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Ionic Permeabilities Induced by *Bacillus thuringiensis* in Sf9 Cells

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Abstract. The effect of *Bacillus thuringiensis* insecticidal toxins on the monovalent cation content and intracellular pH (pH_i) of individual Sf9 cells of the lepidopteran species *Spodoptera frugiperda* (fall armyworm) was monitored with the fluorescent indicators potassium-binding benzofuran isophthalate (PBFI) and 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF). The sequential removal of K^+ and Na^+ from the medium, in the presence of CryIC, a toxin which is highly active against Sf9 cells, caused sharp shifts in the fluorescence ratio of PBFI, demonstrating a rapid efflux of these ions. In Sf9 cells, pH_i depends strongly on the activity of a K^+/H^+ exchanger. In the absence of toxin, removal of K^+ from the external medium resulted in a reversible acidification of the cells. In the presence of CryIC, pH_i equilibrated rapidly with that of the bathing solution. This effect was both time- and concentration-dependent. In contrast with CryIC, CryIIIA, a coleopteran-specific toxin, and CryIA(*a*), CryIA(*b*) and CryIA(*c*), toxins which are either inactive or poorly active against Sf9 cells, had no detectable effect on pH_i. *B. thuringiensis* endotoxins thus appear to act specifically by increasing the permeability of the cytoplasmic membrane of susceptible cells to at least H^+, K^+ and Na^+.

Key words: *Bacillus thuringiensis* — Insecticidal toxins — Sf9 cells — PBFI — Monovalent cations — Intracellular pH

Introduction

During sporulation, *Bacillus thuringiensis* accumulates protein crystals in the form of parasporal bodies. The parasporal proteins produced by different strains of this gram-positive bacterium include a variety of insecticidal toxins that act specifically on different species of Lepidoptera, Coleoptera and Diptera (Aronson, Beckman & Dunn, 1986; Höffe & Whiteley, 1989). Following their ingestion by insect larvae, these so-called δ-endotoxins are solubilized in the midgut and activated by intestinal proteases. Because of their increasing use as alternatives to chemical pesticides in forestry and agriculture, these toxins have received considerable attention in recent years (Brousseau & Masson, 1988; English & Slatin, 1992; Gill, Cowles & Pietrantonio, 1992; Lambert & Peferoen, 1992; Aronson, 1993; Knowles & Dow, 1993). A large number of *B. thuringiensis* toxin genes have been cloned and sequenced, and the three-dimensional structure of two of these, CryIIIA (Li, Carroll & Ellar, 1991) and CryIA(*a*) (Grochulski et al., 1994), has been elucidated by X-ray diffraction analysis. The different toxin proteins have been classified by Höfte and Whiteley (1989) into 13 different types based on their deduced amino acid sequence and host range. Several new toxin types have since been reported and their number will undoubtedly continue to increase for some time (Gill et al., 1992; Aronson, 1993).

Although it is generally agreed that *B. thuringiensis* toxins act by disrupting the ion gradients and osmotic balance across the plasma membrane of midgut cells in susceptible insects (Knowles & Ellar, 1987), their precise mode of action is still incompletely understood (English & Slatin, 1992; Gill et al., 1992; Lambert & Peferoen, 1992; Aronson, 1993; Knowles & Dow, 1993). Ion channel formation by different Cyt (Knowles et al., 1989, 1992) and Cry (Slatin, Abrams & English, 1990; Schwartz et al., 1993) toxins has been demonstrated in artificial lipid bilayer membranes. Activation of ion channels by CryIC has also been demonstrated in cultured lepidopteran cells (Sf9) using the patch-clamp technique (Schwartz et al., 1991). In each case, the channels were either cation- or anion-selective, although

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the selectivity could depend on pH (Schwartz et al., 1991). On the other hand, CryIA(*a*) was shown, with a fluorometric technique and a potential-sensitive probe, to increase the permeability of *Bombyx mori* brush-border membrane vesicles to several monovalent and divalent cations (Uemura et al., 1992) and CryIA(*c*) was shown, with a light-scattering assay, to increase the permeability of *Manduca sexta* midgut brush-border membrane vesicles to cationic, anionic and neutral solutes (Carroll & Ellar, 1993).

Disruption of the pH and K^+ gradients across the plasma membrane is expected to have devastating effects on the cell's bioenergetics and transport ability (Sacchi et al., 1986; Wolfersberger, 1989, 1992). In the present study, we have therefore used a microfluorescence technique to demonstrate that CryIC induces a markedly enhanced permeability to H^+, K^+ and Na^+ in the plasma membrane of Sf9 cells, a cloned ovarian cell line derived from the fall armyworm *Spodoptera frugiperda*. The ability to cause pH to equilibrate across the cell membrane was also shown to be specific for CryIC, the only toxin which, among those tested, is highly toxic to Sf9 cells.

Materials and Methods

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) 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/min. Before use, cells were allowed to settle onto circular 22-mm diameter No. 1 glass coverslips (Fisher Scientific, Montreal, Quebec) for at least 30 min.

Solutions

All chemicals were obtained from Sigma (St. Louis, MO), unless noted otherwise. Experiments were carried out in (mM): 50 KCl, 21 NaCl, 14 $MgCl_2$, 11 $MgSO_4$, 6.8 $CaCl_2$, 3.9 glucose, 2.2 fructose, 120 sucrose, and 10 1,4-piperazinediethanesulfonic acid (Pipes)/Tris, pH 6.5 (G* medium). The pH and composition of this medium were modified as required for individual experiments. When KCl or NaCl were omitted, they were replaced by N-methyl-D-glucamine-HCl to maintain the ionic strength of the solutions constant. The osmolality of all solutions was measured with a DigiMatic Model 3D2 osmometer (Advanced Instruments, Needham Heights, MA) and adjusted to 370 mOsmol/kg H_2O with sucrose.

CryIA(*a*), CryIA(*b*), CryIA(*c*), CryIC and CryIIIA toxins from *B. thuringiensis* were purified and activated as described previously (Masson et al., 1989; Schwartz et al., 1991, 1993).

Microfluorescence Measurements

For measurements of intracellular cation concentrations with potassium-binding benzofuran isophthalate (PBFI) (Molecular Probes, Eugene, OR), the cells were loaded by incubation with 10 μM of the membrane-permeant acetoxymethyl ester derivative of PBFI and 0.25% (w/v) Pluronic F-127 (Molecular Probes) in G* medium for 60 min at room temperature (20–23°C) and rinsed with PBFI-free G* medium. Experiments were carried out at room temperature on single cells in a custom-made coverslip holder fitted to the stage of an Olympus IMT-2 inverted microscope (Olympus Optical, Tokyo) coupled to a PTI spectrofluorometer (Photon Technology International, South Brunswick, NJ). Measurements were done at 340- and 380-nm excitation wavelengths alternating at a frequency of 100 Hz. The sample was illuminated through a 40×-epifluorescence objective (UVFL40, N.A. 0.85, Olympus). The emitted light was collected by the same objective, passed through a 505-nm interference filter (10-nm bandwidth), and its intensity was recorded by a photon-counting photomultiplier detector mounted on the microscope. Background fluorescence was subtracted from the raw data and data points were recorded every 1 sec.

The same apparatus was used for intracellular pH measurements. The cells were loaded by incubation with 5 μM of the acetoxymethyl ester derivative of 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) (Molecular Probes) in G* medium for 20 min at room temperature and rinsed with BCECF-free G* medium. Fluorescence intensities were measured at the excitation wavelengths of 450 and 500 nm and the emission wavelength of 530 nm. At the end of each experiment, the dependence of the intracellular BCECF fluorescence ratio on pH_i was calibrated with the nigericin-high K^+ technique (Thomas et al., 1979). pH_i was varied from about 5.25 to 7.75 by incubating the cells successively in a series of solutions containing 150 mM KCl, 2 mM $MgCl_2$, 6 μM nigericin and 10 mM of either 4-morpholineethanesulfonic acid (Mes)/Tris (pH 5.25–6.0) or Pipes/Tris (pH 6.25–7.75). Because, in Sf9 cells, pH_i often falls below the range where the fluorescence ratio of BCECF is a linear function of pH, the calibration values were fitted to a parabola:

$$F_{500}/F_{450} = a\,[(pH)^2 + b\,(pH) + c].$$

For each experiment, this equation and the values obtained for *a, b* and *c* were used to convert the fluorescence ratio measurements into pH_i values.

Results

Effect of CryIC on the Intracellular Content of K^+ and Na^+

The use of fluorescent probes for estimating the intracellular concentration of monovalent cations in Sf9 cells was evaluated by exposing PBFI-loaded cells to different concentrations of K^+ and Na^+ in the presence of gramicidin D to equilibrate either cation across the cell membrane (Fig. 1). Although PBFI was designed as a probe for measuring K^+ concentrations, it discriminates poorly between K^+ and Na^+ ions (Minta & Tsien, 1989). In addition, the sensitivity of PBFI decreased sharply at concentrations of either monovalent cation above approximately 40 mM (Fig. 1).

In spite of these limitations, PBFI could still be used to demonstrate qualitatively a strong effect of CryIC on the plasma membrane permeability for K^+ and Na^+ in Sf9 cells (Fig. 2). In the absence of toxin, the fluorescence

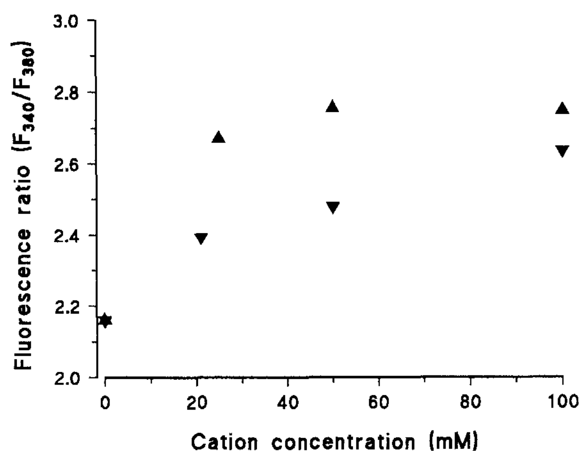

Fig. 1. Dependence of PBFI fluorescence ratio on the intracellular concentration of K^+ and Na^+. Fluorescence intensities were measured at the excitation wavelengths of 340 and 380 nm and the emission wavelength of 505 nm in the presence of 5 μM gramicidin D. The concentrations of K^+ (▲) and Na^+ and Na^+ (▼) were varied in the absence of the other cation. The ionic strength of the medium was kept constant by the additon of N-methyl-D-glucamine-HCl.

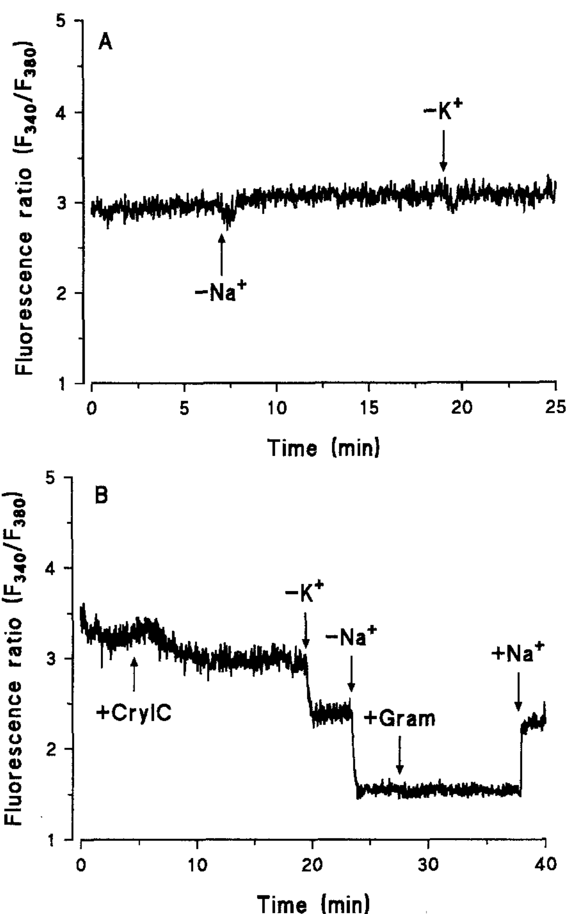

Fig. 2. Effect of CryIC on the monovalent cation content of Sf9 cells. The fluorescence ratio was monitored in PBFI-loaded cells in the absence (*A*) or presence (*B*) of 50 μg/ml CryIC. K^+ and Na^+ were successively replaced by N-methyl-D-glucamine-HCl and 5 μM-gramicidin was added as indicated (+Gram). Representative tracings are shown.

ratio remained unchanged following the replacement of Na^+ and K^+ by N-methyl-D-glucamine (Fig. 2*A*). In intact cells, the intracellular concentration of monovalent cations thus appears to remain sufficiently high to saturate the binding sites on the PBFI molecule. However, in the presence of toxin, the fluorescence ratio of PBFI shifted rapidly after K^+ and Na^+ were removed from the medium, demonstrating a substantial increase in the permeability of the membrane for both cations (Fig. 2*B*). Subsequent addition of gramicidin D to further increase the permeability of the membrane for these cations had no detectable effect.

Effect of CryIC on the Intracellular pH of Sf9 Cells

As shown previously (V. Vachon et al., *submitted*), the resting pH_i of Sf9 cells incubated in G* medium (pH = 6.5) in the absence of toxin is about 6.3. Removal of K^+ from the incubation medium results in a reversible acidification of the cells to pH 5.4 (Fig. 3*A*). This acidification and the alkalinization that follows restoration of K^+ is due to the presence of a strong K^+/H^+ exchange activity in the plasma membrane of Sf9 cells (V. Vachon et al., *submitted*). In the presence of CryIC, pH_i equilibrated with the pH of the medium. Removal of external K^+ was followed by a transient acidification of the cells and the pH returned rapidly to its original level. Conversely, increasing the external K^+ lead to a transient alkalinization of the cells with or without toxin. The extent of cell acidification in CryIC-treated cells following removal of extracellular K^+ and the rate of pH_i recovery, as well as the size and temporal behavior of the alkaline overshoot upon restoration of K^+, depended on both the concentration (compare Fig. 3*B* and 3*D*) and the time of exposure to the toxin (compare Fig. 3*C* and 3*D*).

To test whether the enhanced fluxes of H^+ ions in the presence of CryIC could be the consequence of an increase in the permeability to other ions, experiments similar to that illustrated in Fig. 3*D* were performed using media in which either Na^+ was replaced by N-methyl-D-glucamine or Cl^- was replaced by cyclamate. In both cases, these changes in medium composition had no detectable effect on pH_i and the results (*not shown*) were essentially identical to that shown in Fig. 3*D*. The toxin cannot increase diffusion of H^+ across the cell membrane by simply allowing Na^+ to exchange for protons since this would cause an acidification of the cells, upon addition of the toxin, rather than the observed initial alkalinization. In addition, the alkalinization which follows the acidification of the cells after removal of K^+ from the

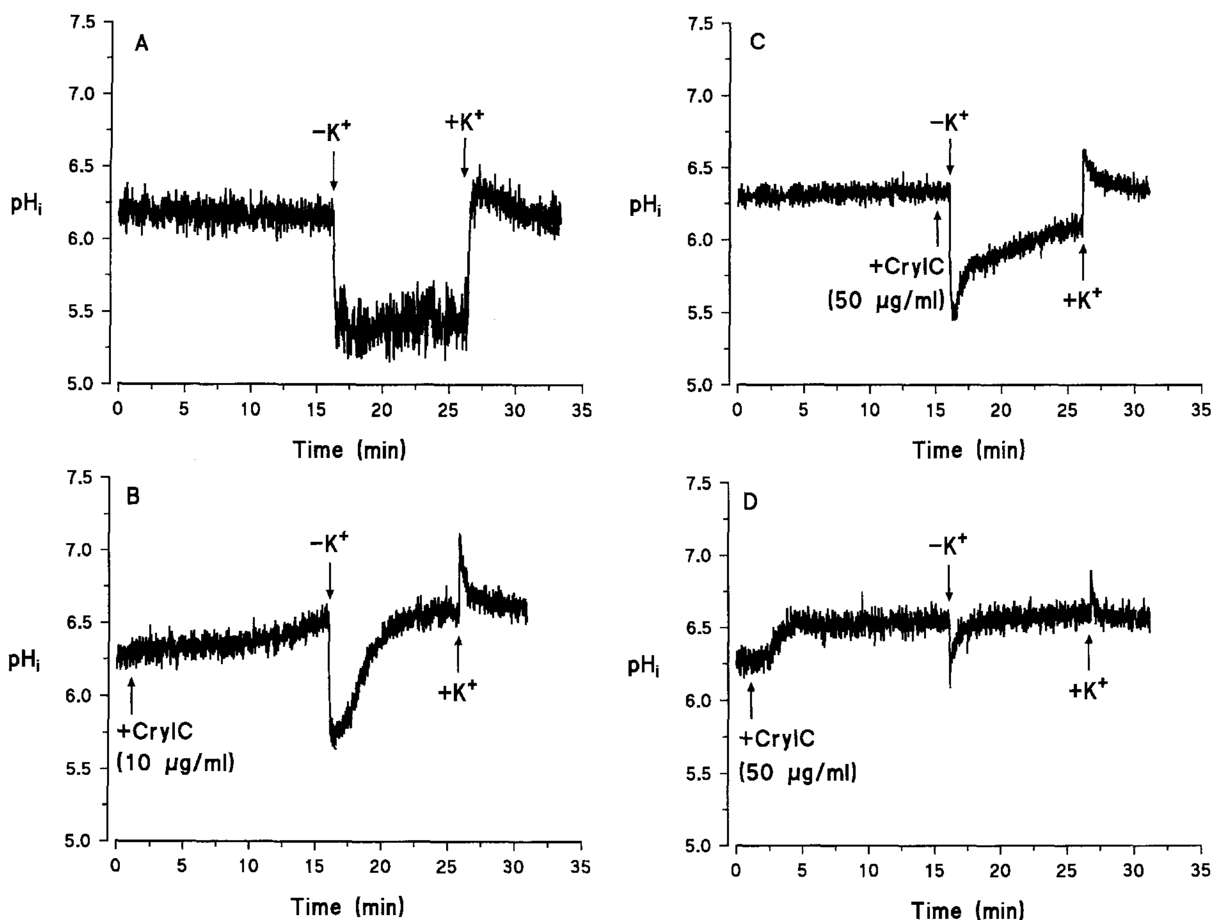

Fig. 3. Concentration- and time-dependent effects of CryIC on the intracellular pH of Sf9 cells. Experiments were carried out in G* medium at pH 6.5. After 16 min of incubation, KCl was replaced by N-methyl-D-glucamine-HCl for another 10 min after which the cells were returned to G* medium. The toxin was added at 0 (*A*), 10 (*B*) or 50 (*C* and *D*) μg/ml after 1 (*B* and *D*) or 15 (*C*) min. Intracellular pH was monitored as described in Materials and Methods. Representative tracings are shown.

medium would not be possible in the absence of extracellular Na^+. A similar argument can be made to exclude the possibility that the toxin increases the permeability of the membrane for H^+ by simply increasing its permeability for K^+: the cells could not realkalinize, in exchange for K^+ ions, following removal of K^+ from the medium. Also, the toxin cannot increase diffusion of H^+ across the cell membrane by simply allowing Cl^- to act as an accompanying ion, since the acidification which follows the alkalinization of the cells after reintroduction of K^+ in the medium would not be possible in the absence of extracellular Cl^-.

In the absence of extracellular K^+, changes in the pH of the external medium have little effect on the intracellular pH of intact Sf9 cells (V. Vachon et al., *submitted*). As shown in Fig. 4*A*, however, after prolonged incubation in a K^+-free medium, the cells were unable to maintain the strong pH gradient imposed across their cell membranes. After a relatively long lag period, pH_i rose gradually before stabilizing at a level well below that of the external medium. These results cannot be accounted for by a simple passive diffusion of H^+ across the cell membrane, and the mechanism by which this alkalinization takes place is still unclear. It was nevertheless independent of changes in the external pH as it also occurred during prolonged incubation at constant external pH or when the order of such changes was reversed. In contrast, in the presence of CryIC, and regardless of the presence or absence of extracellular K^+, changes in external pH were followed immediately by changes in pH_i which equilibrated within a few minutes with that of the external medium (Fig. 4*B*).

Other *B. thuringiensis* Toxins

To test whether the ability of *B. thuringiensis* to increase the permeability of the cell membrane to H^+ ions was related to their toxicity, experiments similar to that shown in Fig. 3*D* were carried out with the coleopteran-specific CryIIIA and the lepidopteran-specific CryIA(*a*), CryIA(*b*) and CryIA(*c*) toxins. In contrast with CryIC,

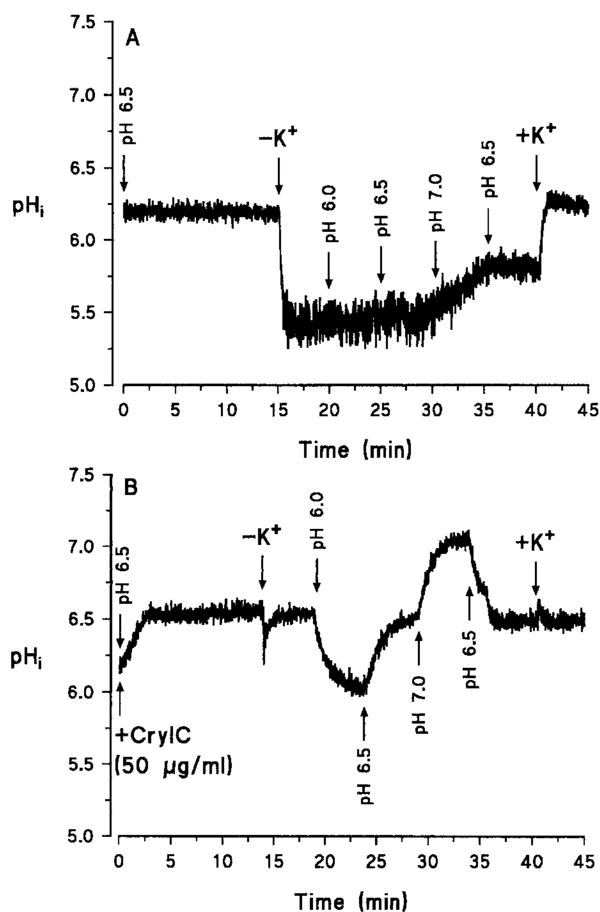

Fig. 4. Effect of changes in the extracellular pH of Sf9 cells in the absence (*A*) or presence (*B*) of CryIC. Experiments were carried out in G* medium at the indicated pH values. During the indicated interval, KCl was replaced by N-methyl-D-glucamine-HCl. Representative tracings are shown.

these toxins were found to be either inactive or very poorly active against Sf9 cells (Schwartz et al., 1993), as evaluated using the lawn assay described by Gringorten et al. (1990). Incubation of Sf9 cells with either of these toxins had no significant effect on their intracellular pH measured in the presence or absence of extracellular K^+ (Fig. 5).

Discussion

The results of the present study clearly demonstrate that CryIC allows the rapid diffusion and equilibration of K^+, Na^+ and H^+ ions across the plasma membrane of Sf9 cells. They also strongly suggest an increase in the permeability of the membrane for anions since the efflux of large amounts of these cations would not be expected to occur without the simultaneous efflux of counterions. In fact, CryIC has previously been shown to activate

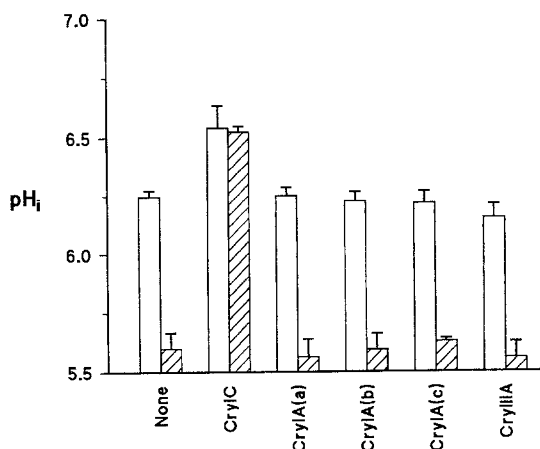

Fig. 5. Effect of different *B. thuringiensis* toxins on the intracellular pH of Sf9 cells. Experiments were carried out using the protocol described for Fig. 3*D*. Each toxin was added at 50 μg/ml. The intracellular pH was measured after 15 min of incubation in the presence of K^+ (open bars) and 10 min after subsequent removal of K^+ from the extracellular medium (hatched bars).

anion-selective channels in the plasma membrane of these cells (Schwartz et al., 1991).

These findings provide strong support in favor of the currently held view that *B. thuringiensis* toxins act by increasing the permeability of the cell membrane to small ions (Knowles & Ellar, 1987; Gill et al., 1992). Because these experiments measured ionic contents of the cells, they did not address directly the somewhat more controversial question of the mechanisms by which these increases in membrane permeability are brought about. The enhanced permeability to K^+, Na^+ and H^+ appears to be due to the formation of channels that allow the diffusion of all three ionic species, but we cannot exclude the possibility that several more specific channels are formed. In addition, the channels could either be formed by the toxin itself or result from the activation of endogenous channels in the cell membrane. In any case, the observed effects of the toxin were irreversible since, following an initial period of exposure to the toxin, the cell membrane remained permeable to these ions after the extracellular medium was replaced with toxin-free media.

Active transport of amino acids in the insect midgut, the actual target tissue for *B. thuringiensis* toxins, is mediated by K^+-dependent symporters located in the apical membrane of columnar cells (Giordana, Sacchi & Hanozet, 1982; Giordana et al., 1989). The driving force for this transport activity is provided predominantly by the electrical component of the transmembrane electrochemical K^+ gradient which is sustained by a vacuolar-type H^+-ATPase coupled with an electrogenic K^+/H^+ antiporter, both located in the apical membrane of goblet cells (Wieczorek et al., 1989, 1991; Chao, Moffett & Koch, 1991). Na^+ gradient-driven amino acid transport

has also been demonstrated in isolated insect midgut brush-border membrane vesicles (Giordana et al., 1989; Wolfersberger, 1989; Sacchi et al., 1994). Because membrane transport phenomena have not been studied extensively in Sf9 cells, the involvement of these and other transporters remains to be established and characterized. Disruption of K^+, Na^+ and H^+ gradients across their cell membranes would nevertheless be expected to destroy their ability to carry out absorptive transport of nutrients. *B. thuringiensis* toxins are indeed known to inhibit amino acid transport in midgut brush-border membrane vesicles (Sacchi et al., 1986; Wolfersberger, 1989). In the insect midgut, such enhanced permeability to these ions would rapidly destroy cellular functions by causing the collapse of the strong electrical potential across the luminal membrane (Dow & Harvey, 1988; Moffett & Koch, 1988) and the equilibration of pH_i with the very high pH of the lumen (Dow, 1984, 1992; Wolfersberger, 1992).

While membrane permeabilizing effects of *B. thuringiensis* toxins have been demonstrated in whole cells and in a variety of artificial systems, spectrofluorescence techniques offer the advantage of being simple, rapid and accurate. They have been used previously to measure the effect of different δ-endotoxins on the electrical potential of membrane vesicles (Uemura et al., 1992) and on the Ca^{2+} content of living cells (Schwartz et al., 1991). In the present study, the efflux of K^+ and Na^+ ions was demonstrated with PBFI (Minta & Tsien, 1989). Unfortunately, however, kinetic analyses of this efflux were somewhat limited by the fact that PBFI becomes saturated at the relatively high concentrations of these ions in Sf9 cells. Similar experiments were carried out with the related probe sodium-binding benzofuran isophthalate (SBFI) (Minta & Tsien, 1989). Although SBFI was more selective than PBFI, its fluorescence ratio also saturated at the high cation concentrations found in the cell (*results not shown*). On the other hand, with appropriate calibration (V. Vachon et al., *submitted*), intracellular pH could be measured accurately over a wide range with BCECF, even at the relatively low intracellular pH of these insect cells. The ability of different toxins to cause the intracellular pH to equilibrate with that of the external medium correlates well with their toxicity towards Sf9 cells. The dissipation of large pH gradients across the cell membrane in the presence of *B. thuringiensis* toxins provides a sensitive method for evaluating toxin activity. As these techniques could be easily adapted for use with other cell lines or insect tissues, such as isolated midguts, this approach could provide the basis for the development of a rapid and sensitive method for comparing the activity of different *B. thuringiensis* toxins and for screening different toxins for potential toxicity towards a given cell type or tissue.

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