are derived from 4 to 9 experiments.

the diameter of the pores lies between 1.0 and 1.2 nm, the effective hydrodynamic diameters of sucrose and raffinose estimated by viscosimetry [41,42].

In the case of KCl, little shrinking of the cells was observed, probably because the rate of influx of this solute was comparable to that of water efflux. In agreement with this interpretation, preincubation of the cells with Cry1C reduced, in a dose-dependent manner, the extent to which they shrank following a sudden increase in the extracellular KCl concentration (Fig. 4). Diffusion of KCl through the toxin-induced channels thus appears to be sufficiently rapid to prevent the cells from shrinking to a full extent before osmotic equilibrium is reached. Increases in the KCl permeability of the cell membrane were observed specifically with Cry1C (Fig. 5) and did not occur when the cells were exposed to Cry1Aa, Cry1Ab or Cry1Ac, three toxins which are known to be inactive against Sf9 cells [25,37]. The toxin preparations used for these experiments were nevertheless shown to be functional by demonstrating their ability to permeabilize brush border membrane vesicles isolated from the midgut of *Lymantria dispar*, an insect which is susceptible to all four toxins (results not shown).

In the experiments presented so far, the recorded volume changes are the net result of cell shrinking, due to the hypertonic shock, and cell swelling, due to

the influx of the test solute. Under these conditions, the tracings provide a good index of cell membrane permeability, but only allow a semi-quantitative analysis of the rates of diffusion of different solutes. In subsequent experiments, the initial shrinking of the cells which accompanies the hypertonic shock was therefore avoided by changing the extracellular medium for an isotonic solution prepared by replacing an appropriate amount of sucrose with the test solute. Following such raises in the extracellular concentration of glucose, the volume of the cells increased as a linear function of time for about 45 s and leveled off gradually afterwards (Fig. 6(a)). The initial rate of cell swelling depended strongly on the toxin concentration to which the cells had been previously exposed. It rose rapidly with toxin concentration but soon approached a maximum value as the toxin concentration was raised above about 5 μg/ml (Fig. 6(b)).

The ionic selectivity of the pores formed by Cry1C was analyzed, using this approach, by measuring the rates of diffusion of different electrolytes (Fig. 7). In order that the measured rates be comparable, the toxin concentration was adjusted so that the swelling rates were both sufficiently high for accurate measurements and well below saturating levels. Cells were therefore exposed to 3 μg/ml toxin for the measurement of the rate of diffusion of KCl (Fig. 7(a)) and to 5 μg/ml for the measurement of the

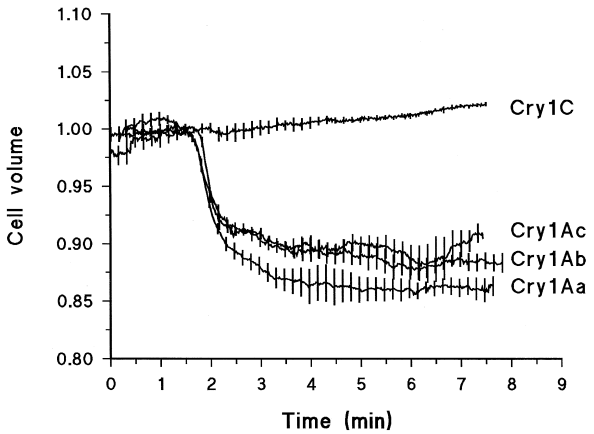

Fig. 5. Effect of different *B. thuringiensis* toxins on the KCl permeability of the plasma membrane of Sf9 cells. Cells were preincubated 15 min with 10 μg/ml of Cry1Aa, Cry1Ab, Cry1Ac or Cry1C. The extracellular medium was suddenly replaced by G* medium in which the KCl concentration was raised from 50 to 100 mM. Values are derived from 4 to 6 experiments.

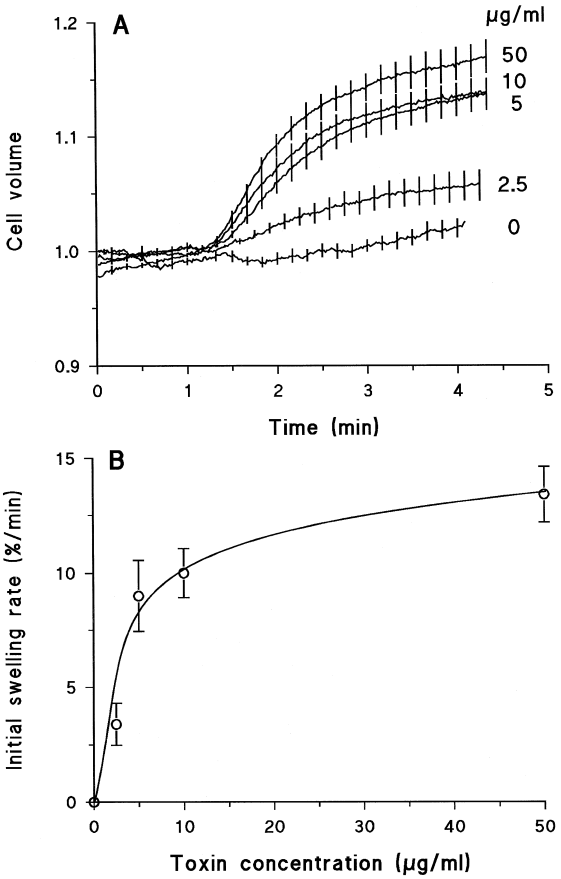

Fig. 6. Effect of toxin concentration on the rate of swelling of Sf9 cells. Cells were preincubated 15 min in G* medium with the indicated concentrations of Cry1C. Cell volume was monitored during replacement of the extracellular medium with an isotonic solution of similar composition except that 50 mM sucrose was replaced by 50 mM glucose (A). Dose-response curve (B). Values are derived from 5 to 10 experiments.

rates of diffusion of the other substrates (Fig. 7(b)). Diffusion of *N*-methyl-D-glucamine chloride was assayed at both toxin concentrations to allow comparison of the values obtained under both conditions. The relative rates of cell swelling in the presence of KCl (4.57%/min), *N*-methyl-D-glucamine chloride (2.45%/min at 3 μg/ml toxin and 3.15%/min at 5 μg/ml toxin), potassium gluconate (2.96%/min) and *N*-methyl-D-glucamine gluconate (1.27%/min) were 1.0, 0.54, 0.50 and 0.22, respectively. In such experiments, the rate of cell swelling is limited by the rate of diffusion of the slowest ionic species [35]. Because *N*-methyl-D-glucamine and gluconate, two glucose derivatives of similar molecular weight (195.22 and 196.16, respectively), have comparable

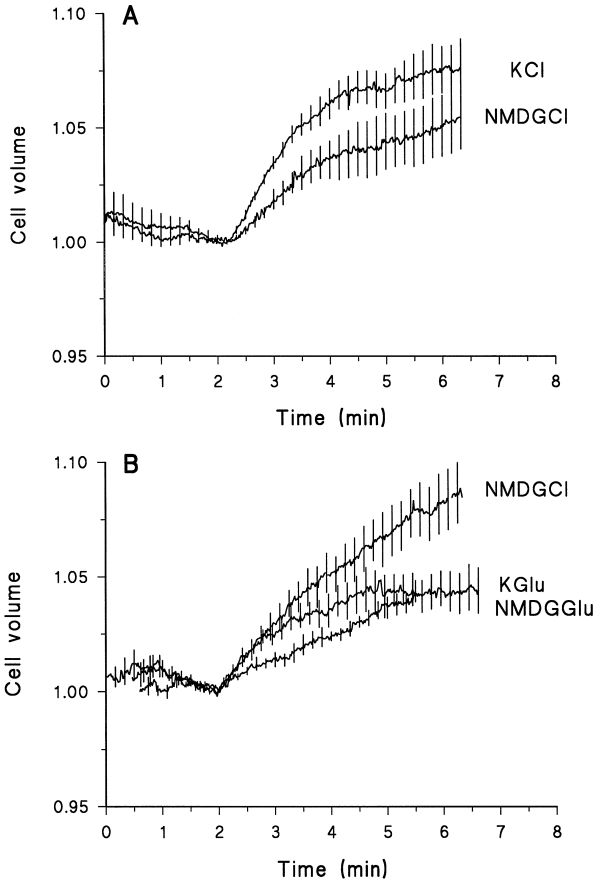

Fig. 7. Effect of Cry1C on the permeability of the plasma membrane of Sf9 cells to different ions. Cells were preincubated 15 min with 3 (A) or 5 (B) μg/ml Cry1C in G* medium. Cell volume was monitored during replacement of the extracellular medium with an isotonic solution of similar composition except that 50 mM sucrose was replaced by 25 mM KCl, *N*-methyl-D-glucamine chloride (NMDGCl), potassium gluconate (KGlu) or *N*-methyl-D-glucamine gluconate (NMDGGlu). Values are derived from 4 to 7 experiments.

sizes, these results indicate that the rate at which different solutes diffuse through the channels formed by Cry1C depends mainly on their size rather than on their electrical charge.

4. Discussion

The results of the present study clearly demonstrate that Cry1C increases the permeability of the plasma membrane of Sf9 cells to a number of cationic, anionic and neutral solutes. This effect is strongly dose-dependent and specific, other toxins to which Sf9 cells are known to be resistant having no detectable effect. Cry1C has previously been shown, using patch-clamp and microspectrofluorescence techniques, to trigger a calcium surge and activate small anionic channels in the plasma membrane of Sf9 cells [39] and to increase the permeability of their cell membrane to K^+, Na^+ and H^+ ions [37]. The use of a technique based on osmotic swelling has the advantage, over spectrofluorescence techniques, of allowing a wide variety of solutes to be tested for their ability to diffuse across the plasma membrane without the need for specific probes.

The pores induced by Cry1C in the plasma membrane of Sf9 cells allowed the diffusion of glucose ($M_r = 180$) and, at a slower rate, sucrose ($M_r = 342$), but were impermeable to raffinose ($M_r = 504$). From these results, their diameter was estimated to be 1.0–1.2 nm. Although a number of *B. thuringiensis* toxins have been shown to increase the permeability of different membranes to relatively large solutes such as L-alanine ($M_r = 89$) [21,34], uracil ($M_r = 112$) [34] and sucrose [33,35], little attention has been devoted to the size of their pores. Knowles and Ellar [36] have estimated the size of the pores formed by different *B. thuringiensis* toxins from the protection afforded by solutes of various sizes against the osmotic lysis of Cf1 cells derived from the spruce budworm *Choristoneura fumiferana*. The pores formed by the toxins of *B. thuringiensis* var. *kurstaki* have a diameter of 1.0–1.2 nm, while those formed by the toxins of var. *aizawai* and by CytA from var. *israelensis* have a diameter of 1.2–2.0 nm [36]. Unfortunately, because var. *kurstaki* and var. *aizawai* produce a number of Cry toxins, it is not possible, except in the case of CytA, to attribute the pore sizes estimated in these experiments to individual toxins. Nevertheless, these results, along with those of the present study, suggest that different *B. thuringiensis* toxins may form pores of different sizes. Such differences could contribute significantly, along with other factors such as those related to the number of pores formed in the membrane, to their different levels of toxicity for a given insect.

Cry1C was also shown to allow the diffusion of two relatively large ions, *N*-methyl-D-glucamine and gluconate. Our finding of similar rates of diffusion for *N*-methyl-D-glucamine chloride and potassium gluconate provides strong evidence indicating that the

ionic selectivity of the pores is at most very low. This result is somewhat surprising in view of the fact that Cry1C has previously been shown to form rather strongly cation-selective channels, not only in receptor-free planar lipid bilayer membranes [25], but also in brush border membrane vesicles isolated from the midgut of *Spodoptera frugiperda* [31], the insect species from which Sf9 cells were originally derived. It should be noted however that Sf9 cells are of ovarian origin and therefore differ, in many respects, from the cells lining the midgut lumen. In particular, the receptor for Cry1C may differ significantly in both cell types. It cannot be excluded that the use of relatively large toxin concentrations, to detect maximal effects of the toxins on membrane permeability, may have contributed to these differences. In addition, the small ionic selectivity of the pores may, at least partially, be caused by the use of a slightly acidic pH (6.5), close to that which is optimal for the growth of Sf9 cells [25]. These differences nevertheless indicate that the biophysical properties of the pores induced by *B. thuringiensis* toxins depend on the type of membrane in which they are formed and the experimental conditions under which they are analyzed.

At present, the events leading to membrane insertion and pore formation by *B. thuringiensis* toxins, following their binding to specific receptors, remain poorly understood [9,10]. In particular, the precise role of the receptors in these post-binding events needs to be clarified. The results of the present study suggest that the receptors, and possibly the lipid environment of the membrane, may not only play a major role in determining the specificity of the toxins and increasing their membrane insertion rate, but also exert a strong influence on the properties of the pores. A detailed comparison of the size and ionic selectivity of the pores formed in different membranes, in the presence and absence of receptors, should contribute to a better understanding of the role played by the receptors in the mode of action of *B. thuringiensis* toxins.

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