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Interaction between Calcium Ions and *Bacillus thuringiensis* Toxin Activity against Sf9 Cells (*Spodoptera frugiperda*, Lepidoptera)†

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The effects of calcium ions and modulators of calcium movement on *Bacillus thuringiensis* insecticidal protein toxicity were investigated with Sf9 cells (*Spodoptera frugiperda*, fall armyworm) by a new *B. thuringiensis* toxicity assay based on measurement of fluorescence of ethidium homodimer, a high-affinity DNA stain. CryIC toxicity was substantially stimulated by extracellular calcium in a dose-dependent way (in the millimolar range), while toxicity enhancement could not be replicated when calcium was replaced by barium. This incremental toxicity was reduced by cobalt and lanthanum ions, two inorganic-calcium transport inhibitors. Methoxyverapamil, a voltage-dependent calcium channel blocker, and nifedipine, an inhibitor of dihydropyridine-sensitive L-type calcium channels, had no effect on CryIC toxin activity, but BAY K 8644, an L-type calcium channel activator, increased CryIC activity at high concentrations of extracellular calcium. While A23187, a calcium ionophore, and TMB-8, an inhibitor of intracellular-calcium mobilization, did not change CryIC-induced mortality, thapsigargin, an inhibitor of calcium uptake in intracellular stores, and more particularly trifluoperazine, which inhibits calcium-calmodulin-dependent processes, increased CryIC-mediated toxicity. The incremental effect of extracellular calcium on CryIC-induced toxicity was consistent with an increased concentration of intracellular calcium.

Bacillus thuringiensis is the major source of environment-friendly, biological pesticides currently used in Canada to control forest pests (53). *B. thuringiensis* products, in addition to efficiently and economically controlling agricultural and forestry insect pests, are playing a growing role worldwide in public health protection and veterinary medicine by providing a powerful weapon against insects that are vectors of several serious human and animal diseases. *B. thuringiensis* is a gram-positive soil bacterium that produces during sporulation a large intracellular crystal containing one or more toxic proteins. These inclusions, once activated in the gut, display a high level of specificity against lepidopteran, dipteran, or coleopteran insect larvae (22). Recently discovered *B. thuringiensis* toxins have been shown to be active against protozoan pathogens, parasitic liver flukes, and acarians (7). At least 15 genes for *B. thuringiensis* proteins have been identified and grouped into six different classes and several subclasses, based primarily on genetic data and their toxicity spectra against insects and other organisms (14). Although little resistance to *B. thuringiensis* toxins has been observed in the past, several cases of field resistance to the pesticide have been reported recently, thus prompting new investigations of the origin of the *B. thuringiensis* resistance process and ways to prevent or delay its occurrence (46).

The exact mode of action of *B. thuringiensis* proteins is not well understood (9, 19) and, with recent reports of insect resistance to the pathogen, its elucidation is becoming imperative. Following solubilization and activation of the crystal in the target insect gut, the toxin interacts with specific surface receptors of midgut cells and forms pores in the cell membrane. This disrupts the ion balance of the cells, resulting in cell swelling and eventual lysis (19). It is generally accepted that the presence of specific receptors on the apical membrane plays an essential role in the specificity of the pathogen (19). Toxin potency may depend on receptor abundance and its affinity for the protein. It may also depend on the ability of the toxin to form efficient pores (9, 11, 25).

Major breakthroughs that may lead to a better understanding of the mode of action of the toxin have been made recently. The three-dimensional structures of CryIIIA, a coleopteran-specific *B. thuringiensis* toxin, and of CryIA(a), a lepidopteran-specific *B. thuringiensis* toxin, have been elucidated (11, 25). Membrane proteins that bind CryIA toxins have been purified, and their genes have been cloned (18, 28, 40, 52). This allows novel approaches with rationally designed proteins for structure-function studies (44, 55) and physiological investigations of cellular responses to *B. thuringiensis* toxin exposure.

So far, only limited attention has been given to *B. thuringiensis* toxin interaction with physiological processes at the cell level, possibly due to lack of appropriate cellular models. Until we reported the first midgut epithelial cell culture (2), only a few *B. thuringiensis* toxin-susceptible insect cell lines were 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 less than that of the insects from which they originate, they allow appropriate detection of entomocidal activity of activated *B. thuringiensis* products with reasonable species and interspecies selectivity (17).

Midgut epithelial cells in culture are difficult to immobilize, and their physiological properties have not yet been fully characterized (2). On the other hand, among the available *B. thuringiensis* toxin-sensitive cell lines, Sf9 cells are extensively used for heterologous protein expression with the baculovirus system (26) and functional studies have provided information on several physiological mechanisms in these cells (15, 16). There-

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fore, Sf9 cells constitute a simple and convenient model for the study of *B. thuringiensis*'s mechanism of action at the cellular level. Recently, the cellular effects of CryI toxins on Sf9 cells have been the object of intense scrutiny in our laboratories (42, 43, 50, 51). Sf9 cells cloned from the IPLB-Sf21AE parent line derived from pupal ovarian tissue of *Spodoptera frugiperda* are sensitive to CryIC and, to a much lesser extent, to CryIA(b) toxins (43). There is a K^+-H^+ exchanger in Sf9 cells (50), and CryIC induces the rapid diffusion and equilibration of K^+, Na^+, and H^+ ions across the plasma membrane (51). In an earlier study, we demonstrated that CryIC triggers an intracellular calcium surge within seconds following toxin exposure and, thereafter, that the pathogen activates anion-selective channels in the cell membrane (42). We observed a similar CryIC-mediated calcium response in UCR-SE1a cells, a *Spodoptera exigua* cell line (30). These data suggest that cellular calcium changes related to toxin exposure represent an early step in the activity of the toxin. However, the origin of this calcium surge and its contribution to toxicity are unknown. Interestingly, calcium salts have been shown to act synergistically with *B. thuringiensis* toxins against several lepidopteran pests in vivo (6, 29, 32, 37–39) and a recent in vivo study of the interaction of caffeine with *B. thuringiensis* toxin activity against the bertha armyworm suggests that the observed augmented toxicity is mediated by deregulation of cellular-calcium transport processes (31).

Extracellular calcium is one of several modulators of cellular-calcium homeostasis, and proper regulation of intracellular-calcium concentration is of paramount importance to cellular function (3). Cellular-calcium concentration is controlled by the finely tuned interaction between the transport of calcium ions across membranes and their compartmentalization in cellular organelles (34). Disruption of calcium homeostasis, as a result of enhanced calcium influx, reduced efflux, compromised uptake and release by intracellular-calcium stores, or inadequate calcium buffering, will likely result in several cytotoxic effects (33, 45).

While *B. thuringiensis* toxin exposure triggers an immediate intracellular-calcium surge in susceptible cells (30, 42), little is known about the interaction between extracellular or intracellular calcium and *B. thuringiensis*-induced toxicity at later stages of the intoxication process. In this study, we investigated the effects of extracellular-calcium and cellular-calcium transport modulators on the toxicity induced by 3 h of exposure of Sf9 cells to CryIC toxin. Toxicity was assessed by a newly designed, efficient, fluorescence assay based on nucleus staining by ethidium homodimer, a DNA-intercalating probe (8, 27). We demonstrate that a significant dose-dependent increase in CryIC-induced toxicity occurred when calcium ions were added to the medium. However, the presence of extracellular calcium was not required for CryIC activity. The physiological mechanisms which may underlie this interaction between extracellular calcium and toxicity were examined with several agents which are known to modulate the intracellular-calcium concentration. Cobalt and lanthanum ions reduced efficiently calcium-dependent CryIC-induced toxicity. On the other hand, BAY K 8644, thapsigargin, and particularly trifluoperazine (TFP) augmented substantially the activity of the toxin.

MATERIALS AND METHODS

Cells. Sf9 cells (ATCC CRL1711) were seeded into spinner flasks to an initial density of about 10^6 cells/ml in Grace's medium supplemented with 3.33 mg of yeastolate/liter, 3.33 mg of lactalbumin hydrolysate/liter, and 10% heat-inactivated fetal bovine serum. Cells were incubated at 27°C with constant stirring at 50 to 100 rpm. In preparation for the experiments, cell aliquots were transferred to 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, N.Y.) filled with supplemented Grace's medium and kept at 27°C in an air incubator for 48 h or more. Experiments were conducted when cells were firmly attached and had reached 80 to 90% confluence.

Experimental procedures. (i) Solutions. The calcium-free minimal bath solution (0MBS) contained 50 mM KCl, 21 mM NaCl, 58.4 mM NMDG-Cl (*N-methyl*-D-glucamine-chloride), 14 mM $MgCl_2$, 11 mM $MgSO_4$, 3.9 mM D-glucose, 20 mM PIPES [1,4-piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], and 2 mM EGTA. Osmolarity was adjusted to 390 mosM with sucrose, and pH was adjusted to 6.4 with NaOH. Calcium-rich MBSs with final calcium concentrations of 6.8 mM (6.8MBS), 13.6 mM (13.6MBS), and 27.2 mM (27.2MBS) were obtained by adding to 0MBS 8.8, 15.6, or 29.2 mM $CaCl_2$, respectively, while the concentration of NMDG-Cl was reduced accordingly to keep the total chloride ion concentration constant.

Concentrated stock solutions of test agents were prepared in 0MBS or, when required, in dimethyl sulfoxide (DMSO), ethanol, or acetone, from which working solutions at various dilutions in 0MBS were derived. This allowed the delivery of constant volumes (10 μl) to each test well to obtain the desired final concentrations of the reagents, which were within the range of those reported in several channel and cell-signalling studies (4, 13, 15, 16, 30, 41, 42, 47, 48, 54). Ethidium homodimer-1 stock solution (12 mM) was prepared in DMSO, from which a 150 μM working solution in 0MBS was derived. The cell-staining solution (final concentration, 6 μM) was obtained by diluting the proper amount of working solution in 0MBS, 6.8MBS, 13.6MBS, or 27.2MBS.

(ii) Viability assay. Sf9 cell viability was assessed with ethidium homodimer-1, a membrane-impermeant fluorescent probe which undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids (10). Compared to other commonly used nucleic acid stains, like ethidium bromide, for example, ethidium homodimer is more impermeable to viable cell membranes, binds 100 to 1,000 times better to nucleic acids, and fluoresces more once it is bound to DNA (8). The compromised membranes of intoxicated or dying cells become permeable to the probe, which enters the cells and their nuclei. Under 528-nm excitation, nuclei are intensely stained red (617-nm emission). Therefore, the ethidium homodimer fluorescence measurement provides a simple way to monitor cells whose health is deteriorating but which have not reached the terminal state of cellular disruption.

Experiments were conducted with a multiwell-plate fluorescence scanner (Cytofluor 2300; Millipore Corp., Bedford, Mass.) equipped with a 530- by 30-nm half-bandwidth excitation filter and a 620- by 40-nm half-bandwidth emission filter. After two washes in 6.8MBS, cells were preincubated for 30 min at 27°C in 180 μl of the staining solution alone/well, i.e., 6 μM ethidium homodimer-1 in 0MBS, 6.8MBS, 13.6MBS, or 27.2MBS. The calcium modulators were then added to the wells (10-μl aliquots from the proper working solutions), and the same volume of 0MBS was added to the control wells. Following a 30-min incubation of the cells at 27°C, 10-μl aliquots of the appropriate toxin working solutions were added to the test wells to attain the desired final toxin concentrations and equivalent volumes of toxin-free media were added to the control wells. The multiwell plate was immediately placed in the fluorescence plate reader for the first set of fluorescence readings (the time zero [t_0] measurement). The purpose of the t_0 measurement was twofold. First, it was used to ascertain good viability of the cells at the beginning of the experiment, i.e., with or without calcium modulators and within minutes following toxin (or vehicle only) addition to the wells. This was done by verifying that the fluorescence intensity levels were homogeneously distributed through the entire well plate. Second, it provided for each well the reference level of fluorescence to which the next set of readings was compared.

Time-course experiments were conducted to monitor the fluorescence level for a 5-h period after addition of *B. thuringiensis* toxins to test wells and to compare it to that of control wells (data not shown). These experiments established that for CryIC doses of less than 25 μg/ml, ethidium homodimer fluorescence of intoxicated cells increased continuously over this period of time. For higher doses (up to 50 μg/ml), fluorescence reached a maximum after approximately 4 h and decreased thereafter, indicative of stained DNA leakage in the medium, i.e., cell lysis. Measurements of toxin effect in the absence or the presence of calcium-modulating agents were therefore conducted after a 3-h exposure (t_{180} measurement), which provided a signal sufficiently intense to be detected over the baseline (t_0 measurement). This approach minimized both the amount of time the cells spent outside the incubator and the amount of data to analyze.

After completion of the t_0 measurement, the plate was returned to the incubator for 180 min, after which the t_{180} set of readings was taken. Reading of an entire 96-well plate took about 5 min. In each experimental session, two microtiter plates were generally read in succession and under identical experimental conditions.

(iii) Intracellular-calcium measurement. Separate control experiments were conducted (i) to determine the intracellular-calcium level in Sf9 cells at the time of *B. thuringiensis* toxin addition and (ii) to assess, with octopamine as a physiological stimulant, the functional competence of the cells after a 30-min incubation in medium containing various levels of calcium. Octopamine, an insect neurohormone, mobilizes calcium from intracellular stores in Sf9 cells (15). The cytosolic-calcium concentration was measured with Fura 2 as described previously (42). Briefly, cells cultured on glass coverslips were loaded for 30 min in 6.8MBS with 1 μM Fura 2–acetoxymethyl ester, the calcium-insensitive, mem-

brane-permeant form of Fura 2, a calcium-sensitive fluorescence indicator. After a 10-min incubation in dye-free 6.8MBS, intracellular Fura 2-AM was hydrolyzed into membrane-impermeant Fura 2 by cellular esterases and thus trapped intracellularly. Upon binding of Fura 2 to intracellular calcium (dissociation constant at room temperature, 135 nM), the excitation spectrum of the probe displayed a dose-dependent shift towards lower wavelengths with no change in the emission peak (505 nm). This spectral property of Fura 2 allowed us to use it for high-sensitivity, usually artifact-free ratiometric determination of intracellular-calcium concentration with dual excitation at 340 and 380 nm (12).

Coverslips were mounted in a custom-made experimental chamber containing 1 ml of 0MBS, 6.8MBS, 13.6MBS, or 27.2MBS for 30 min, during which the fluorescence levels of single cells or a small group of cells were measured with a microscope-based, dual-excitation photometric instrument (Photon Technology International, Monmouth Junction, N.J.). Calcium concentrations were derived from fluorescence data with calibration buffers (12).

Toxins. Toxins were produced, activated, purified, and tested for purity as described previously (42). They were stored in lyophylized form and reconstituted to a final concentration of 1 mg/ml in high-purity water with 10 mM Tris at pH 10.0. Diluted working solutions of various concentrations were prepared in the same buffer, from which 10-μl aliquots were taken and added to the wells to attain the desired final toxin concentrations. CryIC's 50% cytotoxic dose for Sf9 cells is 40 μg/ml (42), and for IPLB-Sf21AE, the parental cell line, it is 15.8 μg/ml (17). Therefore, doses in the 0.5- to 50-μg/ml range were used in this study.

Chemicals. Grace's insect cell culture medium, yeastolate, lactalbumin hydrolysate, and fetal bovine serum were purchased from Gibco BRL (Life Technologies, Burlington, Ontario, Canada). PIPES, Tris, TFP dimaleate, sodium dodecyl sulfate (SDS), octopamine, and A23187 (calcimycin from *Streptomyces chartreusis*) were obtained from Sigma, St. Louis, Mo. TMB-8 [8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride], thapsigargin (a naturally occurring sesquiterpene lactone), D600 (methoxyverapamil, a phenethylamine derivative), nifedipine, and BAY K 8644 (two 1,4-dihydropyridines) were purchased from Calbiochem Corp., La Jolla, Calif. EGTA was from Fluka (Caledon Lab, Georgetown, Ontario, Canada). Dehydrated DMSO was purchased from Aldrich Chemicals, Milwaukee, Wis. Ethidium homodimer-1 and Fura 2-AM were obtained from Molecular Probes, Eugene, Oreg.

Data analysis. Data were stored and analyzed on a personal computer. Toxicity was determined by comparing the fluorescence intensity level in each well at t_{180} with that in the same well at t_0, i.e., immediately after addition of the toxin. A toxicity index was defined as the ratio of these two values. Due to variability in toxicity index values obtained under identical experimental conditions but in different experimental sessions, intersession data pooling was not possible. Toxicity index values for each condition were therefore determined in quadruplicate with preparations obtained from the same-day cultures. They are given as means ± standard errors of the means. CryIC toxicity in the presence of reagents known to modulate calcium transport and/or intracellular-calcium concentration was compared to that of the toxin alone under various concentrations of external calcium by two-factor, multiple-replicate analysis of variance (ANOVA) ($P < 0.05$). Percent changes of toxicity under identical conditions of external-calcium concentrations were calculated as 100 × [(toxicity index in the presence of the calcium modulator/toxicity index in the absence of the calcium modulator) − 1]. The significance of these changes was assessed from post hoc analysis (Tukey's honest significant difference test; $P < 0.05$) of the two-factor, multiple-replicate ANOVA results.

RESULTS

To study the interaction between calcium ions and toxicity in Sf9 cells exposed for 3 h to *B. thuringiensis* toxins, we developed a 96-well-plate fluorescence assay based on ethidium homodimer staining of nuclear DNA in compromised cells. Control experiments showed that the fluorescence level from cells that were not exposed to an active *B. thuringiensis* toxin remained essentially constant during the course of an experiment, whether the cells were incubated in MBS alone or in MBS with calcium modulators. This confirms that cell viability was only minimally affected by time and bath composition.

To be useful for screening new *B. thuringiensis* products and, more generally, for the study of the *B. thuringiensis* toxin mode of action, our assay should discriminate between active and inactive *B. thuringiensis* toxins and provide dose-response data. Therefore, intoxication experiments were conducted with CryIA(a), CryIA(b), CryIA(c), CryIC, CryIIA, and CryIIIA proteins. Among these toxins, only CryIC is active against *Spodoptera* species in vivo (17, 37, 38). Similarly, *Spodoptera frugiperda* cell lines are highly sensitive to CryIC (17, 42). CryIA(b) is moderately toxic to Sf9 cells (43) and IPLB-Sf21AE cells (17), and the other toxins have no effect on *Spodoptera* cell lines. By our ethidium homodimer assay in 6.8MBS, which corresponds to the calcium concentration of Grace's culture medium, it was found that among the proteins tested, only CryIC was active against Sf9 cells (Fig. 1A). A positive control was provided by 0.025% SDS, an anionic surfactant which disrupts cell membranes (1). Figure 1B shows the dose-response curve of CryIC from which a 50% effective dose of approximately 15 μg/ml was derived. This value is consistent with those obtained by other methods (17, 42). The assay clearly discriminates between active and inactive toxins and relates the level of toxicity to the dose of an active toxin to which cells are exposed.

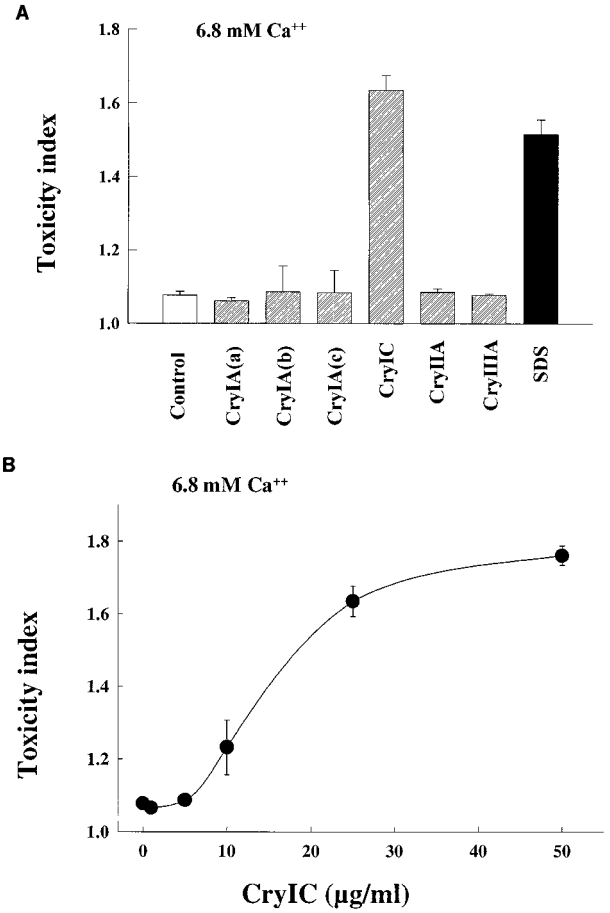

FIG. 1. (A) Specific activities of *B. thuringiensis* toxins against Sf9 cells. Six different Cry toxins (25 μg/ml; hatched bars) were tested in 6.8MBS. Control experiments were conducted under the same conditions but without toxin (open bar) or without toxin but in the presence of 0.025% SDS (filled bar). (B) CryIC dose-response curve for cells incubated in 6.8MBS.

Effect of extracellular-calcium and barium ions. In the absence of toxin, increasing the extracellular-calcium concentration from 0 to 27.2 mM did not affect Sf9 cell viability (Fig. 2). Similarly, CryIA(a), which was inactive in 6.8 mM external calcium (Fig. 1A), had no effect at any of the other calcium concentrations tested (Fig. 2). This result was also obtained with CryIA(b), CryIA(c), CryIIA, and CryIIIA (not shown). However, CryIC toxicity was significantly affected by extracellular calcium (Fig. 2). Toxin-mediated Sf9 mortality increased with the external-calcium concentration in a dose-dependent manner. In the absence of extracellular calcium, a residual toxicity which was also dose dependent (Fig. 2) was observed.

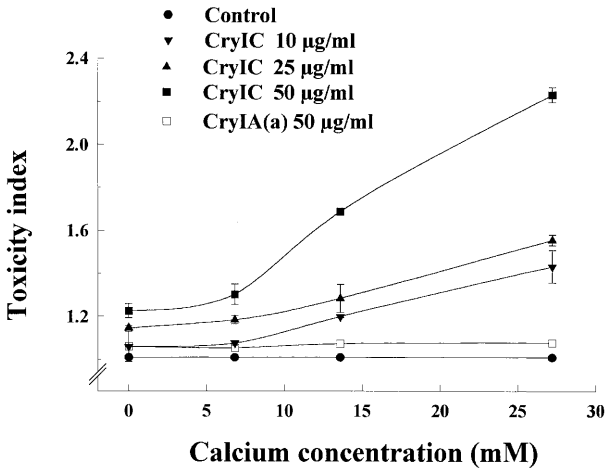

FIG. 2. Calcium dose-response curves of CryIC intoxication of Sf9 cells. Control experiments were conducted in the absence of the toxin (control) or in the presence of CryIA(a) toxin.

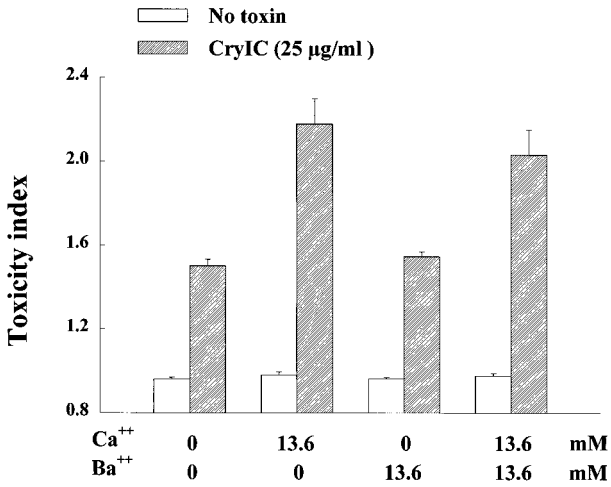

FIG. 4. Effect of barium ions on CryIC toxin-induced mortality.

Information on the influence of changes in extracellular-calcium concentration on the level of free intracellular calcium and on the functional competence of the cells was required for a more complete interpretation of the results of CryIC-induced toxicity experiments with calcium modulators. Therefore, Fura 2 experiments were conducted on single Sf9 cells incubated for 30 min in 0MBS, 6.8MBS, 13.6MBS, or 27.2MBS. Raising extracellular calcium from 0 to 27.2 mM induced a 2.4-fold increase in the basal concentration of intracellular calcium (Fig. 3). After a 30-min incubation in 6.8MBS, 13.6MBS, or 27.2MBS, cells displayed calcium transients in response to 50 μM octopamine (Fig. 3, insets). The cells were therefore physiologically competent. While a similar response was observed shortly after removal of calcium from the bath (not shown), the ligand failed to elicit a calcium signal after a 30-min incubation in 0MBS (Fig. 3, inset), indicative of depletion of an octopamine-sensitive intracellular pool.

Barium is not a substrate for calcium transporters or pumps in mammalian systems (41). This divalent cation, however, is a potassium channel blocker (21) and permeates certain classes of calcium channels better than calcium (13). While maintaining a constant ionic strength, we conducted experiments in which calcium was replaced by barium (Fig. 4). In the absence of toxin, viability of the cells was not affected by the presence of the divalent ion. Total substitution of calcium by barium did not affect CryIC residual toxicity, i.e., the toxicity observed in the absence of calcium. However, this maneuver failed to induce the amplifying effect on toxicity observed with increasing extracellular-calcium concentrations. With an equimolar mixture of calcium and barium in the bath, CryIC caused the same mortality as with calcium alone.

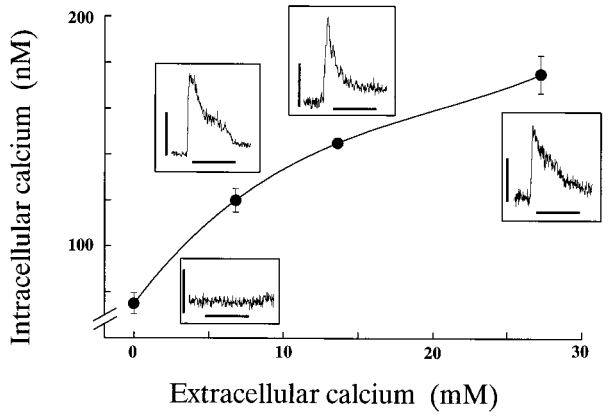

FIG. 3. Effect of extracellular-calcium concentration on Sf9 cell intracellular-calcium concentration. The graph represents the dose response of basal intracellular calcium after a 30-min incubation under each extracellular-calcium-concentration condition. Insets near datum points show calcium signals triggered by exposure of the cells to 50 μM octopamine after a 30-min incubation under each extracellular-calcium concentration condition. Octopamine responses were recorded at the beginning and at the end of each experiment. Horizontal bars, 200 s; vertical bars, 150 nM.

Effect of agents which modulate calcium transport through the cell membrane. Interaction between CryIC toxicity and calcium movement across the plasma membranes of Sf9 cells was investigated with cobalt and lanthanum ions, D600, nifedipine, BAY K 8644, and A23187. Cobalt and lanthanum ions are general inhibitors of calcium transport across cell membranes (49). D600 blocks voltage-dependent calcium channels (48). Nifedipine and BAY K 8644 are dihydropyridines which affect specifically L-type calcium channels, a particular class of voltage-dependent calcium channels. Nifedipine is a potent inhibitor (13), and BAY K 8644 activates these channels (49). A23187 is a calcium ionophore that transports this ion across cell membranes, thus equilibrating concentrations of extracellular and intracellular calcium (36).

(i) Cobalt and lanthanum ions. In the absence of toxin, cobalt and lanthanum ions had hardly any effect on cell viability (Fig. 5A and B), but both cations reduced significantly, at any dose (in the millimolar range) and in a dose-dependent manner, the toxic effect of CryIC, with the inhibition being more efficient under conditions of high-concentration extracellular calcium (Fig. 5C and D). The residual activity of the toxin in the absence of extracellular calcium was also substantially reduced by cobalt and lanthanum (Fig. 5A and B).

(ii) Voltage-dependent calcium channel modulators. In an attempt to characterize the source of calcium-CryIC toxicity interaction, experiments were conducted with D600, nifedipine, and BAY K 8644. When tested alone, these agents were not toxic to Sf9 cells (data not shown). Table 1 summarizes the effect on CryIC toxicity of the compounds tested under conditions with three concentrations of extracellular calcium. The activity of the toxin was unaffected by 50 μM D600 or nifedipine. On the other hand, CryIC-mediated cell mortality was

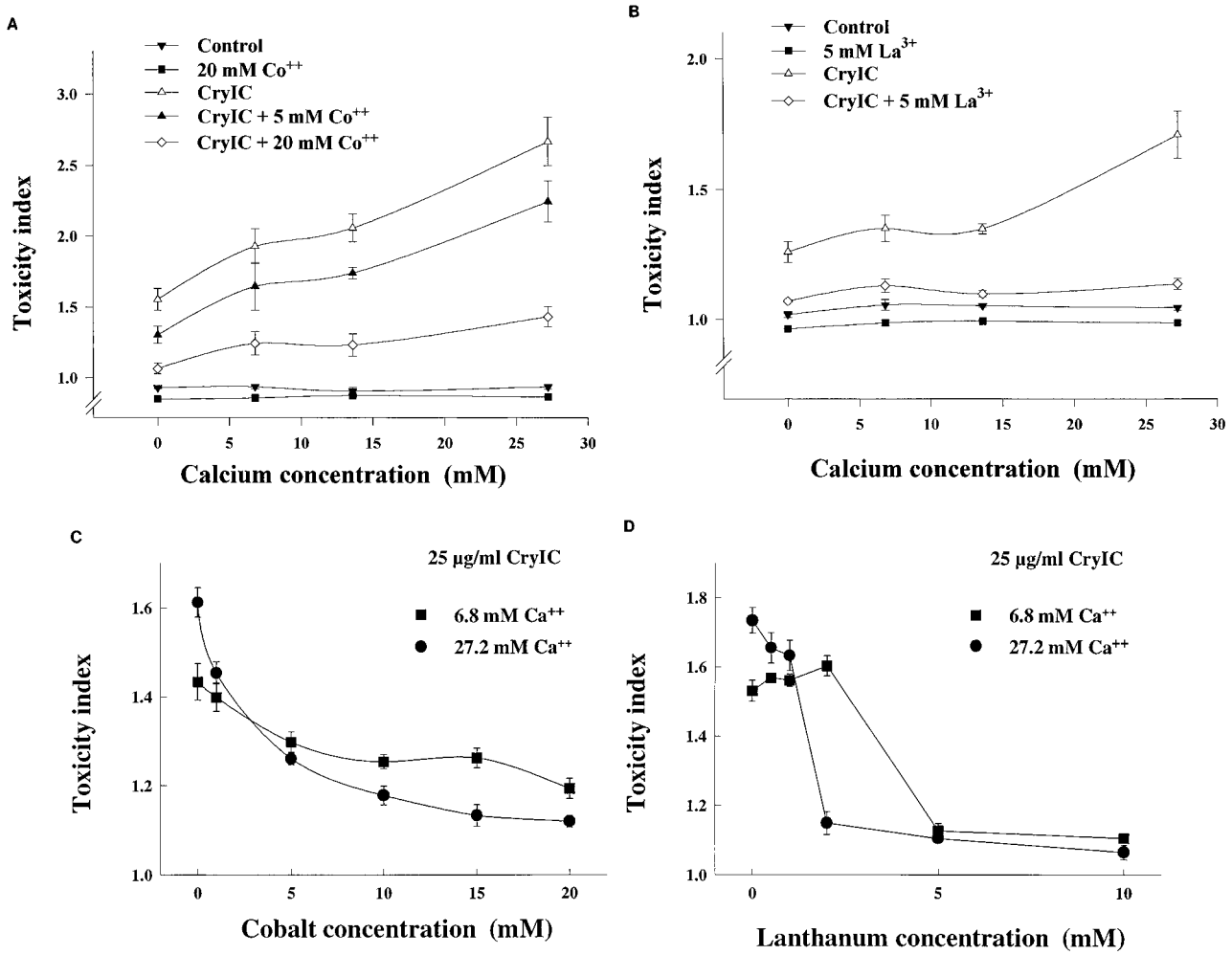

FIG. 5. Effects of cobalt and lanthanum ions on CryIC-mediated toxicity in Sf9 cells. (A) Calcium dependence of cobalt's effect on cell mortality induced by 25 μg of CryIC/ml. (B) Calcium dependence of lanthanum's effect observed under the same conditions as those of panel A. Control experiments were conducted in the absence of toxin, cobalt, and lanthanum (A and B). (C) Cobalt dose-response relationships to intoxication with 25 μg of CryIC/ml. (D) Lanthanum dose-response relationships obtained under the same conditions as those in panel C.

substantially augmented by 40 μM BAY K 8644, particularly in high-concentration extracellular calcium.

(iii) Calcium ionophore A23187. The results of the above-described experiments indicated that external-calcium-related incremental toxicity may be related to an increase in the concentration of intracellular calcium. In general, calcium ionophores provide a simple way to raise intracellular calcium. In control experiments, it was found that 40 μM A23187 did not

TABLE 1. Effects of calcium modulators on CryIC-induced toxicity

Calcium modulator	Dose (mM)	Overall effect on CryIC toxicity[a]			Toxicity change (%)[b] at extracellular calcium concn (mM):		
		Decrease	Increase	No effect	0	6.8	27.2
Cobalt	20	x			−56.9	−59.3[c]	−65.0[c]
Lanthanum	5	x			−53.3	−56.2[c]	−73.5[c]
D600	0.05			x			
Nifedipine	0.05			x			
BAY K 8644	0.04		x		−0.5	+38.5	+61.1[c]
A23187	0.04			x			
TMB-8	0.1			x			
Thapsigargin	2.7×10^{-3}		x		−3.8	+41.8	+27.7[c]
TFP	0.1		x		+348.2[c]	+212.1[c]	+104.3[c]

[a] Two-factor, replicate-measure ANOVA ($P < 0.05$).
[b] +, percent toxicity increase; −, percent toxicity decrease.
[c] Significantly different by Tukey's test ($P < 0.05$); from post hoc ANOVA data (see Materials and Methods).

affect cell viability. The same dose of the calcium ionophore failed to affect CryIC-induced toxicity (Table 1). This unexpected result may be explained by a reduced efficiency of the ionophore under the slightly acidic pH conditions used in this study (36) or, alternatively, by the capability of cells to downregulate the ionophore-induced calcium surge.

Effect of agents that modulate calcium movement of intracellular pools and calcium signalling. (i) TMB-8 and thapsigargin. The above-described data indicated that CryIC toxicity enhancement in the presence of calcium may result from extracellular-calcium influx through a channel-like pathway. To test the possibility that calcium-related CryIC toxicity changes may also be linked to the release of calcium from intracellular-calcium-sequestering organelles, experiments were conducted with thapsigargin and TMB-8. These compounds modulate calcium transport across the membranes of intracellular calcium stores. Thapsigargin inhibits the calcium-ATPase of intracellular pools, therefore increasing the level of cytosolic calcium by preventing calcium ion uptake in cellular stores (47). TMB-8 is an antagonist of calcium release from the endoplasmic reticulum (4). Sf9 viability was unaffected by these two agents in the absence of the toxin (data not shown). While 100 μM TMB-8 had no effect on CryIC-mediated mortality, 2.7 μM thapsigargin enhanced significantly the activity of the toxin, particularly under conditions of high concentrations of external calcium (Table 1).

(ii) TFP. Other calcium-dependent enzymes or signalling pathways may also be involved in calcium-toxin interaction in Sf9 cells. The calcium-binding protein calmodulin is expressed in Sf9 cells (23). Because of the critical role played by calcium-calmodulin-dependent processes in intracellular calcium homeostasis as well as in the regulation of other second messengers and enzymes in several cell systems (3), experiments were conducted with TFP, a widely used inhibitor of calcium-calmodulin-dependent processes in mammalian systems (54). While 100 μM TFP alone did not affect Sf9 viability (data not shown), the inhibitor augmented dramatically CryIC-induced mortality of the cells (Table 1).

DISCUSSION

The results of this study clearly show that (i) CryIC-induced toxicity was markedly augmented, in a dose-dependent manner, by the presence of calcium ions in the extracellular milieu; (ii) this effect was specific to calcium, as it could not be induced by barium, either in the absence or in the presence of calcium ions; (iii) residual CryIC-induced toxicity occurred in the absence of extracellular calcium; (iv) intracellular calcium mobilization from TMB-8-sensitive pools was not required for CryIC-mediated toxicity; (v) cobalt and lanthanum ions reduced the calcium-dependent increase in CryIC toxicity; (vi) BAY K 8644 and thapsigargin increased the effect of the *B. thuringiensis* protein; and (vii) TFP strongly stimulated CryIC-mediated toxicity.

Fluorescence and patch-clamp studies of Sf9 cells have disclosed several early CryIC-related events. Within seconds following addition of activated CryIC toxin, a sudden surge of calcium was observed (42). This signal, which we attributed to the docking of the toxin to an acceptor molecule, was also recorded in UCR-SE1a cells from *Spodoptera exigua* (30) and in Cf1 cells from *Choristoneura fumiferana* (35). The signal appears to be a general, early response of susceptible insect cells to the detection of specific toxins. However, because in these experiments no attempt was made to relate the calcium surge to toxicity, it is possible only to speculate that this calcium signal may participate in the process of toxin intercalation into the cell membrane. Nevertheless, in the next 5 to 10 min following toxin exposure, anion-selective channel activation was induced by the toxin (42) and the cells became permeable to sodium, potassium, and protons (51). Our work with ethidium homodimer fluorescence shows that cell damage was undetectable after 5 to 10 min of exposure to CryIC toxin (t_0 measurement). However, 3 h later, a marked level of basal toxicity was recorded under physiological calcium conditions (i.e., in 6.8MBS) and significant incremental toxicity was reached by addition to the incubating medium of calcium ions or compounds which modulate the concentration of intracellular calcium. It is important to note that in the absence of CryIC toxin, there was no detectable cell damage caused by either a 60-min incubation in media containing nonphysiological levels of calcium (i.e., 0MBS, 13.6MBS, or 27.2MBS) or a 30-min exposure to calcium modulators.

Our results are consistent with an important role for calcium ions in the establishment of the CryIC toxicity level. We propose that under normal physiological conditions, basal CryIC toxicity is mainly due to increased permeability of the cells to ions and metabolites and that calcium-dependent incremental toxicity is caused by an increase in the concentration of intracellular calcium. This is supported by the following lines of evidence. As demonstrated by the Fura 2 experiments, while octopamine triggered a calcium surge immediately after calcium omission in the bath, which demonstrates calcium release from intracellular stores, this signal was no longer observed after 30 min under the same conditions. This indicates that octopamine-sensitive calcium pools were then depleted. These pools are thought to be sensitive to inositol phosphates and are blocked by TMB-8 (15). On the other hand, while calcium omission did not affect cell viability, CryIC was toxic in 0MBS, which suggests that under these conditions toxicity was either independent of calcium or linked to calcium pools which were not part of the octopamine signalling cascade. This is further supported by the fact that thapsigargin, but not TMB-8, stimulated CryIC-induced toxicity. Cobalt and lanthanum ions, which inhibit calcium influx, reduced markedly the calcium-related incremental effect on CryIC toxicity, and compounds like BAY K 8644 and thapsigargin, which cause a rise in the concentration of intracellular calcium, amplified the effect of the toxin. Furthermore, TFP was a very potent cofactor to toxicity. This compound inhibits several calcium-calmodulin-dependent mechanisms, including calcium-ATPase and kinase activities, protein phosphorylation, and dephosphorylation, all of which play critical roles in the maintenance of proper cellular function. It is proposed that calcium-calmodulin-dependent mechanisms are activated by a *B. thuringiensis* toxin-mediated rise in intracellular calcium and provide some protection to the cell. Inhibition by TFP, which becomes more efficient in the presence of high-concentration intracellular calcium, will therefore result in augmented toxicity. However, the possibility that the effect of TFP, a hydrophobic and liposoluble compound, is also related to cell membrane perturbation (54), thus promoting toxin insertion into the cell membrane, cannot be excluded.

The mechanism responsible for CryIC-mediated variation in the concentration of intracellular calcium and the nature of the pathway(s) utilized by calcium ions to enter Sf9 cells are unclear. Elevated levels of calcium may be responsible for facilitated toxin partitioning into the plasma membrane due to Ca^{2+}-promoted lateral phase separation (24) and/or for the development of cytotoxic effects as a result of calcium overload (33). Calcium may flow through endogenous channels activated by toxin-induced membrane depolarization or intracellular-messenger production. Insensitivity to D600 and nifedi-

pine suggests that voltage-dependent calcium channels are not involved, which is supported by other studies of Sf9 cells (16). However, CryIC-mediated toxicity was enhanced by BAY K 8644, an L-type calcium channel activator, which may indicate that insect cell sensitivity to voltage-dependent calcium channel modulators is different from that of mammalian cells. Alternatively, calcium may enter cells through toxin-made ion-selective channels which are not inhibited by calcium (43). In fact, CryIA(c), another lepidopteran *B. thuringiensis* toxin, formed calcium-permeable, lanthanum-blockable channels in planar lipid bilayers (35). According to the data of our study, the toxin-made channels would be cobalt and lanthanum inhibitable and may possess a BAY K 8644 activation site. Finally, the possibility that the rise in the concentration of intracellular calcium was due to reduced calcium extrusion from the cells or sequestration in cellular organelles, i.e., to the inhibition of active calcium transporters, consistent with the results of the TFP experiments, cannot be excluded.

Little is known about the interaction between *B. thuringiensis* toxins and cellular function. Physiological responses to *B. thuringiensis* intoxication may play an important role, either protective or aggravating, in the sequence of events which finally provokes cell swelling and lysis. Increased cyclic AMP levels upon toxin exposure were observed in cells from *Mamestra brassicae*, the cabbage moth, but the authors of the study concluded that this was a secondary effect which was unrelated to cytotoxicity (20). Our work adds further support to the concept that the mode of action of *B. thuringiensis* toxins may include several steps affecting cellular function, in addition to the well-documented membrane permeabilization process. While control experiments conducted without *B. thuringiensis* toxin established that none of the ions or compounds used in this study was toxic per se, some of these agents affected toxin-induced mortality, suggesting that in addition to forming calcium-permeable pathways in intoxicated cells, CryIC profoundly influenced intracellular mechanisms involved in Sf9 cell calcium homeostasis. It remains to be established whether the cellular processes involved were direct targets of the toxin or whether they were affected by intracellular messengers as a result of toxin-mediated plasma membrane permeabilization.

There is a possibility that calcium modulation of cytotoxicity is a general property of pore-forming toxins. However, in the case of *B. thuringiensis* toxins and Sf9 cells, we demonstrate that the effect is specifically related to CryIC, the only toxin among those tested here which kills Sf9 cells, while all of the toxins form pores in planar phospholipid bilayers (11, 19, 43, 44). Furthermore, it was found that modulation of CryIC toxicity is specific to calcium and not barium, which rules out a simple nonselective permeabilization mechanism. This interaction between toxicity and calcium appears therefore to represent an important feature of *B. thuringiensis* proteins which may provide a new insight into their mechanism of action and an added tool for improved toxin formulation.

Incorporation of phagostimulants and other adjuvants in *B. thuringiensis* formulations has been attempted to enhance potency, to increase host susceptibility, and, ultimately, to reduce field application costs of the biopesticide (5). In particular, calcium salts were quite effective in increasing the in vivo toxic effect of *B. thuringiensis* var. *kurstaki*, var. *entomocidus*, or var. *aizawai* against the cotton leafworm (37–39), the Indian mealworm (6), the European corn borer (29), and the bertha armyworm (32). These results are in agreement with our data demonstrating the amplifying effect of extracellular calcium on CryIC toxicity to Sf9 cells. Moreover, our work demonstrates clearly at the cellular level the important role played by calcium metabolism in *B. thuringiensis* toxin potency. This is consistent with the results of a recent in vivo study of the synergistic interaction between caffeine and *B. thuringiensis* toxins, which suggested that intracellular-calcium transport mechanisms were disrupted (31).

In conclusion, our study shows that calcium ions from both extracellular space and intracellular organelles participate in *B. thuringiensis* protein toxicity and, on a more practical standpoint, that the potency of the pathogen may be substantially improved by incorporating calcium salts and/or appropriate intracellular calcium modulators in *B. thuringiensis* formulations. It is expected that this work will promote alternative approaches for the elucidation of the mode of action of *B. thuringiensis* toxins by further examination of the cellular mechanisms by which calcium ions interact with *B. thuringiensis* toxin activity and that it will stimulate new interest for studies aimed to improve *B. thuringiensis* toxin potency by means of agents which promote the maintenance of high levels of calcium in target cells.

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