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BACILLUS THURINGIENSIS INSECTICIDAL CRY1AB TOXIN DOES NOT AFFECT THE MEMBRANE INTEGRITY OF THE MAMMALIAN INTESTINAL EPITHELIAL CELLS: AN IN VITRO STUDY

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SUMMARY

The mammalian intestinal epithelium has been found, based on in vivo experiments, to be resistant to insecticidal Cry toxins, which are derived from *Bacillus thuringiensis* and fatally damage insect midgut cells. Thus, the toxins are commonly used as a genetic resource in insect-resistant transgenic plants for feed. However, Cry toxins bind to the cellular brush border membrane vesicle (BBMV) of mammalian intestinal cells. In this study, we investigated the affinity of Cry1Ab toxin, a lepidopteran-specific Cry1-type toxin, to the cellular BBMV of two mammalian intestinal cells as well as the effect of the toxin on the membrane potential of three mammalian intestinal cells compared to its effects on the silkworm midgut cell. We found that Cry1Ab toxin did bind to the bovine and porcine BBMV, but far more weakly than it did to the silkworm midgut BBMV. Furthermore, although the silkworm midgut cells developed severe membrane potential changes within 1 h following the toxin treatment at a final concentration of 2 μg/ml, no such membraneous changes were observed on the bovine, porcine, and human intestinal cells. The present in vitro results suggest that, although Cry1Ab toxin may bind weakly or nonspecifically to certain BBMV components in the mammalian intestinal cell, it does not damage the cell's membrane integrity, thus exerting no subsequent adverse effects on the cell.

Key words: Cry1Ab toxin; silkworm; cattle; pig; human; membrane potential.

INTRODUCTION

During its sporulation phase, *Bacillus thuringiensis* produces crystals composed of insecticidal Cry proteins that are toxic to various kinds of insects belonging to Lepidoptera, Diptera, and Coleoptera. More than 100 Cry proteins have been identified and classified based on amino acid sequence identity, according to the proposal by Crickmore et al. (1998). The toxic action of Cry protein in target insects has been well investigated, especially that of lepidopteran-specific Cry1 toxins (Bouclas and Pendland, 1998; Schenepf et al., 1998; Aronson and Shai, 2001). When susceptible insect larvae ingest the crystals, the Cry protoxins are solubilized and activated to Cry toxins by midgut proteases. The Cry toxins attack the insect midgut epithelial cell by binding to receptors on the cell, invading the cell membrane, and forming somewhat cation-selective ion channels that result in the disintegration of the membrane and osmotic cell lysis. However, in vivo experiments (McClintock et al., 1995; Kuiper et al., 2001) have shown that the Cry toxins are harmless to humans and farm animals because of the lack of both a Cry protein activation process (Okunuki et al., 2002) and Cry toxin receptors in the mammalian small intestine (Sacchi et al., 1986). Consequently, the Cry toxins have been used worldwide as a component of pesticides (McClintock et al., 1995) and as a genetic resource for developing insect-resistant, genetically modified (GM) plants (Betz et al., 2000).

Nevertheless, we detected trace amounts of the Cry1Ab toxin, a Cry1-type toxin, in the gastrointestinal contents of calves that were fed GM corn Bt11 (Chowdhury et al., 2003). In addition, the Cry1Ab toxin is easily digested by gastric fluids, but resistant to intestinal fluids (Okunuki et al., 2002). Therefore, it is likely that the intestinal epithelium of the GM–corn-fed animals is still exposed to the corn-derived Cry toxin. However, there have been no detailed investigations to determine whether this toxin affects the intestinal epithelium in farm animals.

In this study, we investigated whether Cry1Ab toxin has the binding affinity to the bovine and porcine intestinal brush border membrane vesicle (BBMV), the Cry toxin–binding region in insect midgut cells. We also examined how the toxin affects the membrane potential of bovine, porcine, and human intestinal epithelial cells using *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4[3]$), a voltage-dependent fluorescent dye. We found that Cry1Ab toxin bound to the bovine and porcine BBMV, but very weakly. However, the toxin did not damage the membrane potentials of the bovine, porcine, and human intestinal epithelial cells. Therefore, we concluded that Cry1Ab toxin does not impair the membrane integrity of the mammalian intestinal epithelial cell, and thus exerts no subsequent adverse effects on the cell.

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Materials and Methods

Cry1Ab toxin preparation. Crystalliferous Cry1Ab toxin was purified from an *Escherichia coli* recombinant strain harboring a *cry1Ab* gene of *B. thuringiensis* serovar *kurstaki* HD-1 and activated to Cry1Ab toxin as described by Agrawal et al. (2002). Briefly, the crystal protein was solubilized in 50 m*M* sodium carbonate buffer (pH 10.5) containing 10 m*M* dithiothreitol at 37° C for 2 h with vigorous shaking. The solubilized crystal protein was activated with trypsin at a trypsin:protoxin ratio of 1:50 (by mass) at 37° C. The activated Cry1Ab toxin was purified by ion-exchange liquid chromatography using a Q-Sepharose anion exchanger. Fractions containing the purified toxin were pooled and dialyzed against phosphate-buffered saline (PBS). Protein concentration was determined by DC Protein Assay (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard.

BBMV purification. BBMV is frequently used in binding assays for Cry toxins because it is the region in insects that contains receptors for the toxin. Fifth-instar larvae of the silkworm *Bombyx mori* (race No. 606 of the National Institute of Agrobiological Science), two healthy Holsteins (50 and 84 mo old), one healthy Japanese Black cow (85 mo old) and three healthy crossbred pigs (Large White × Landrace, 7 mo old) were used for the purification of BBMV in accordance with National Institute of Animal Health guidelines for animal use. The silkworm BBMV was purified from the fifth-instar larvae midgut according to the method described by Wolfersberger et al. (1987). The bovine and porcine BBMV were prepared from the small intestine as described by Hauser et al. (1980). The final pellet was stored at −80° C. BBMV quality was tested by measuring the aminopeptidase activity (Knight et al., 1994).

Assays of Cry1Ab toxin binding to BBMV. Two assays of Cry1Ab toxin binding to BBMV were done: a coprecipitation assay and a BIAcore assay.

For the coprecipitation assay, BBMV (30 μg proteins) were incubated with Cry1Ab toxin in 100 μl of PBS containing 10% Block Ace (Dainippon Pharmaceutical, Osaka, Japan). After a 1-h incubation at room temperature, the sample was centrifuged for 10 min at 13,000 × *g* to separate the bound toxin from the free one. The pellet containing the bound toxin was washed three times with PBS containing 10% Block Ace, and the resulting pellet was suspended in 20 μl of 2% sodium dodecyl sulfate (SDS) loading buffer with incubation at 100° C for 5 min. After the incubation, the suspension underwent SDS-5–20% polyacrylamide gel electrophoresis (PAGE). Following electrophoresis and transfer to the nitrocellulose membrane (Bio-Rad), the membrane was treated with the anti-Cry1A polyclonal antibody for 1 h at room temperature, and then with horseradish peroxidase–conjugated anti-rabbit polyclonal antibody (OEM Concepts, Toms River, NJ) for 0.5 h at room temperature. The immune complexes were revealed with ECL Plus Western Blotting Detection system (Amersham Biosciences, Buckinghamshire, U.K.).

For the BIAcore assay, we quantified the amounts of Cry1Ab toxin bound to the silkworm, bovine, and porcine BBMV using a BIAcore system instrument (Pharmacia Biosensor AB, Uppsala, Sweden), an optical biosensor based on the principles of surface plasmon resonance (Plant et al., 1995). The sensor chip L1 consists of a carboxymethyldextran hydrogel derivatized with lipophilic alkyl chain anchors on a gold film (Pharmacia Biosensor AB), and lipid bilayers were formed on the sensor chip by fusion of lipid bilayer vesicles by following the manufacturer's protocol. To immobilize the BBMV on the sensor chip, the chip was washed with 20 m*M* 3-[(3-Cholamidopropyl)dimethyl-amino]propanesulfonic acid (CHAPS), and BBMV were injected into the BIAcore system. The lipid layer was then washed with 50 m*M* sodium hydroxide. The degree of surface coverage was determined from the amount of lipid bound at a stable level after the sodium hydroxide wash. Cry1Ab (20 μg/ml) was injected over the immobilized membrane surface for 5 min. The Cry1Ab binding assay was performed repeatedly, after washing the surface with 20 m*M* CHAPS and immobilizing new BBMV. The resonance unit (RU) is an arbitrary unit used by the BIAcore system, and there is a linear relationship between the mass molecule bound to the sensor chip and the observed RU. A reagent flow rate of 5 μl/min was used in all experiments. The amount of binding Cry1Ab toxin to BBMV was calculated as follows.

The amount of binding Cry1Ab toxin = (the amount of binding Cry1Ab toxin to the immobilized BBMV on the L1 chip / the amount of immobilized BBMV on the L1 chip) × 100.

Analyses of the Cry1Ab toxin effect on the membrane potential. The effects of the Cry1Ab toxin were analyzed in silkworm midgut (SM) cells, bovine gut (BG) cells, porcine gut (PG) cells, and in a human intestinal epithelial (HIE) cell line.

We obtained SM cells using the methodology described by Baines et al. (1994), with some modifications. The midguts were removed from fifth-instar larvae of the silkworm *Bombyx mori* (race No.606 of the National Institute of Agrobiological Science), rinsed several times in Grace's insect medium (JRH Biosciences, Lenexa, KS) containing antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 250 μU/ml amphotericin B), and incubated in 4000 U/ml collagenase-Type I at room temperature for 2 h. The SM cells were then collected by centrifugation (500 × *g*, 1 min) and diluted (1 × 10^5 cells/ml) for use in the experiments.

We acquired BG and PG cells as described by Föllmann et al. (2000). Briefly, specimens from the bovine and porcine intestine were obtained from freshly slaughtered animals. A 15-cm-long section of the intestine was isolated and washed several times in PBS containing antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 250 μU/ml amphotericin B). The intestinal epithelium was scraped from the underlying tissue with a glass slide. The scraped tissue was incubated in 100 U/ml collagenase-Type I at room temperature for 1 h. The BG and PG cells were then collected by centrifugation (130 × *g*, 5 min) and diluted (1 × 10^5 cells/ml) for use in the experiments.

We obtained the HIE cell line from Applied Cell Biology Research Institute and cultured it according to the supplier's instructions. The HIE cells (1 × 10^5 cells/ml) were cultured within a multiwell plate (SUMILON CELLTIGHT C-1 plate 96F, Sumitomo Bakelite, Akita, Japan) in CS-2.0 Serum-Free Medium with Rocket Fuel (Cell Systems, Kirkland, WA) for the first 5 d. For the subsequent 5 d, the cells were cultured in a differentiation medium consisting of Dulbecco modified Eagle medium (Nissui Pharmaceutical, Tokyo, Japan) and Entero-Stim Differentiation Medium (Becton Dickinson, Bedford, MA), with a one-to-one ratio, containing 1 ml of MITO Serum Extender (Becton Dickinson) per liter of the medium. Both the CS-2.0 Serum-Free Medium and the differentiation medium contained antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 250 μU/ml amphotericin B).

The membrane potentials of the SM, BG, and PG cells were measured using $DiBAC_4(3)$ (DOJINDO, Kumamoto, Japan), a voltage-dependent fluorescent dye, in accordance with the manufacturer's instructions. Fluorescence was recorded at the 485/510 nm excitation/emission wavelength pairs using Fluoroskan Ascent FL (Thermo Labsystems, Helsinki, Finland). After attaining the desired incubations with 2 μg/ml Cry1Ab toxin or PBS in the sterile 1.5-ml tube (Eppendorf, Hamburg, Germany), we washed the cells twice in a buffer containing 20 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sufonic acid (HEPES), pH 7.4, 120 m*M* sodium chloride (NaCl), 2 m*M* potassium chloride (KCl), 2 m*M* calcium chloride ($CaCl_2$), 1 m*M* magnesium chloride ($MgCl_2$), 5 m*M* glucose, and 5 μM $DiBAC_4(3)$. The cells were transferred to a 96F Nunclon™ *delta surface, white microwell plate* (Nunc, Roskilde, Denmark) with 180 μl of the buffer and then incubated for 30 min at 37° C in a 5% carbon dioxide (CO_2) humidified atmosphere. Subsequently, the microwell plate was set in Fluoroskan Ascent FL, and then relative fluorescence units (RFU) of each well were measured after adding 20 μl of 1 *M* KCl. The assay was repeated three times, using four wells per incubation.

The membrane potential of HIE cells also was measured as above. After attaining the desired incubations with 2 μg/ml Cry1Ab toxin or PBS in the multiwell plate used to culture the HIE cells, we washed the cells twice in a buffer containing 20 m*M* HEPES, pH 7.4, 120 m*M* NaCl, 2 m*M* KCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* glucose, and 5 μM $DiBAC_4(3)$. The cells were then incubated for 30 min in 180 μl of the buffer at 37° C in a 5% CO_2 humidified atmosphere and measured. The assay was repeated three times, using four wells per incubation.

Statistical analysis. StatView (SAS Institute, Inc., Cary, NC) was used to evaluate the data in this study. The amount of Cry1Ab toxin per immobilized BBMV was compared using a one-way analysis of variance. Differences were considered significant for P values less than 0.05.

Results

Binding assays. In the coprecipitation assay, Cry1Ab toxin bound to the bovine and porcine BBMV less strongly than to the silkworm BBMV (Fig. 1). There was almost no difference in binding intensity among individual fractions of the bovine and porcine BBMV. Subsequently, we quantified the amounts of Cry1Ab toxin bound to the silkworm, bovine, and porcine BBMV by BIAcore assay. The amounts of Cry1Ab toxin bound to the silkworm BBMV averaged 3.203 (Cry1Ab-RU/BBMV-RU), whereas the amounts of Cry1Ab

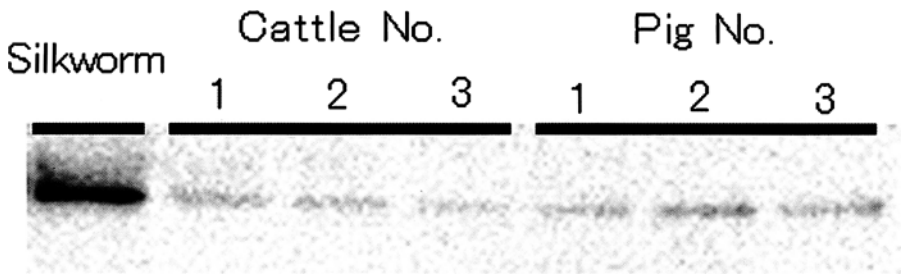

FIG. 1. Binding of Cry1Ab toxin to the silkworm, bovine, and porcine BBMV in the coprecipitation assay. Cry1Ab toxin bound to the silkworm BBMV more strongly than to the bovine and porcine BBMV. Lane silkworm: BBMV (30 μg) of silkworms, lane cattle 1: BBMV (30 μg) of a healthy Holstein cow (50 mo old), lane cattle 2: BBMV (30 μg) of a healthy Holstein cow (84 mo old), lane cattle 3: BBMV (30 μg) of a healthy Japanese Black cow (85 mo old), lane pig 1: BBMV (30 μg) of a healthy crossbred pig (Large White × Landrace, 7 mo old), lane pig 2: BBMV (30 μg) of a healthy crossbred pig (Large White × Landrace, 7 mo old), lane pig 3: BBMV (30 μg) of a healthy crossbred pig (Large White × Landrace, 7 mo old)

TABLE 1

THE AMOUNTS OF CRY1AB TOXIN BINDING TO BBMV

Species	The amounts of Cry1Ab toxin per immobilized BBMV[a]
Silkworm	3.203 ± 1.689
Cattle	1.196 ± 0.368
Pig	1.973 ± 0.349

[a] Each value represents the mean value of three independent experiments ± standard deviation.

toxin bound to the bovine and porcine BBMV were 1.196 (Cry1Ab-RU/BBMV-RU) and 1.973 (Cry1Ab-RU/BBMV-RU), respectively (Table 1). Although there were no statistically significant differences among the amounts of the toxin bound to the silkworm, bovine, and porcine BBMV, the BIAcore results quantitatively confirmed the coprecipitation findings that the toxin affinity is weak in the bovine and porcine BBMV.

Evaluation of the effect of Cry1Ab toxin on the membrane potential of the SM, BG, PG, and HIE cells. The membrane potential changes of the SM, BG, and PG cells after 1-h incubation of Cry1Ab toxin are presented in Fig. 2. In the SM cells, the RFU of Cry1Ab toxin-treated cells rose sharply after the KCl addition. It remained high during the observation period and did not return to the basal level that preceded the KCl addition, whereas the RFU of PBS-treated cells gradually increased and then started decreasing toward the basal level. The maximum RFU of the Cry1Ab-treated cells obviously exceeded that of the PBS-treated cells (left panel, Fig. 2). In the BG and PG cells, there was no difference in RFU change between the toxin- and PBS-treated cells (middle and right panels, Fig. 2), indicating that the toxin treatment does not impair the membrane potential.

The HIE cells also had no substantial changes in the membrane potential when compared to the controls. As Fig. 3 indicates, both the Cry1Ab-treated and the PBS-treated cells had the same RFU change patterns even after a 48-h incubation with the toxin. Moreover, we observed no significant changes in the morphology and LDH release of the Cry1Ab toxin-treated cells even at the longest incubation (48 h, data not shown). These results suggest that Cry1Ab toxin did not damage the membrane integrity of the HIE cells.

DISCUSSION

Cry toxins bind to the receptors on the membrane of the midgut cell in susceptible insects, thereby creating an ionophore that results in disintegration of the membrane and leads to swelling and disruption of the cell (Aronson and Shai, 2001; Bouclas and Pendland, 1998; Schenepf et al., 1998). Thus, both the membrane binding and membrane disintegration are essential factors in understanding the mode of action of Cry toxins on target cells. Although it is known that Cry toxins bind to the cellular BBMV of the mammalian cells, there have been no reported analyses of its effect on the cells. In this study, we investigated in vitro whether Cry1Ab toxin, a lepidopteran-specific Cry toxin, affects the mammalian intestinal epithelium, with special reference to the membrane binding and disintegration by the toxin.

First, both coprecipitation and BIAcore assays confirmed that

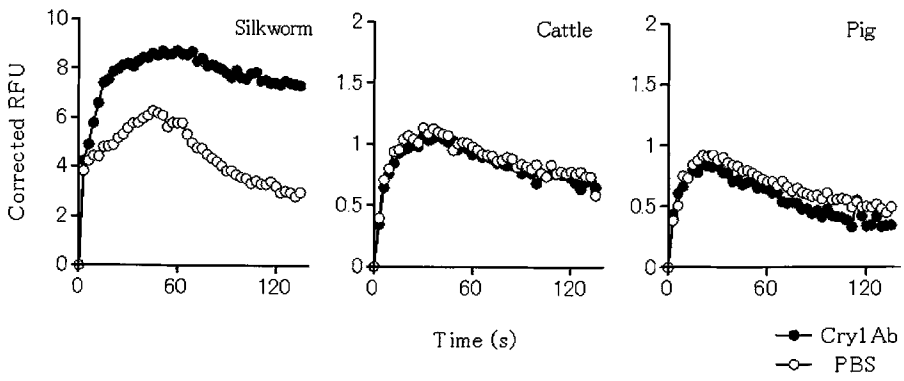

FIG. 2. Effect of Cry1Ab toxin on the membrane potentials of the SM, BG, and PG cells. All assays were performed after 1-h incubation of Cry1Ab toxin. In the SM cells, the relative fluorescence unit (RFU) change pattern of the PBS-treated cells contrasts with the RFU change pattern of the Cry1Ab toxin-treated cells, which hover at the high RFU. In the BG and PG cells, there is no difference between the Cry1Ab toxin- and the PBS-treated cells. The RFUs were normalized to zero by the addition of 1*M* KCl for comparative purposes. The data are representative of three independent experiments. ●, Cry1Ab toxin-treated cells; ○, PBS-treated cells.

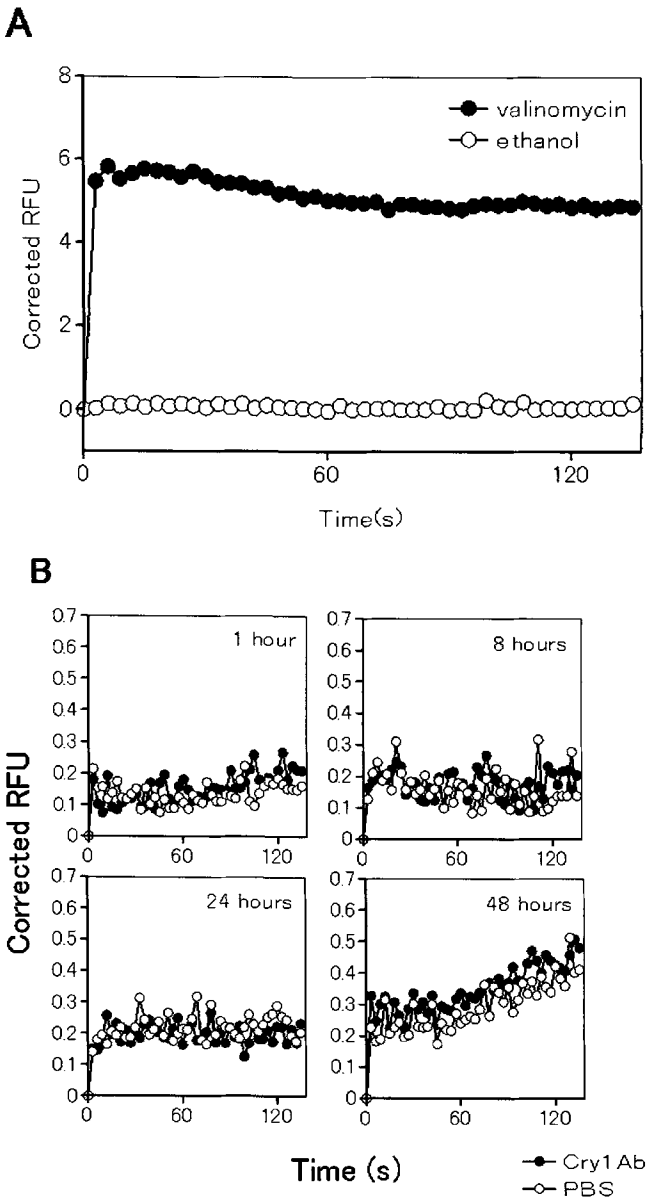

FIG. 3. (*A*) Effect of valinomycin on the membrane potential of the HIE cells for 1-h incubation. The RFU change pattern of the ethanol-treated cells contrasts with the RFU change pattern of the valinomycin-treated cells, which hover at the high RFU. The data represent means of three independent experiments. •, valinomycin-treated cells; ○, ethanol-treated cells. FIG. 3 (*B*) Effect of Cry1Ab toxin on the membrane potentials of the HIE cells for various incubation periods. Each incubation period is shown at right upper corner of each graph. The RFU change patterns of the Cry1Ab toxin- and the PBS-treated cells were similar, indicating no specific RFU responses because of the toxin treatment. The data represent means of three independent experiments. •, Cry1Ab-treated cells; ○, PBS-treated cells.

Cry1Ab toxin binds to the bovine and porcine intestinal BBMV less strongly than to the silkworm midgut BBMV. It has been reported that Cry toxins can bind nonspecifically to the rat intestinal BBMV (Hofmann et al., 1988) and the pig kidney BBMV (Lee et al., 1992). Identification of the toxin-binding bovine and porcine BBMV proteins is under way in our laboratory, but we do not preclude the possibility that the binding in this study might have been, at least in part, nonspecific. In any case, it seems likely that the Cry1Ab-specific membrane receptors in those mammalian gut cells are, if any, very few or negligible.

Second, to determine the direct effect of Cry1Ab toxin on the membrane integrity of mammalian intestinal epithelial cells, we compared the membrane potentials among Cry1Ab toxin–treated SM, BG, PG, and HIE cells, using $DiBAC_4(3)$, a voltage-dependent fluorescent dye. The dye is internalized into the cytoplasm by depolarization and subsequently externalized by hyperpolarization (Epps et al., 1994). The $DiBAC_4(3)$ assay thus can detect the membrane potential changes triggered by ion influx aberration. On the Cry1Ab toxin–treated SM cells, the RFU level increased rapidly and remained high during the observation period. Those RFU changes would reflect the ion influx aberration because of Cry1Ab toxin–induced *ionophore* formation on the membrane of the cell (Aronson and Shai, 2001; Bouclas and Pendland, 1998; Schenepf et al., 1998). In contrast, in the BG, PG, and HIE cells, the Cry1Ab toxin treatment did not affect the membrane potential patterns. Incidentally, we have preliminarily verified that the BG, PG, and HIE cells augment RFU levels in response to exposure to valinomycin, a K^+-selective ionophore used as a positive control, confirming that the $DiBAC_4(3)$ assay can work on those mammalian cells. Moreover, we observed no significant changes in the morphology and LDH release of the HIE cells even after a 48-h incubation with the toxin. Similar findings have been obtained on Cry1Ab-treated primary cultured bovine hepatocytes (Shimada et al., 2003). These results allowed us to confirm that Cry1Ab toxin does not affect the membrane potentials of those mammalian gut cells.

To our knowledge, this is the first study to have verified in vitro the harmlessness of the toxin on the membrane function in mammalian cells. This phenomenon might be related to the lack or scarcity of the Cry1Ab toxin–specific receptors in the BBMV region of those cells.

In conclusion, although Cry1Ab toxin may bind weakly or nonspecifically with cellular proteins in the BBMV, we find that it does not damage the membrane integrity of the mammalian intestinal epithelial cell, and thus produces no subsequent adverse effect on the cell. As the use of Cry toxins is expected to spread in the agricultural and industrial fields, we propose that understanding the molecular basis of the harmless nature and nonspecific binding of Cry toxins to mammalian cells could contribute to confirming the safety of the toxins in commercial use.

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