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# Cry1Ac, A *BACILLUS THURINGIENSIS* TOXIN, TRIGGERS EXTRACELLULAR $Ca^{2+}$ INFLUX AND $Ca^{2+}$ RELEASE FROM INTRACELLULAR STORES IN Cf1 CELLS (*CHORISTONEURA FUMIFERANA*, LEPIDOPTERA)

L. POTVIN[1], R. LAPRADE[2] AND J. L. SCHWARTZ[1,2,*]

[1]*Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec, Canada H4P 2R2* and [2]*Groupe de Recherche en Transport Membranaire, Université de Montréal, Montreal, Quebec, Canada H3C 3J7*

*Author for correspondence (e-mail: jean-louis.schwartz@bri.nrc.ca)

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## Summary

**Intracellular $Ca^{2+}$ concentration was measured in single Cf1 cells (*Choristoneura fumiferana*, spruce budworm) loaded with Fura-2, a $Ca^{2+}$-sensitive fluorescent probe. Cf1 cells displayed $Ca^{2+}$ surges in response to Cry1Ac and Cry1C proteins, two Cf1-toxic *Bacillus thuringiensis* products, but not to Cry1Aa and Cry3A, which are not toxic to Cf1 cells. In the presence of extracellular $Ca^{2+}$, the toxin-induced $Ca^{2+}$ response was insensitive to methoxyverapamil, a voltage-dependent $Ca^{2+}$ channel blocker, but was abolished by lanthanum, a general inhibitor of $Ca^{2+}$ transport. In the absence of external $Ca^{2+}$, Cry1Ac induced a small intracellular $Ca^{2+}$ transient which was inhibited by TMB-8, a blocker of $Ca^{2+}$ release from inositol-1,4,5-trisphosphate-sensitive pools. Under these conditions, thapsigargin, which inhibits intracellular $Ca^{2+}$-ATPases, elicited a $Ca^{2+}$ surge when applied alone. However, subsequent addition of Cry1Ac failed to induce a $Ca^{2+}$ signal, indicating a depletion of intracellular $Ca^{2+}$ pools. In Cf1 cells, therefore, bioactive *B. thuringiensis* toxins triggered intracellular $Ca^{2+}$ surges which were mainly due to the influx of extracellular $Ca^{2+}$ through toxin-made pores, as confirmed by planar lipid bilayer experiments. Furthermore, TMB-8- and thapsigargin-sensitive $Ca^{2+}$ stores contributed to the Cry1Ac-induced $Ca^{2+}$ signal.**

Key words: intracellular $Ca^{2+}$, *Bacillus thuringiensis*, lepidopteran cell line, ion channel, $Ca^{2+}$ transport, Fura-2, planar lipid bilayer.

## Introduction

The inclusion bodies produced during sporulation by *Bacillus thuringiensis*, a Gram-positive soil bacterium, are highly specific gut poisons causing insect death within a few hours of ingestion (Höfte and Whiteley, 1989; Gill *et al.* 1992). Several formulated products based on *B. thuringiensis* toxins are currently used as efficient tools for the control of agro-forestry insect pests (Cannon, 1996) and of the insect vectors of several human and animal diseases (Federici, 1995). The exact mechanism of action of *B. thuringiensis* toxins is not well understood (Gill *et al.* 1992; Knowles, 1994). Following ingestion and solubilisation by intestinal secretions in the insect midgut, the crystal proteins are cleaved by gut proteases. The resulting products are 60–65 kDa activated proteins which bind to specific sites of the brush-border membrane of the columnar cells lining the gut lumen. This triggers a cascade of poorly elucidated events leading to the death of the insect. It is believed that the pore-related increased permeabilisation of the target cells and the resulting cellular ionic and metabolite imbalance constitute the critical steps leading to cell disruption. With the recent elucidation of the atomic structures of Cry3A, a coleopteran-specific toxin (Li *et al.* 1991), and Cry1Aa, a lepidopteran-specific toxin (Grochulski *et al.* 1995), a better understanding of the molecular mode of action of these proteins should emerge. This will be essential to deal with insect resistance to *B. thuringiensis* insecticides, the most serious problem which these products will inevitably face (Tabashnik, 1994).

So far, only limited attention has been given to the interactions of *B. thuringiensis* toxin with physiological processes at the cell level, possibly because of the lack of appropriate cellular models. Only a few *B. thuringiensis* toxin-susceptible insect cell lines are available for physiological studies. While these cells are not the natural targets of the pathogens and their sensitivity to the crystal proteins is several orders of magnitude lower than that of the insects from which they originate, they allow appropriate detection of the entomocidal activity of activated *B. thuringiensis* products with reasonable species and interspecies selectivity (Johnson, 1994; McCarthy, 1994*b*). Several physiological mechanisms have been investigated in Sf9 cells from the fall armyworm (*Spodoptera frugiperda*, Lepidoptera) (Hu *et al.* 1994*a*,*b*), UCR-SE-1a cells from the beet armyworm (*Spodoptera exigua*, Lepidoptera) (Monette *et al.* 1994) and Cf1 cells from the spruce budworm (*Choristoneura fumiferana*, Lepidoptera)

(Gole *et al.* 1987; Orr *et al.* 1988; Gupta and Downer, 1993). These cells, because of their sensitivity to *B. thuringiensis* toxins, provide simple and convenient models for studies of the mechanism of action of the toxin at the cellular level. Recently, the cellular effects of Cry1 toxins on Sf9 cells have been the object of intense scrutiny in our laboratories (Schwartz *et al.* 1991; Vachon *et al.* 1995*a*,*b*; Monette *et al.* 1997; Villalon *et al.* 1997). Cry1C induced cell swelling and caused the rapid diffusion and equilibration of $K^+$, $Na^+$ and $H^+$ across the plasma membrane of Sf9 cells (Vachon *et al.* 1995*b*; Villalon *et al.* 1997). The toxin triggered an intracellular $Ca^{2+}$ surge within seconds of toxin exposure and thereafter activated anion-selective channels in the cell membrane (Schwartz *et al.* 1991). We observed a similar Cry1C-mediated $Ca^{2+}$ response in UCR-SE-1a cells (Monette *et al.* 1994). Furthermore, it was established that Cry1C toxicity to Sf9 cells was substantially stimulated by extracellular $Ca^{2+}$ in a dose-dependent manner and that this effect was related to an increased concentration of intracellular $Ca^{2+}$ (Monette *et al.* 1997). These data suggested that cellular $Ca^{2+}$ changes related to toxin exposure represent an early step in the activity of the toxin and may be a general response of susceptible insect cells to the detection of *B. thuringiensis* toxins. Furthermore, they supported the concept of a synergetic interaction between $Ca^{2+}$ and *B. thuringiensis* toxins, as demonstrated in several lepidopteran pests *in vivo* (for a review, see Dent, 1993) and by a recent *in vivo* study on the interaction of caffeine with *B. thuringiensis* toxin activity against the bertha armyworm (*Mamestra configurata*, Lepidoptera), suggesting that the augmented toxicity was mediated by the deregulation of cellular $Ca^{2+}$ transport processes (Morris *et al.* 1994).

In this report, we used Fura-2, a $Ca^{2+}$ fluorophore, to examine the effects of *B. thuringiensis* toxins on the intracellular $Ca^{2+}$ concentration of Cf1 cells, a *B. thuringiensis* toxin-sensitive cell line (for a review, see McCarthy, 1994*a*). We demonstrated that active toxins triggered $Ca^{2+}$ surges which were produced largely by the influx of extracellular $Ca^{2+}$ through toxin-made membrane pores but also by a small component originating from the release of $Ca^{2+}$ from intracellular stores. Furthermore, planar lipid bilayer experiments conducted in the present study demonstrated that the pores induced by Cry1Ac, which are permeable to $K^+$ (Slatin *et al.* 1990; Schwartz *et al.* 1997), also allowed the passage of $Ca^{2+}$.

## Materials and methods

### *Cells*

Cf1 cells are derived from trypsin-treated larval tissue of the spruce budworm *Choristoneura fumiferana*. They were obtained from S. Sohi (Natural Resources Canada, Sault Sainte Marie, Ontario, Canada) and grown in Grace's medium supplemented with 0.25 % (w/v) tryptose and 10 % (v/v) heat-inactivated foetal bovine serum. Cultures were maintained in 25 $cm^2$ plastic tissue culture flasks (Sarstedt Inc, Newton, North Carolina, USA) at 27 °C and were subcultured every 3–4 days to a final concentration of $1.5\times10^6$ to $2\times10^6$ cells $ml^{-1}$. In preparation for the experiments, 500 µl of cells from cultures at 80–90 % confluence were deposited on glass coverslips (24 mm in diameter) in supplemented Grace's medium at room temperature (20–22 °C). Attachment to the coverslips occurred within 2 h to a final confluence of 80–90 %.

### *Solutions*

The normal bath solution (NBS) was a simplified Grace's solution containing 50 mmol $l^{-1}$ KCl, 21 mmol $l^{-1}$ NaCl, 6.8 mmol $l^{-1}$ $CaCl_2$, 14 mmol $l^{-1}$ $MgCl_2$, 11 mmol $l^{-1}$ $MgSO_4$, 3.9 mmol $l^{-1}$ D-glucose and 20 mmol $l^{-1}$ Pipes. Osmolarity was adjusted to 380 mosmol $l^{-1}$ with sucrose, and pH was set to 6.4 with NaOH. $Ca^{2+}$-free NBS (0NBS) was obtained by replacing $CaCl_2$ with 5 mmol $l^{-1}$ EGTA. Concentrated stock solutions of test agents were prepared in NBS, 0NBS or, when required, dimethylsulphoxide (DMSO). Working solutions were obtained by dilution in NBS or 0NBS. Fura-2/AM (Fura-2 acetoxymethyl ester) stock solution (1 mmol $l^{-1}$) was prepared in DMSO and was used at a final concentration of 2 µmol $l^{-1}$ in NBS.

### *Cell $Ca^{2+}$ measurements*

Intracellular $Ca^{2+}$ concentration in single cells or small group of cells was determined using Fura-2 as described previously (Schwartz *et al.* 1991). Briefly, cells were loaded with Fura-2/AM, the $Ca^{2+}$-insensitive, membrane-permeant form of Fura-2, a fluorescent $Ca^{2+}$ indicator, in NBS for 1 h at room temperature. Cells were washed three times and incubated for 10 min in NBS to achieve intracellular Fura-2/AM hydrolysis by cellular esterases into Fura-2, which remained trapped intracellularly. Upon binding to $Ca^{2+}$ (135 nmol $l^{-1}$ dissociation constant at 20 °C), the excitation spectrum of Fura-2 undergoes a dose-dependent shift towards lower wavelengths with no change in emission peak (505 nm). This spectral property of Fura-2 was used for high-sensitivity, largely artefact-free determination of cellular $Ca^{2+}$ concentration, which can be derived from the ratio of Fura-2 fluorescence intensities measured at 340 nm and 380 nm excitation (Grynkiewicz *et al.* 1985). Coverslips were mounted in a custom-made experimental chamber containing 0.5 ml of NBS or 0NBS and located on the stage of an IMT-2 inverted fluorescence microscope (Olympus Optical Co, Tokyo, Japan) equipped with a 40×, 0.85 NA epifluorescence objective and attached to a dual-excitation photometric instrument (Photon Technology Instrument, Monmouth Junction, New Jersey, USA). Test compounds were added to the chamber after a 2 min control period. Diluted working solutions of *B. thuringiensis* toxins were prepared in NBS or 0NBS and added to the chamber to a final concentration of 0.35 µmol $l^{-1}$. For calibration, the maximum and minimum fluorescence ratios were determined at the end of each experiment by the sequential addition of 20 µmol $l^{-1}$ ionomycin and 20 mmol $l^{-1}$ EGTA. All experiments were performed at room temperature.

Autofluorescence of Cf1 cells at either wavelength was at least 10 times lower than that of Fura-2 loaded cells and, therefore, fluorescence levels were not corrected for

background. The fluorophore was uniformly distributed in the cell cytosol, with no sign of compartmentalisation in the nucleus or cytoplasmic organelles. The basal level of intracellular $Ca^{2+}$ in NBS was 82±14 nmol $l^{-1}$ (mean ± S.E.M., $N$=23).

### *Planar lipid bilayer*

Reconstitution of *B. thuringiensis* toxins in planar lipid bilayers has been described in detail elsewhere (Schwartz *et al.* 1993). Briefly, phospholipid membranes were formed from a 7:2:1 lipid mixture of phosphatidylethanolamine, phosphatidylcholine and cholesterol painted on a 250 µm circular aperture in a Delrin wall separating two low-volume chambers (4 ml *trans*, 3.5 ml *cis*). Under the experimental conditions used in the present study, membranes had a capacitance of approximately 150–200 pF and remained stable for hours. Channel activity, following injection of 0.3 µmol $l^{-1}$ activated protein near the membrane in the *cis* chamber, was monitored by step changes in the current recorded when test voltages were applied to the planar lipid bilayer. All experiments were performed at room temperature in solutions containing either 50 or 450 mmol $l^{-1}$ $CaCl_2$ and buffered with 10 mmol $l^{-1}$ Tris, pH 9.0. Single-channel currents were recorded with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, California, USA). Analysis was performed on a personal computer using pClamp and Axotape software (Axon Instruments).

### *Toxins and chemicals*

Toxins were produced, activated, purified and tested for purity as described previously (Masson *et al.* 1989). They were stored in lyophilised form and reconstituted to a final concentration of 1 mg $ml^{-1}$ in high-purity water with 10 mmol $l^{-1}$ Tris at pH 10.0. Grace's insect cell culture medium was purchased from Gibco BRL (Life Technologies, Burlington, Ontario, Canada). Foetal bovine serum was obtained from PDI Bioscience (Aurora, Ontario, Canada). Tryptose was purchased from Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK. TMB-8 [8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride], thapsigargin (a naturally occurring sesquiterpene lactone) and EGTA were obtained from Sigma, St Louis, Missouri, USA. D600 (methoxyverapamil, a phenethylamine derivative) and ionomycin-free acid (from *Streptomyces conglobatus*) were purchased from Calbiochem Corp, La Jolla, California, USA. Anhydrous DMSO was obtained from Aldrich Chemicals, Milwaukee, Wisconsin, USA. Fura-2/AM was purchased from Molecular Probes, Eugene, Oregon, USA. Lipids were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA.

## Results

### *Effects of* B. thuringiensis *toxins*

The spruce budworm is susceptible to several Cry toxins (Van Frankenhuyzen *et al.* 1991, 1993), some of which also show *in vitro* activity against Cf1 cells (Schwartz *et al.* 1993; McCarthy, 1994*a*), as summarised in Table 1. Cell $Ca^{2+}$ measurements were conducted with 0.35 µmol $l^{-1}$ Cry1Aa, Cry1Ab, Cry1Ac and Cry1C, four lepidopteran-specific toxins, and 0.35 µmol $l^{-1}$ Cry3A, a coleopteran-specific toxin. In NBS, i.e. in the presence of 6.8 mmol $l^{-1}$ extracellular $Ca^{2+}$, neither Cry1Aa ($N$=9) nor Cry3A ($N$=6) elicited a $Ca^{2+}$ surge in Cf1 cells (results not shown). However, the cells responded to Cry1Ac, Cry1Ab and Cry1C exposure (Fig. 1). Cry1Ac triggered a large, sustained $Ca^{2+}$ surge. A 260 % increase in fluorescence ratio was observed. The signal reached 90 % of its maximum amplitude after 132±95 s (mean ± S.E.M., $N$=35). Cry1C also induced a sustained $Ca^{2+}$ surge, but the fluorescence ratio was only 20–60 % greater than the basal level and it took 405±110 s (mean ± S.E.M., $N$=13) to reach 90 % of the final level. The response to Cry1Ab was transient. Its peak amplitude was between those of Cry1Ac and Cry1C (a 130 % increase) and the time to 90 % peak was 221±123 s (mean ± S.E.M., $N$=18).

Table 1. *$Ca^{2+}$ response to, and toxicity of, Cry toxins*

| Activated *Bacillus thuringiensis* toxin | Cf1 cells: Intracellular $[Ca^{2+}]$ change in response to 0.35 µmol $l^{-1}$ toxin | Cf1 cells: *In vitro* toxicity: effective dose[b] (ng) | *Choristoneura fumiferana* larvae: *In vivo* toxicity: frass failure dose[c] (ng $larva^{-1}$) |
|---|---|---|---|
| Cry1Aa | No change | Non-toxic | 12.6 |
| Cry1Ab | ++ | 500 | 13.2 |
| Cry1Ac | +++ | 0.1 | 27.9 |
| Cry1C | + | 6.0 | 23.6 |
| Cry3A | No change | Non-toxic | Non-toxic |

[a]+++, large surge, short delay; ++, smaller surge, longer delay; +, smallest surge, longest delay.
[b]Lawn assay toxicity threshold (Schwartz *et al.* 1993).
[c]Force-feeding assay (Van Frankenhuyzen *et al.* 1991, 1993).

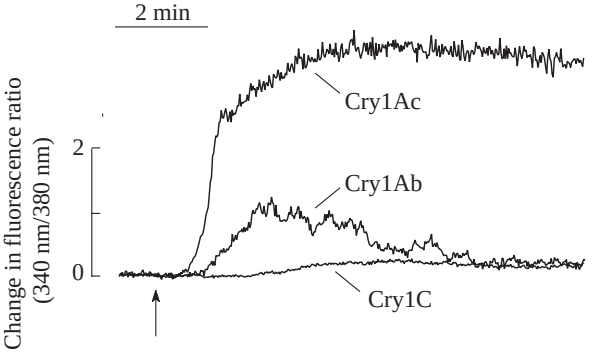


Fig. 1. $Ca^{2+}$ surges in single, Fura-2 loaded Cf1 cells in response to exposure to Cry toxin. The cells were bathed in a $Ca^{2+}$-rich physiological saline solution (NBS). Cry1Ab, Cry1Ac or Cry1C (0.35 µmol $l^{-1}$) was added to the bath at the time indicated by the arrows. Traces are representative of 35 experiments with Cry1Ac, 18 with Cry1Ab and 13 with Cry1C.

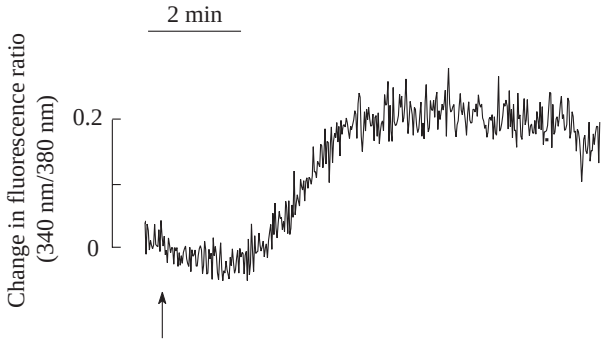


Fig. 2. $Ca^{2+}$ transient in response to exposure to Cry1Ac toxin in the absence of extracellular $Ca^{2+}$. The cell was bathed in 0NBS (see Materials and methods). Toxin addition is indicated by the arrow. The $Ca^{2+}$ surge is much smaller than that observed with $Ca^{2+}$ in the bath. The trace is representative of seven experiments.

In the absence of extracellular $Ca^{2+}$, Cry1Ac triggered a small, but significant, $Ca^{2+}$ surge (Fig. 2). The response was sustained over the measurement period. Its amplitude was 20 % greater than the basal fluorescence ratio ($N$=7). When tested in 0NBS, Cry1Aa, Cry1Ab, Cry1C and Cry3A did not affect the intracellular $Ca^{2+}$ concentration of the cells.

### *Effects of $Ca^{2+}$ transport modulators on Cry1Ac-induced $Ca^{2+}$ surges*

#### *$Ca^{2+}$ transport through the cell membrane*

$Ca^{2+}$ entry through the plasma membrane was investigated using D600, $Co^{2+}$, $Ni^{2+}$ and $La^{3+}$. D600 is an efficient blocker of voltage-dependent $Ca^{2+}$ channels (Triggle, 1990). $Co^{2+}$, $Ni^{2+}$ and $La^{3+}$ are general inhibitors of $Ca^{2+}$ transport across cell membranes (Tsien, 1990). The addition of 50 μmol $l^{-1}$ D600, either before or after Cry1Ac application, had no effect on the sustained [$Ca^{2+}$] elevation observed in the presence of extracellular $Ca^{2+}$ ($N$=11, results not shown).

Intracellular $Ca^{2+}$ concentration was unaffected by 5 mmol $l^{-1}$ $Co^{2+}$ or 5 mmol $l^{-1}$ $Ni^{2+}$ when added to NBS in the absence of Cry1Ac toxin. However, following Cry1Ac application in $Co^{2+}$- or $Ni^{2+}$-containing NBS, both 340 nm and 380 nm fluorescence intensities decreased (results not shown). It was verified in control experiments that the ions alone did not induce cell swelling or dye leakage. Thus, it appeared that, in the presence of Cry1Ac, $Ni^{2+}$ and $Co^{2+}$ entered the cells and quenched the $Ca^{2+}$ fluorophore. In fact, it has been reported that $Ni^{2+}$ and $Co^{2+}$ can enter melanotrophs through $Ca^{2+}$ channels and that Fura-2 is indeed quenched by these ions (Shibuya and Douglas, 1992).

When 5 mmol $l^{-1}$ $LaCl_3$ was added to NBS before the toxin, Cf1 cell $Ca^{2+}$ concentration was unaffected by exposure to Cry1Ac (Fig. 3). When $LaCl_3$ was added during the plateau phase of the $Ca^{2+}$ response to Cry1Ac, the surge was immediately interrupted, but the intracellular $Ca^{2+}$ concentration did not return completely to the baseline.

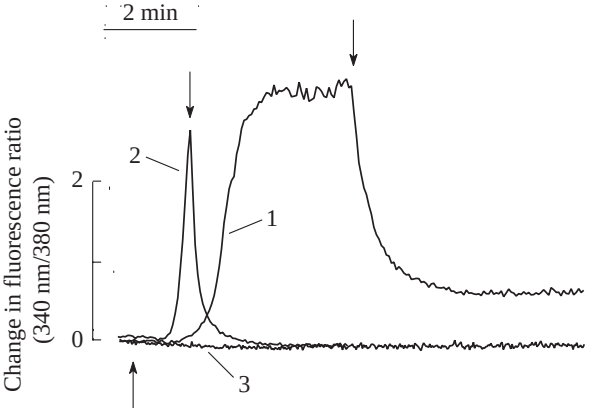


Fig. 3. Effect of $La^{3+}$ on the Cry1Ac-induced $Ca^{2+}$ response in single Cf1 cells. The cells were bathed in $Ca^{2+}$-rich physiological saline solution (NBS). Toxin addition is shown by the leftmost, upward-pointing arrow. Trace 1: $LaCl_3$ (5 mmol $l^{-1}$) was added to the bath during the plateau phase of the $Ca^{2+}$ surge (rightmost, downward-pointing arrow). It immediately inhibited the $Ca^{2+}$ response. Trace 2: the same effect was observed when $La^{3+}$ was added during the fast rising phase of the surge (middle, downward-pointing arrow). Trace 3: in this experiment, the cell was bathed in $La^{3+}$-containing NBS and then exposed to Cry1Ac. The trivalent cation prevented the development of the $Ca^{2+}$ surge. Traces are representative of five experiments.

#### *Cry1Ac channels are permeable to $Ca^{2+}$*

Cry1Ac forms $K^+$-selective channels in planar lipid bilayers (Slatin *et al.* 1990; Schwartz *et al.* 1997) and permeabilises Cf1 cells (Knowles and Ellar, 1987; B. Escriche, N. De Decker and E. Van Kerkhove, personal communication). The hypothesis that Cry1Ac channels are also permeable to divalent ions was tested in experiments using planar lipid bilayers. Under both symmetrical and non-symmetrical conditions (50 mmol $l^{-1}$ $CaCl_2$ in the *cis* chamber and either 50 mmol $l^{-1}$ or 450 mmol $l^{-1}$ $CaCl_2$ in the *trans* chamber), and with 0.3 μmol $l^{-1}$ Cry1Ac added to the *cis* chamber, discrete current jumps were observed (Fig. 4A, upper trace, non-symmetrical conditions). Under non-symmetrical conditions, the zero-current voltage was shifted to the right by 17 mV (Fig. 4B), consistent with $Ca^{2+}$ selectivity. The conductance of the channel was 141±27 pS ($N$=6) under symmetrical conditions and 185±32 pS (means ± S.E.M., $N$=5) under non-symmetrical conditions (Fig. 4A). The addition of 5 mmol $l^{-1}$ $CoCl_2$ to the *cis* chamber affected the kinetics of the channel dramatically without blocking it (Fig. 4A, middle trace). However, subsequent addition of 5 mmol $l^{-1}$ of $LaCl_3$ abolished channel activity (Fig. 4A, lower trace). These data demonstrate that $Ca^{2+}$ passes through Cry1Ac channels and that the trivalent ion $La^{3+}$ is an efficient blocker of the channel. They suggest that $Co^{2+}$ enters the channel and introduces at least one additional short-lived state to the kinetic behaviour of the channel: current jumps of similar amplitude but significantly shorter duration.

#### *Cell $Ca^{2+}$ mobilisation*

The results described above obtained using 6.8 mmol $l^{-1}$

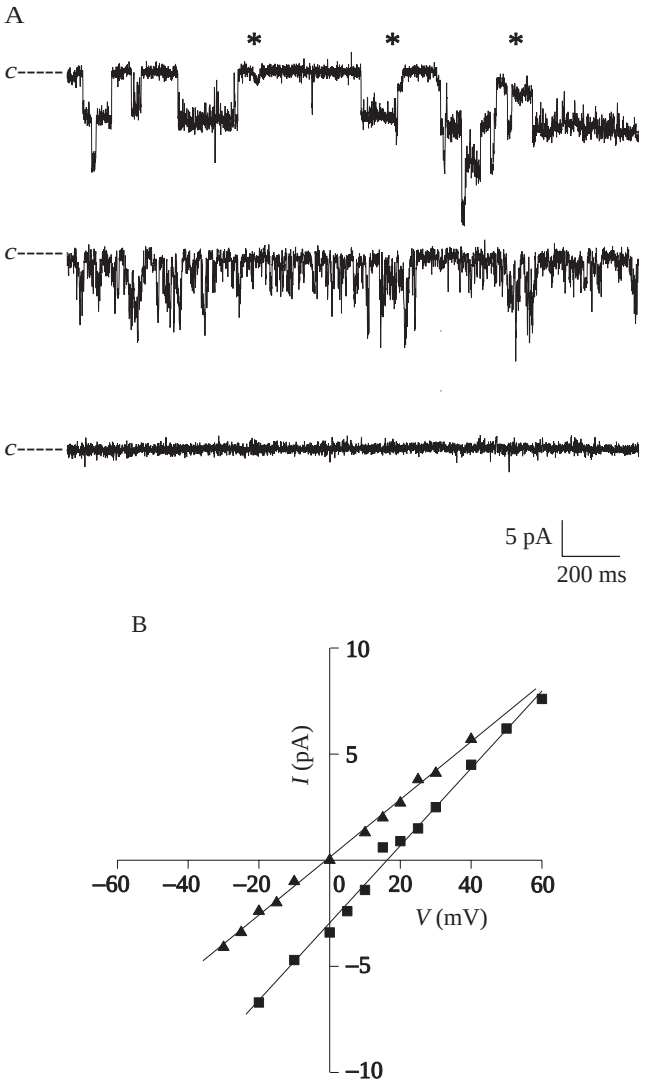


Fig. 4. (A) Single-channel current flowing through Cry1Ac toxin channels formed in planar lipid bilayers under non-symmetrical conditions (50 mmol $l^{-1}$ $CaCl_2$ in the *cis* chamber, 450 mmol $l^{-1}$ $CaCl_2$ in the *trans* chamber). The membrane potential was held at −20 mV (with respect to the *trans* chamber, which was held at ground potential). The three traces are from the same experiment in which 5 mmol $l^{-1}$ $CoCl_2$ and 5 mmol $l^{-1}$ $LaCl_3$ were sequentially added to the *cis* side of the membrane. The upper trace shows distinct current jumps, indicative of channel switching between the open and the closed states (indicated by the letter *c* and the dashed line to the left of the traces). There were at least three equally conducting channels in the phospholipid membrane. Subconducting levels were also observed (asterisks). The middle trace shows the effects of $Co^{2+}$ on Cry1Ac channel activity. Less than 60 s after addition of the divalent ion, channel current kinetics was significantly affected and assumed fast transitions between the open and closed states. Subsequent addition of $La^{3+}$ resulted in complete current inhibition, as shown in the lower trace. The data are representative of three experiments. (B) Current–voltage relationships for the Cry1Ac channel. The amplitude of the current (*I*) was plotted against applied voltage (*V*) for an experiment conducted under symmetrical (50 mmol $l^{-1}$ $CaCl_2$ in the *cis* chamber, 50 mmol $l^{-1}$ $CaCl_2$ in the *trans* chamber, triangular symbols) and under non-symmetrical (50 mmol $l^{-1}$ $CaCl_2$ in the *cis* chamber, 450 mmol $l^{-1}$ $CaCl_2$ in the *trans* chamber, square symbols) conditions. The *I–V* relationships were rectilinear ($r^2 > 0.98$) over the range of voltage tested and were shifted to the right when an ionic gradient was established across the channel, indicative of channel selectivity to $Ca^{2+}$. The channel conductance was 141±27 pS under symmetrical conditions (*N*=6) and 185±32 pS under non-symmetrical conditions (*N*=5) (means ± S.E.M.).

$Ca^{2+}$ in the bath indicated that the $Ca^{2+}$ surge observed in response to Cry1Ac exposure was probably due to the influx of the divalent ion through toxin-made pores. However, a $Ca^{2+}$ signal was also recorded when the toxin was applied to the cells in the absence of extracellular $Ca^{2+}$, suggesting that $Ca^{2+}$ was released from intracellular pools into the cytosol. In an attempt to identify the origin of the Cry1Ac-induced signal recorded in 0NBS, experiments were conducted using thapsigargin and TMB-8. These compounds modulate $Ca^{2+}$ transport across the membranes of $Ca^{2+}$ stores. Thapsigargin inhibits the $Ca^{2+}$-ATPase of $Ca^{2+}$ pools, thus increasing the level of cytosolic $Ca^{2+}$ by preventing $Ca^{2+}$ uptake into cellular stores (Thastrup *et al.* 1990). TMB-8 is an antagonist of $Ca^{2+}$ release from the endoplasmic reticulum (Chiou and Malagodi, 1975). Fig. 5 shows the effect of thapsigargin. In NBS, 100 nmol $l^{-1}$ thapsigargin elicited a large $Ca^{2+}$ transient. Subsequent addition of Cry1Ac resulted in a sustained elevation of cell [$Ca^{2+}$] similar to that observed with the toxin alone. In 0NBS, thapsigargin triggered a transient small rise in [$Ca^{2+}$]. Subsequent addition of Cry1Ac had no effect on the intracellular $Ca^{2+}$ concentration, which remained at the basal level. The same experimental protocol was used with TMB-8

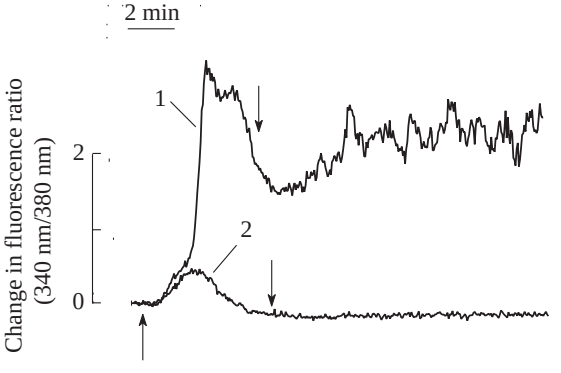


Fig. 5. Effect of thapsigargin on Cf1 intracellular [$Ca^{2+}$] and the Cry1Ac-induced $Ca^{2+}$ surge. Thapsigargin (100 nmol $l^{-1}$) was applied to the cells at the time indicated by the leftmost, upward-pointing arrow. Trace 1: in the presence of extracellular $Ca^{2+}$, the agent triggered a large $Ca^{2+}$ transient. Subsequent application of 0.35 μmol $l^{-1}$ Cry1Ac (downward-pointing arrow) elicited a second $Ca^{2+}$ surge. Trace 2: in the absence of extracellular $Ca^{2+}$, a small $Ca^{2+}$ transient was induced by thapsigargin. There was no further $Ca^{2+}$ response to the addition of Cry1Ac toxin at the time indicated by the downward-pointing arrow. Traces are representative of six experiments.

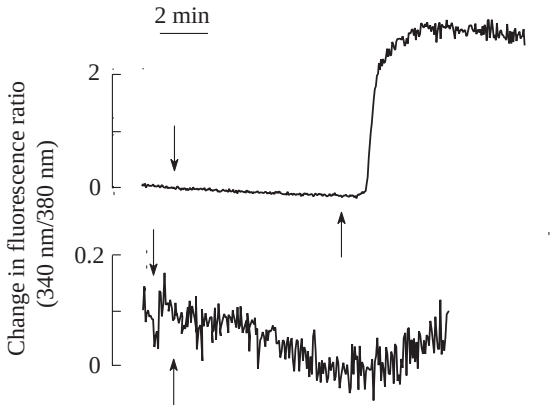


Fig. 6. Response to Cry1Ac in TMB-8-treated cells. Top trace: in $Ca^{2+}$-rich physiological solution, 50 μmol $l^{-1}$ TMB-8 was applied to the cell (downward-pointing arrow). The addition of 0.35 μmol $l^{-1}$ Cry1Ac (upward-pointing arrow) evoked a large $Ca^{2+}$ surge. The trace is representative of three experiments. Bottom trace: when the cell was bathed in $Ca^{2+}$-free medium and 50 μmol $l^{-1}$ TMB-8 was added to the bath (downward-pointing arrow), 0.35 μmol $l^{-1}$ Cry1Ac (upward-pointing arrow) failed to trigger a $Ca^{2+}$ signal. The trace is representative of three experiments.

(50 μmol $l^{-1}$), and the results are illustrated in Fig. 6. In NBS, there was no response to TMB-8 alone, and Cry1Ac induced its typical $Ca^{2+}$ surge in the presence of the drug. In 0NBS, TMB-8 had no effect on cell [$Ca^{2+}$]. Further addition of Cry1Ac failed to trigger a $Ca^{2+}$ surge.

## Discussion

This study demonstrates that cytotoxic *B. thuringiensis* proteins triggered $Ca^{2+}$ surges in Cf1 cells, and the results are similar to previous studies in our laboratory in which we reported comparable $Ca^{2+}$ responses in Sf9 cells (Schwartz *et al.* 1991) and UCR-SE-1a cells (Monette *et al.* 1994) exposed to Cry1C toxin, to which both cell lines are susceptible (McCarthy, 1994*a*,*b*). Table 1, which summarises Cf1 toxicity data (Van Frankenhuyzen *et al.* 1991; Schwartz *et al.* 1993), and the results of the present study show that non-cytotoxic proteins (Cry1Aa and Cry3A) failed to elicit a change in Cf1 intracellular $Ca^{2+}$ concentration, whereas Cry1Ab, Cry1Ac and Cry1C, which are active against the cell line, induced $Ca^{2+}$ surges in the cells. The correlation between toxicity and the extent of intracellular $Ca^{2+}$ activity was not perfect: Cry1C, which is more toxic than Cry1Ab, induced smaller $Ca^{2+}$ surges in the cells. However, of the three proteins, Cry1Ac, the most toxic to Cf1 cells, triggered the largest $Ca^{2+}$ response, suggesting that the increase in cell [$Ca^{2+}$] participates in the mode of action of the toxin. This is consistent with our previous work on Sf9 cells, which demonstrated that Cry1C toxicity was related to extracellular $Ca^{2+}$ concentration in a dose-dependent manner and that this effect was influenced by several $Ca^{2+}$ transport modulators, implying that changes in intracellular [$Ca^{2+}$] may be related to cytotoxicity (Monette *et al.* 1997).

The results of this study clearly show that the Cry1Ac-induced $Ca^{2+}$ surge had two components: a large $Ca^{2+}$ surge was recorded in the presence of $Ca^{2+}$ in the bath, and a small transient $Ca^{2+}$ signal took place in a $Ca^{2+}$-free environment. Our data indicate that the large surge was due to the influx of $Ca^{2+}$ into the cell. $La^{3+}$, which is known to inhibit the cell $Ca^{2+}$ extrusion mechanisms (Triggle, 1990), had no effect on cell [$Ca^{2+}$] in the absence of Cry1Ac. However, $La^{3+}$ prevented the $Ca^{2+}$ surge when added to the bath before the toxin and terminated the toxin-induced response when applied after the toxin. This suggests that the trivalent ion blocked the pathway used by $Ca^{2+}$ to enter the cells. Experiments with D600, a general inhibitor of voltage-dependent $Ca^{2+}$ channels (Tsien, 1990), showed that such channels were not involved in toxin-induced $Ca^{2+}$ influx, either because Cf1 cells do not possess voltage-dependent $Ca^{2+}$ channels or because, under our experimental conditions, the cells were fully depolarised and the channels were inactivated. Indeed, preliminary whole-cell patch-clamp experiments in our laboratory and elsewhere (B. Escriche, N. De Decker and E. Van Kerkhove, personal communication) indicated that the resting potential of Cf1 cells was close to 0 mV. While other $Ca^{2+}$-permeable channels that could be activated by Cry1Ac and inhibited by $La^{3+}$ may exist in Cf1 cells, the more likely explanation for the large rise in $Ca^{2+}$ response to toxin exposure is that $Ca^{2+}$ entered the cells through the pores formed by the insertion of toxin into the cell membrane. Several studies have shown that Cry toxins formed ion channels in planar lipid bilayers (Slatin *et al.* 1990; Schwartz *et al.* 1993, 1997; English *et al.* 1991; Von Tersch *et al.* 1994; Grochulski *et al.* 1995; Lorence *et al.* 1995) and single insect cells (Schwartz *et al.* 1991; Monette *et al.* 1994). The toxins also permeabilised liposomes (Yunovitz and Yawetz, 1988; Haider and Ellar, 1989; English *et al.* 1991; Butko *et al.* 1994), midgut brush-border membranes vesicles (Sacchi *et al.* 1986; Hendrickx *et al.* 1989; Wolfersberger, 1989; Uemura *et al.* 1992; Carroll and Ellar, 1993; Lorence *et al.* 1995; Martin and Wolfersberger, 1995) and isolated midguts (Harvey and Wolfersberger, 1979; Liebig *et al.* 1995; Peyronnet *et al.* 1997). In the present study, we have provided evidence that divalent ions entered toxin-exposed cells, most probably through the toxin pores, as demonstrated by Fura-2 quenching by $Ni^{2+}$ and $Co^{2+}$. Furthermore, using planar lipid bilayers, we have demonstrated that Cry1Ac channels are indeed permeable to $Ca^{2+}$.

Interestingly, in the absence of extracellular $Ca^{2+}$, Cry1Ac induced a small $Ca^{2+}$ transient in Cf1 cells, a clear indication that the effect of the toxin on intracellular [$Ca^{2+}$] could not be solely attributed to the influx of $Ca^{2+}$ through toxin-made pores. Such a response when $Ca^{2+}$ was omitted from the bath was also observed in a previous study on the effects of Cry1C on UCR-SE-1a cells (Monette *et al.* 1994). It has been reported that, in both the Cf1 cell line and in a *Mamestra brassica* (cabbage moth) cell line, adenylate cyclase was activated by *B. thuringiensis* proteins that are toxic to these cells (Knowles and Farndale, 1988). However, in the *M. brassica* cells, this effect could not be related to the cytolytic mechanism, and it

was suggested that the toxins interacted with cell membrane components, possibly lipids, thus affecting adenylate cyclase activity. In Cf1 cells, hormone-mediated receptor responses include the activation of adenylate cyclase and phospholipase C (Orr *et al.* 1988). Adenylate cyclase, which produces cyclic AMP, is sensitive to intracellular $Ca^{2+}$ concentration and protein kinase C, a $Ca^{2+}$-activated, phospholipid-dependent enzyme. This protein is stimulated by diacylglycerol, one of the two second messengers produced by phosphoinositide metabolism (Rasmussen and Barrett, 1984). Protein kinase C has recently been characterised in Cf1 cells (Gupta and Downer, 1993). Our data on *B. thuringiensis*-induced $Ca^{2+}$ mobilisation from thapsigargin- and TMB-8-sensitive stores indicate that the inositol trisphosphate second messenger may also be produced in Cf1cells. The mechanism by which $Ca^{2+}$ is released from organelles in response to toxin exposure has yet to be investigated. It is tempting to speculate that, upon binding to a specific, as yet poorly identified, surface receptor (Knowles and Ellar, 1986), which may be coupled to both adenylate cyclase and phospholipase C, *B. thuringiensis* toxins induce intracellular $Ca^{2+}$ signalling and the production of protein kinase C, which in turn modulates cyclic AMP levels, as observed by Knowles and Farndale (1988). Further studies are needed to examine the role that such signals may play in the mode of action of the *B. thuringiensis* protein.

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