Parasporin-1, a novel cytotoxic protein from *Bacillus thuringiensis*, induces Ca\(^{2+}\) influx and a sustained elevation of the cytoplasmic Ca\(^{2+}\) concentration in toxin-sensitive cells * 

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Parasporin-1 is a novel non-insecticidal inclusion protein from *Bacillus thuringiensis* that is cytotoxic to specific mammalian cells. In this study, we investigated the effects of parasporin-1 on toxin-sensitive cell lines to elucidate the cytotoxic mechanism of parasporin-1. Parasporin-1 is not a membrane-pore-forming toxin as evidenced by measurements of lactate dehydrogenase release, propidium iodide penetration, and membrane potential in parasporin-1-treated cells. Parasporin-1 decreased the level of cellular protein and DNA synthesis in parasporin-1-sensitive HeLa cells. The earliest change observed in cells treated with this toxin was a rapid elevation of the intracellular free-Ca\(^{2+}\) concentration; increases in the intracellular Ca\(^{2+}\) levels were observed 1-3 min following parasporin-1 treatment. Using four different cell lines, we found that the degree of a cell's sensitivity to parasporin-1 was positively correlated with the size of the increase in the intracellular Ca\(^{2+}\) concentration. The toxin-induced elevation of the intracellular Ca\(^{2+}\) concentration was markedly decreased in low-Ca\(^{2+}\) buffer and was not observed in Ca\(^{2+}\)-free buffer. Accordingly, the cytotoxicity of parasporin-1 decreased in the low-Ca\(^{2+}\) buffer and was restored by the addition of Ca\(^{2+}\) to the extracellular medium. Suramin, which inhibits trimeric G-protein signaling, suppressed both the Ca\(^{2+}\) influx and the cytotoxicity of parasporin-1. In parasporin-1-treated
HeLa cells, degradation of pro-caspase-3 and poly(ADP-ribose) polymerase (PARP) was observed. Furthermore, synthetic caspase inhibitors blocked the cytotoxic activity of parasporin-1. These results indicate that parasporin-1 activates apoptotic signaling in these cells as a result of the increased Ca$^{2+}$ level and that the Ca$^{2+}$ influx is the first step in the pathway that underlies parasporin-1 toxicity.

Pathogenic bacteria produce a wide variety of protein toxins and toxin-like molecules. These toxins and toxin-like molecules have been extensively studied to understand the diseases caused by pathogenic bacteria and to find effective preventive treatments. It is known that bacterial toxins affect the enzymic or non-enzymic activities of specific host molecules, which are often critical for cell function, resulting in inhibition or excess activation of these targets. Although bacterial toxins differ in their modes of action, they often show strict target specificities when compared with chemically synthesized drugs. Thus, bacterial toxins can be used as powerful therapeutic agents or as tools in biological studies (1).

*Bacillus thuringiensis* is a gram-positive, spore-forming bacterium that produces parasporal inclusions during sporulation. The inclusions often contain one or more insecticidal proteins that are toxic to the larvae of certain insects and, in some cases, to nematodes, mites, and protozoa (2). There is a remarkable diversity of *B. thuringiensis* strains and inclusion proteins. Previous studies have identified a number of non-insecticidal *B. thuringiensis* strains in natural environments, even outnumbering the insecticidal strains (3, 4).

Among the non-insecticidal *B. thuringiensis* strains, we found strains that produced a novel class of inclusion proteins. These inclusion proteins were non-hemolytic and were cytotoxic to cultured mammalian cells, including human cancer-cell lines (5). The biochemical characteristics of the cytotoxic proteins, including their cell specificities and cytotoxic activities, were highly heterogeneous; some affected a wide range of human cells, whereas others killed only a few specific cell types (5-8). These proteins do not belong to the same class as the Cyt proteins, which similarly exhibit cytotoxicity against mammalian cells, but also have hemolytic and insecticidal activities. Based on these observations, we classified these proteins into a new "parasporin" protein family, members of which are preferentially cytotoxic to mammalian cells (9). To date, four members of the parasporin family have been identified (10).

Parasporin-1 is produced as an 81-kDa parasporal inclusion protein (pro-parasporin-1) by the A1190 strain of *B. thuringiensis*, one of our isolates. The active form of parasporin-1 is a heterodimer composed of 15- and 56-kDa subunits that
are created when pro-parasporin-1 is cleaved by trypsin at two sites (10). Purified parasporin-1 is highly cytotoxic to certain cell lines, implying that the toxin-sensitive cell lines have a specific receptor for parasporin-1. Although the cytotoxic mechanism of parasporin-1 seems to differ from those of other known membrane pore-forming toxins such as the B. thuringiensis insecticidal Cry and Cyt toxins, the nature of the cytotoxicity and the underlying mechanism have not yet been clarified.

In this study, we have investigated the cytotoxic action of parasporin-1. Among the various changes in cells treated with parasporin-1, the initial effect we observed was an unusually large influx of extracellular Ca\(^{2+}\). The cytotoxicity of parasporin-1 was attenuated by low extracellular concentrations of Ca\(^{2+}\), suggesting that parasporin-1 induces excess Ca\(^{2+}\) influx, resulting in the apoptotic death of the target cell.

**MATERIALS AND METHODS**

*Reagents and cells -* A23187, ionomycin, and the caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK were obtained from CalBiochem (San Diego, CA). Probenecid, valinomycin, nigericin, streptolysin O (SLO), and suramin were purchased from Sigma (Tokyo, Japan), and DiSC\(_3\)(5) was purchased from Molecular Probes (Eugene, OR). Fura-2 acetoxymethyl (AM) ester and Pluronic F-127 were obtained from Dojindo Laboratories (Kumamoto, Japan). MTS assay kits (Cell Titer96\textsuperscript{®} AQueous One Solution Cell Proliferation Assay) and Polybuffer 96 were obtained from Promega (Madison, WI) and Amersham Pharmacia Biotech (Piscataway, NJ), respectively. Prepared DMEM and Ca\(^{2+}\)-free DMEM were purchased from Invitrogen Corp. (Carlsbad, CA). Anti-PARP monoclonal antibodies, anti-caspase-3 monoclonal antibodies, and horseradish peroxidase secondary antibodies were purchased from MBL Corp. (Nagoya, Japan). HeLa, Caco-2, and Sawano cells were purchased from Riken Cell Bank (Tsukuba, Japan). Normal uterus smooth muscle cells (UtSMCs) and SmGM medium (specific medium for UtSMCs) were obtained from BioWhittaker Inc. (Walkersville, MD).

*Cell culture -* HeLa and Sawano cells were cultured in 2 mM glutamine-containing MEM supplemented with 10% and 15% FBS, respectively. Caco-2 cells were cultured in MEM supplemented with 10% FBS, 1% non-essential amino acids, and 2 mM glutamine. UtSMCs were incubated in SmGM medium. All cells were incubated at 37°C in 5% CO\(_2\) in air.

*Parasporin-1 purification -* Pro-parasporin-1 was activated with trypsin and parasporin-1 was purified as reported previously (10) with a slight modification. Briefly, samples were digested with trypsin without
phenylmethanesulfonyl fluoride treatment before being fractionated by affinity chromatography on a Hi-trap chelating column (Amersham Pharmacia Biotech). Bound protein was eluted by washing with Buffer A [25 mM Tris-HCl (pH 8.0) and 150 mM NaCl] containing 250 mM imidazole. Fractions were pooled and fractionated again on a Superdex 75 pg column (bed volume 1.6 x 65 cm). Fractions containing the cytotoxic activity of parasporin-1 were pooled and purified further by chromatofocusing on a Mono P HR 5/20 column (Amersham Pharmacia Biotech). Parasporin-1 was eluted using a linear gradient from pH 8 to 7 by washing with 10% (v/v) Polybuffer 96-HCl (pH 7.0). Finally, parasporin-1 was purified with a Superdex 75 HR 10/30 column equilibrated with Buffer B [25 mM Tris-HCl (pH 8.8) and 150 mM NaCl]. Purified parasporin-1 was subjected to SDS-polyacrylamide gel electrophoresis on 10-20% gradient gels (11) and protein bands were detected with silver stain (12). Fig. 1 shows the electrophoresis results for the final fractions of purified parasporin-1, which contained 15- and 56-kDa proteins. The purified parasporin-1 samples were used throughout the experiments described in this paper. Protein concentrations were determined with a Bradford protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard.

Lactate dehydrogenase release assay - The ability of purified parasporin-1 to cause lactate dehydrogenase (LDH) release was evaluated using HeLa cells. After the trypsinized HeLa cells were suspended in the culture medium at a density of 2.2 x 10^5 cell/ml, 90 µl of the suspension was dispensed into each well of a 96-well plate (2 x 10^4 cells/well). After 16 h, the HeLa cells were treated with parasporin-1 (10 µg/ml) or SLO (2 µg/ml). LDH activity was measured using a CytoTox 96 assay kit (Promega) according to the manufacturer's instructions. The relative value (%) of LDH release was obtained by comparing the activity measured in the samples to a maximal value, which was obtained after freeze-thaw lysing HeLa cells.

Propidium iodide influx assay - HeLa cells were cultured for 16 h in a 96-well plate at a density of 2 x 10^4 cells/well. After washing the cells three times with Hanks’-HEPES buffer [20 mM HEPES-NaOH (pH 7.4), 1.2 mM CaCl_2, 136 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM MgCl_2, 0.41 mM MgSO_4, 0.34 mM Na_2HPO_4, and 5.55 mM glucose], the cells were incubated for 10 min with Hanks’-HEPES buffer containing 5 µg/ml propidium iodide (PI). After the addition of parasporin-1 or SLO, PI entry was monitored by measuring the increase in fluorescence intensity at 612 nm, upon excitation at 485 nm, using a FLEX station (Molecular Device Inc., Sunnyvale, CA). Fluorescence intensity was measured every...
The fluorescent signals were normalized to the maximal fluorescent signal obtained following the addition of 1% Triton X-100.

**Membrane potential measurements** - HeLa cells were seeded in 96-well, black-wall microplates (Corning Inc., Corning, NY) at a density of \(2.0 \times 10^4\) cells/well and cultured at 37°C for 16 h. The cells were washed three times with Hanks’-HEPES buffer and were incubated with Hanks’-HEPES buffer containing DiSC\(_3\)(5) (200 nM) for 30 min in a CO\(_2\) incubator. Cells were excited at 625 nm and the intensity of the light emitted at 670 nm was measured every 30 sec. The base-line level of fluorescence was determined during the initial 3 min of the FLEX station read. Parasporin-1 or SLO was then added to each well and the subsequent changes in the fluorescence intensity of DiSC\(_3\)(5) were monitored. Maximal depolarization was obtained at the end of each experiment by adding pre-mixed valinomycin and nigericin to the final concentrations of 2 and 5 µM, respectively (13). These experiments were performed at 37°C.

**Cytotoxic assay** - HeLa cells were precultured under the same conditions used for the LDH release assay. After pre-culture, the diluted sample of parasporin-1 (10 µl) was added to the culture medium. After a 20-h incubation, the cells were observed under a phase-contrast microscope to estimate the cytotoxicity, and were subjected to an MTS assay to measure cell viability in accordance with the manufacturer’s instructions (Promega). For the calculation of cell viability, the absorbance (590 nm) of HeLa cells treated with 10 µg/ml parasporin-1 in the MTS assay was used as a background value. After subtraction of the background value from the value for each sample, cell viability was calculated as the absorbance value at 590 nm relative to the blank value (100%).

The effect of extracellular Ca\(^{2+}\) on parasporin-1 toxicity was measured in low-Ca\(^{2+}\) and normal-Ca\(^{2+}\) media (10% FBS, 30 µg/ml kanamycin, and 2 mM glutamine in Ca\(^{2+}\)-free DMEM or DMEM, respectively). The Ca\(^{2+}\) concentration of FBS was determined to be 3.4 mM using the laboratory test reagent Calcium E-test Wako (Wako Chemicals, Osaka, Japan). Therefore, the Ca\(^{2+}\) concentrations of normal- and low-Ca\(^{2+}\) media were approximately 2.1 and 0.3 mM, respectively. HeLa cells were precultured in normal- or low-Ca\(^{2+}\) medium and then were treated with parasporin-1 (0-10 µg/ml) for 16 h at 37°C. The viability of the HeLa cells was determined as described above.

**Protein and DNA synthesis measurements** - To measure cellular protein synthesis, HeLa cells were seeded on 12-well tissue culture plates (7.3 x 10\(^4\) cells/well) and cultured for 20 h at 37°C in a CO\(_2\) incubator. After the
cells were twice washed with ice-cold PBS, 0.75 ml of assay medium (Ham's F-12 medium containing 10% FBS, 100 units/ml ampicillin, and 0.1 mg/ml streptomycin) was added. After incubation with parasporin-1 (2.5 µg/ml) for the indicated times, cells were incubated with 380 kBq/ml [3H]-leucine for 10 min in assay medium. For blocking of [3H]-leucine incorporation, 1.5 mg/ml leucine was added (final conc.: 0.75 mg/ml). After washing the cells with ice-cold PBS, the cells were lysed with 0.2 N NaOH and treated with 20% TCA. The amount of TCA-insoluble radioactivity incorporated into proteins was then determined. The rate of protein synthesis is expressed as a percentage of the value obtained from control cultures that did not receive parasporin-1. DNA synthesis was assessed by measuring the incorporation of [3H]-thymidine. After incubation of HeLa cells with 2.5 µg/ml parasporin-1 for the indicated times, HeLa cells were incubated with 380 kBq/ml [3H]-thymidine in assay medium for 10 min. Cells were washed with ice-cold PBS and treated with 20% TCA after lysis with 0.2 N NaOH. The amount of TCA-insoluble radioactivity incorporated into DNA was determined.

**Measurement of intracellular Ca\(^{2+}\) concentrations** - HeLa cells were seeded in 96-well black-wall microplates at a density of 2.0 x 10^4 cells/well and cultured at 37°C for 20 h. The cells were washed three times in wash buffer [Ca\(^{2+}\)-free Hanks'-HEPES buffer containing 2.5 mM probenecid and 1% (w/v) BSA] and were loaded with the intracellular Ca\(^{2+}\)-sensitive fluorescent indicator Fura-2/AM (4.5 µM) for 30 min at 37°C in dye-loading buffer [Hanks'-HEPES buffer containing 2.5 mM probenecid, 1% (v/v) FBS, and 0.05% (w/v) pluronic F-127]. After washing three times with wash buffer, cells were incubated in assay buffer [Hanks'-HEPES buffer containing 2.5 mM probenecid and 1% (w/v) BSA] for an additional 15 min at 37°C in the measurement equipment to allow for hydrolysis of the AM-ester. The fluorescence emitted at 510 nm after excitation with a wavelength that alternated between 340 and 380 nm was recorded every 30 sec using a fluorometric plate reader (FLEX station, Molecular Devices). Base-line intracellular fluorescence was established during the initial 180 sec of the FLEX station read. Parasporin-1 was then added to each well and subsequent changes in the intracellular Ca\(^{2+}\) concentration were monitored. These experiments were carried out at 37°C. Calibration of the maximal fluorescence signals (R\(_{\text{max}}\)) was determined by sequential addition of ionomycin (1 µM) and calcium (2 mM), whereas that of the minimal fluorescence signals (R\(_{\text{min}}\)) was obtained by treating the cells with Triton X-100 (1%) followed by the addition of EGTA (25 mM). The intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) concentration was calculated according to
Gryniewicz et al. (14) using the equation 

\[ [\text{Ca}^{2+}]_i = K_d x \left( \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \right) x \left( \frac{S_{\text{f2}}}{S_{\text{b2}}} \right) \]

where \( K_d \) is the dissociation constant (224 nM for Fura-2) and \( S_{\text{f2}}/S_{\text{b2}} \) is the ratio of the fluorescent intensity of the \( \text{Ca}^{2+} \)-free indicator to the fluorescent intensity of the \( \text{Ca}^{2+} \)-bound indicator measured with excitation at 380 nm.

**Western blot analysis** - After treatment with parasporin-1, cells were washed with TBS [10 mM Tris-HCl (pH 7.5) and 137 mM NaCl] and were fixed in TBS containing 10% TCA for 1 h at 4˚C. For total protein extraction, fixed cells were suspended in lysis buffer [25 mM Tris-HCl (pH 6.8), 10% SDS, 2 mM EDTA, and 20% glycerol]. The protein concentrations in the resulting lysates were determined using a BCA protein assay kit (Sigma). Lysates containing 5 µg of protein were resolved on a 12% acrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with TBS containing 0.5% Tween 20 and 5% skim milk and were then incubated with the different antibodies in TBS containing 0.05% Tween 20 and 0.5% skim milk. Super Signal West Pico Chemiluminescent Substrate (Pierce) and horseradish peroxidase-conjugated secondary antibodies were used for detection of the protein bands.

**RESULTS**

The effects of parasporin-1 on HeLa cell membrane permeability - Some bacterial toxins, including *B. thuringiensis* Cry toxin, form pores in the plasma membranes of target cells, thereby increasing membrane permeability, \( K^+ \) efflux, and membrane depolarization (13, 15-18). To test whether or not parasporin-1 affects membrane permeability, a LDH leakage assay and a PI influx assay were performed. Parasporin-1 did not induce the release of LDH from HeLa cells, whereas the SLO, a membrane pore-forming toxin from *Streptococcus pyogenes*, induced LDH release within 30 min (Fig. 2A). PI influx assays also demonstrated that parasporin-1 does not affect membrane permeability; SLO, but not parasporin-1, induced PI influx (Fig. 2B).

To examine whether parasporin-1 induces plasma membrane depolarization, we analyzed the effects of parasporin-1 on the membrane potential of treated cells using the membrane potential-sensitive fluorescence probe DiSC\(_3\)(5), which has been widely used for this purpose (19). When SLO was added to HeLa cell cultures, a rapid increase in the fluorescence intensity was observed, indicating that membranes of the cells were depolarized (Fig. 2C, right panel). On the other hand, the fluorescence intensity barely increased after treatment with 10 µg/ml parasporin-1 (Fig. 2C, left panel). These results indicate that parasporin-1 does not form pores in the plasma membrane or induce membrane depolarization.
Inhibition of cellular protein and DNA syntheses by parasporin-1 - The effects of parasporin-1 on the synthesis of cellular proteins and DNA were examined. Although the level of protein synthesis in parasporin-1-treated HeLa cells was about 25% of that in control samples (Fig. 3A), the time course of parasporin-1 inhibition was quite different from those of other toxins that inhibit the protein synthesis machinery, such as diphtheria toxin and ricin toxin, which enter the cytoplasm after binding to the plasma membrane. Diphtheria toxin inhibits cellular protein synthesis after a 30-min lag period, which is the time it takes the toxic moiety to reach the cytosol (20). In contrast, parasporin-1 inhibited protein synthesis soon after its addition to the medium (Fig. 3A). Similarly, partial inhibition of DNA synthesis was observed immediately after the addition of parasporin-1 (Fig. 3B). These results suggest that parasporin-1 exerts its toxic effects quickly by a process that does not require the entry of parasporin-1 into the cell's cytoplasm.

Parasporin-1 induces Ca\(^{2+}\) influx and a sustained elevation of the intracellular Ca\(^{2+}\) concentration in toxin-sensitive cells - The nature of parasporin-1 cytotoxicity, i.e. rapid action without a lag time and partial inhibition of DNA and protein synthesis, implied that parasporin-1 may affect the cells by enhancing or suppressing the levels of intracellular second messengers through the binding of this toxin to the cell surface. Because trimeric GTP-binding proteins are often targets of bacterial toxins, intracellular levels of cAMP and Ca\(^{2+}\) were measured in cells treated with parasporin-1. No alterations of the intracellular cAMP levels, which were quantified by competitive ELISA, were observed after a 2-h treatment with parasporin-1 (5 µg/ml) (data not shown). In contrast, intracellular Ca\(^{2+}\) levels were markedly elevated when HeLa cells were treated with parasporin-1. Intracellular Ca\(^{2+}\) levels were measured using Fura-2, a fluorescent Ca\(^{2+}\) indicator. After loading HeLa cells with Fura-2/AM, the cells were treated with various concentrations of parasporin-1 (0-10 µg/ml) and the [Ca\(^{2+}\)]\(_i\) concentration was estimated from the fluorescence intensity. The [Ca\(^{2+}\)]\(_i\) concentration increased soon after the addition of parasporin-1 (within 3 min) in a dose-dependent manner (Fig. 4A).

The elevation of [Ca\(^{2+}\)]\(_i\) levels can be achieved in two different ways: release from an intracellular storage site or influx from the extracellular environment (21, 22). To clarify the mechanism underlying the parasporin-1-induced elevation of the [Ca\(^{2+}\)]\(_i\) concentration, the parasporin-1-induced Ca\(^{2+}\) increase was measured in cells maintained in a Ca\(^{2+}\)-free buffer. In the presence of extracellular Ca\(^{2+}\), parasporin-1 increased [Ca\(^{2+}\)]\(_i\) levels as shown in Fig. 4A. In
Ca\(^{2+}\)-free buffer, however, parasporin-1 did not induce an elevation of the intracellular Ca\(^{2+}\) level (Fig. 4B). This result indicates that parasporin-1 promotes Ca\(^{2+}\) influx from the extracellular buffer.

In a previous study, we showed that parasporin-1 was a highly cell-type specific cytotoxin (10). To examine whether the parasporin-1-induced elevation of intracellular Ca\(^{2+}\) levels correlated with the cytotoxicity of this protein, the effect of parasporin-1 on the elevation of the [Ca\(^{2+}\)]\(_i\) concentration was examined using four cell lines that have different sensitivities to parasporin-1 (Fig. 5A). HeLa cells, which had the highest sensitivity to parasporin-1 among these four cell lines, showed the largest toxin-induced elevation of the [Ca\(^{2+}\)]\(_i\) concentration (Fig. 4A). Caco-2 cells, the least parasporin-1-sensitive cell line, did not show an increase in the [Ca\(^{2+}\)]\(_i\) concentration following parasporin-1 treatment (Fig. 5B). Sawano cells, which are less sensitive to parasporin-1 than HeLa cells, showed a moderate increase in the intracellular Ca\(^{2+}\) level (Fig. 5C). Finally, although the increase in the [Ca\(^{2+}\)]\(_i\) level in UtSMCs, which are less sensitive to parasporin-1 than Sawano cells, induced by 10 \(\mu\)g/ml parasporin-1 was comparable with that observed in Sawano cells, the increase induced by 1 \(\mu\)g/ml parasporin-1 was significantly smaller in UtSMCs than in Sawano cells (Fig. 5C and D). In addition, A549 cells, which have a parasporin-1 sensitivity that is similar to that of UtSMCs, showed an increase in the [Ca\(^{2+}\)]\(_i\) concentration that was similar to that observed in UtSMCs (data not shown). Thus, the degree of the increase in the [Ca\(^{2+}\)]\(_i\) level induced by parasporin-1 was well correlated with the sensitivities of the cell lines to parasporin-1.

**Involvement of extracellular Ca\(^{2+}\) in the cytotoxicity of parasporin-1** - [Ca\(^{2+}\)]\(_i\) levels are tightly regulated by store-operated and capacitative calcium entry mechanisms (23-27). Disruption of Ca\(^{2+}\) homeostasis that leads to a high concentration of cytosolic free Ca\(^{2+}\) induces cell injury and cell death (28). We speculated that the toxicity of parasporin-1 may be caused by the unusual increase in the [Ca\(^{2+}\)]\(_i\) concentration. To examine this possibility, cells were treated with parasporin-1 in Ca\(^{2+}\)-depleted conditions and cell viability and the [Ca\(^{2+}\)]\(_i\) concentration were measured. When HeLa cells were treated with parasporin-1 in low-Ca\(^{2+}\) medium (approximately 0.3 mM Ca\(^{2+}\)), parasporin-1 was markedly less cytotoxic than when normal medium was used (approximately 2.1 mM Ca\(^{2+}\)) (Fig. 6A). The rise in the [Ca\(^{2+}\)]\(_i\) concentration induced by parasporin-1 was smaller in assay buffer containing 0.3 mM CaCl\(_2\) than in assay buffer containing 2.1 mM CaCl\(_2\) (Fig. 6B). The addition of Ca\(^{2+}\) to the low-Ca\(^{2+}\) medium resulted in the recovery of parasporin-1 cytotoxicity (Fig. 6C, left panel), whereas no
restoration of the toxicity was observed after the addition of Mg\textsuperscript{2+} (Fig. 6C, right panel). These results indicate that extracellular Ca\textsuperscript{2+} plays an important role in the toxicity of parasporin-1 and that the cytotoxic effect of parasporin-1 is induced by the excess entry of extracellular Ca\textsuperscript{2+} into the cytosol.

**Suramin inhibits parasporin-1-induced Ca\textsuperscript{2+} influx and the resulting cytotoxicity** - To examine the pathway of Ca\textsuperscript{2+} influx activated by parasporin-1, various antagonists of Ca\textsuperscript{2+} influx were tested. It is known that the entry of a small amount of extracellular Ca\textsuperscript{2+} into a cell can trigger the release of Ca\textsuperscript{2+} from intracellular storage compartments by activating ryanodine receptors in a process called Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR; 40-42). Because dantrolene and ryanodine, which are two antagonists of ryanodine receptors, did not affect the parasporin-1-induced elevation of the cytosolic Ca\textsuperscript{2+} level (data not shown), we determined that CICR did not contribute to the observed increase in the [Ca\textsuperscript{2+}]\textsubscript{i} concentration.

Hormones and growth factors induce Ca\textsuperscript{2+} release from intracellular stores through phospholipase C (PLC)-mediated D-myoinositol-1,4,5-triphosphate (InsP3) generation and InsP3 receptors (43). To test whether PLC activation is involved in the parasporin-1-induced elevation of the [Ca\textsuperscript{2+}]\textsubscript{i} levels, the effect of the PLC inhibitor U73122 on parasporin-1 toxicity was tested. U73122 did not inhibit either the increase in the [Ca\textsuperscript{2+}]\textsubscript{i} concentration or the parasporin-1 toxicity (data not shown), suggesting that PLC activation and InsP3 production do not contribute to the increased cytosolic Ca\textsuperscript{2+} concentration induced by parasporin-1.

Voltage-dependent Ca\textsuperscript{2+} channels (VDCCs) are one of the pathways through which Ca\textsuperscript{2+} enters cells via the plasma membrane. Nimodipine, flunarizine, diltiazem, and verapamil, which are organic, low-molecular-weight VDCC antagonists (29-31), did not inhibit parasporin-1-induced Ca\textsuperscript{2+} influx into HeLa cells when the antagonists were used at a concentration of 50 µM (data not shown). Additionally, La\textsuperscript{3+} (100 µM) and Cd\textsuperscript{2+} (100 µM) did not affect the parasporin-1-induced Ca\textsuperscript{2+} influx (data not shown). These results suggest that VDCCs are not involved in the parasporin-1-induced Ca\textsuperscript{2+} influx.

Another class of Ca\textsuperscript{2+} channels is regulated by hetero-trimeric G-proteins (32). Suramin, which inhibits G-proteins and G-protein-coupled receptors (33, 34), suppressed the parasporin-1-induced Ca\textsuperscript{2+} influx (Fig. 7A). The cytotoxic effect of parasporin-1 was also suppressed by the addition of suramin (Fig. 7B). Because suramin did not inhibit Ca\textsuperscript{2+} influx resulting from ionomycin treatment, it is clear that suramin did not inhibit Ca\textsuperscript{2+} entry in a non-specific manner (data not shown). These results also indicate that the parasporin-1-induced Ca\textsuperscript{2+} influx is the first
step in the pathway that underlies the cytotoxicity of this toxin.

Ca$^{2+}$ ionophores can mimic parasporin-1 cytotoxicity - Ca$^{2+}$ ionophores, such as ionomycin and A23187, are well-known chemical compounds that can increase the [Ca$^{2+}$], concentration. We tested whether or not Ca$^{2+}$ ionophores mimicked the toxicity of parasporin-1 against HeLa cells. HeLa cells were treated for 20 h with various concentrations of parasporin-1 or the Ca$^{2+}$ ionophores A23187 and ionomycin. Both ionophores were cytotoxic to HeLa cells, although concentration of ionophores required for cytotoxicity was much higher than that of parasporin-1 (Fig 8A). As shown in Fig. 8B, the morphology of HeLa cells treated with the Ca$^{2+}$ ionophores was similar to that of the parasporin-1-treated HeLa cells, which supported the idea that parasporin-1 induces cell death as a result of the continuously high [Ca$^{2+}$], level.

Parasporin-1 induces apoptosis in HeLa cells - To examine the involvement of apoptosis in the parasporin-1-induced cell death, the cleavage of pro-caspase-3 and PARP was monitored by western blot analysis. Cisplatin treatment, which induces apoptosis in HeLa cells, was used as positive control (51). The active form of caspase-3 was detected within 8 h of treatment and the level of this protein increased in a time-dependent manner (Fig. 9A, upper panel). The cleavage of PARP also was detected by western blot analysis within 8 h of the treatment (Fig. 9A, lower panel). In addition, the effects of caspase inhibitors on parasporin-1 toxicity were assessed. The general caspase inhibitor Z-VAD-FMK and the caspase-3-specific inhibitor Z-DEVD-FMK were used. The cytotoxicity of parasporin-1 decreased following the addition of either caspase inhibitor (Fig. 9B). These results suggest that caspase-3 was activated as a result of treatment with parasporin-1 and that HeLa cells underwent apoptosis when the cells were exposed to parasporin-1.

DISCUSSION

Parasporin-1 is a newly identified protein that is cytotoxic to mammalian cells. We have studied the cytotoxic effects of parasporin-1 using HeLa cells and other cell lines. Parasporin-1 quickly (within 1-2 min) raised [Ca$^{2+}$], levels in HeLa cells, and this increase in the Ca$^{2+}$ concentration was the earliest effect of parasporin-1 treatment that we detected. Both the initial increase of the [Ca$^{2+}$], concentration and the sustained elevation of the [Ca$^{2+}$], level were only induced in parasporin-1-sensitive cell lines. Furthermore, under low-Ca$^{2+}$ conditions, the cytotoxicity of parasporin-1 was largely attenuated, whereas it was restored by the addition of Ca$^{2+}$ to the media. Ca$^{2+}$ ionophores, such as A23187 and ionomycin, mimicked the cytotoxicity of parasporin-1,
though the required concentrations of these ionophores were much higher than that of parasporin-1. Finally, suramin inhibited not only the parasporin-1-induced Ca\(^{2+}\) influx, but also the cytotoxicity of parasporin-1. From these results, we conclude that parasporin-1 induces an unusually large influx of Ca\(^{2+}\) in the susceptible cells and this sustained increase in the Ca\(^{2+}\) concentration is responsible for the cytotoxicity of parasporin-1.

In addition to the Ca\(^{2+}\) influx, we demonstrated that parasporin-1 affects protein and DNA synthesis. These alterations could be explained by the increased [Ca\(^{2+}\)], concentration. Elevation of the amount of intracellular Ca\(^{2+}\) may induce phosphorylation of eukaryotic elongation factor-2 and decrease the rate of protein synthesis (35-38). Inhibition of protein synthesis could consequently result in inhibition of DNA synthesis.

Several bacterial toxins are known to form membrane pores. Ions, low-molecular weight material, and even macromolecules can penetrate through these non-selective pores. For example, B. thuringiensis Cry toxin forms a membrane pore (39). In insect cells, this toxin increases cell membrane permeability to K\(^{+}\), Na\(^{+}\), and H\(^{+}\), resulting in depolarization of the plasma membrane and depletion of intracellular K\(^{+}\) (15-17). Aerolysin from Aeromonas hydrophila and epsilon toxin from Clostridium perfringens also induce membrane depolarization and K\(^{+}\) efflux from cells (13, 18). Although parasporin-1 induced Ca\(^{2+}\) influx in HeLa cells, this toxin did not increase membrane permeability as measured by PI influx or LDH release. In addition, the membrane potential was not changed by parasporin-1 treatment. These results indicate that parasporin-1 is not a pore-forming toxin.

We have recently described another member of the parasporin family (identified as parasporin-2 in ref. 10) from the A1547 strain of B. thuringiensis (6). In addition to the different cytotoxic spectra of parasporin-1 and parasporin-2, parasporin-2 reduced the membrane potential of treated cells at the same concentration used for the cytotoxic assay (52), indicating that parasporin-1 and parasporin-2 exert their cytotoxic effects on mammalian cells by different mechanisms.

The level of [Ca\(^{2+}\)], is regulated by Ca\(^{2+}\) influx from the extracellular environment and Ca\(^{2+}\) release from intracellular storage sites (21, 22). Parasporin-1 did not raise [Ca\(^{2+}\)], levels in the absence of external Ca\(^{2+}\), indicating that parasporin-1 induces Ca\(^{2+}\) influx, but not Ca\(^{2+}\) release from intracellular storage sites. Pharmacological analysis also demonstrated that Ca\(^{2+}\) release from intracellular storage sites was not involved in the parasporin-1-induced elevation of the [Ca\(^{2+}\)], concentration. Because parasporin-1-induced Ca\(^{2+}\) influx was not inhibited by VDCC antagonists, VDCCs also did not contribute to the Ca\(^{2+}\) influx induced by parasporin-1. The parasporin-1-induced
elevation of the \([\text{Ca}^{2+}]\) levels was not affected by antagonists of ryanodine receptors, PLC, or VDCCs, whereas suramin inhibited both the increase in the \([\text{Ca}^{2+}]\) concentration and the cytotoxicity of parasporin-1. Suramin antagonizes hetero-trimeric G-protein signaling (33, 34), though the specificity of this inhibition has not been clarified (44-49). In any case, suramin (0.6 mM) did not affect the binding of parasporin-1 to HeLa cells (data not shown). We have shown that suramin inhibited both the \(\text{Ca}^{2+}\) influx and the cytotoxic activity, implying that hetero-trimeric G-proteins or G-protein-coupled receptors are involved in the parasporin-1-induced \(\text{Ca}^{2+}\) influx.

In HeLa cells, parasporin-1 induced an sustained increase in the \([\text{Ca}^{2+}]\) level. These results imply that parasporin-1 disrupts \(\text{Ca}^{2+}\) homeostasis in HeLa cells. Intracellular \(\text{Ca}^{2+}\) is an important regulator of apoptosis; a number of studies have shown that calcium homeostasis is involved in apoptosis and that \([\text{Ca}^{2+}]\) levels increase prior to the activation of apoptosis (28, 50). Suppression of parasporin-1 toxicity by synthetic caspase inhibitors and the degradation of apoptosis-related proteins were observed in parasporin-1-treated HeLa cells. Therefore, parasporin-1 likely causes the activation of an apoptotic pathway.

In conclusion, \(\text{Ca}^{2+}\) influx is a key step in the cytotoxic mechanism of parasporin-1 and extracellular \(\text{Ca}^{2+}\) plays an important role in parasporin-1 toxicity. As far as we know, there are no previous reports of a bacterial cytotoxic protein that causes cell death by specifically increasing the concentration of intracellular \(\text{Ca}^{2+}\). Therefore, parasporin-1 seems to induce cell death by a novel mechanism. In this study, we elucidated the primary step of parasporin-1 toxicity. Future studies will attempt to identify the parasporin-1 receptor and clarify the pathway of parasporin-1-induced \(\text{Ca}^{2+}\) influx. Further studies on the cytotoxic mechanism of parasporin-1 should be valuable with respect to our knowledge of cellular \(\text{Ca}^{2+}\) regulation and \(\text{Ca}^{2+}\) channels, and may show that parasporin-1 can be used as a therapeutic cell type-specific toxin.

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FOOTNOTES

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1The following abbreviations are used: Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; SLO, Streptolysin O; DiSC3(5), 3,3’-dipropylthiadicarbocyanine iodide; MEM, minimum essential medium; DMEM, Dulbecco’s modified Eagle’s medium; normal uterus smooth muscle cells, UtSMCs; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; [Ca\(^{2+}\)], intracellular free Ca\(^{2+}\); PI, propidium iodide; FBS, fetal bovine serum; AM, acetoxymethyl ester; MTS, 3-(4,5 dimethylthiazol-2-ly)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; and BSA, bovine serum albumin.
FIGURE LEGENDS

Fig. 1. SDS-PAGE of purified parasporin-1. Purified parasporin-1 was analyzed by SDS-PAGE. Purified parasporin-1 (1 µg) was loaded onto a 10-20% polyacrylamide gel. Protein bands were detected by silver staining. Lane 1, molecular marker; lane 2, purified parasporin-1 after gel filtration analysis. Marker proteins with known molecular weights are shown on the left.

Fig. 2. The effect of parasporin-1 on membrane permeability. (A) An LDH release assay. HeLa cells were treated with parasporin-1 (left panel) or SLO (right panel). After incubation for the indicated times, LDH activity in the medium was measured. (B) Influx of PI into HeLa cells. HeLa cells were incubated with 5 µg/ml PI for 10 min. Parasporin-1 (left panel) or SLO (right panel) was added at the time indicated by the black arrow. The red arrow indicates the time at which Triton X-100 (1%) was added. PI entry was monitored by measuring the cell-associated fluorescence intensity. The data represent three independent experiments. (C) Changes in the membrane potential of parasporin-1-treated cells measured using a membrane-potential-sensitive dye. After HeLa cells were incubated with 200 nM DiSC3(5) for 30 min at 37˚C, they were treated with 10 µg/ml parasporin-1 (left panel) or 2 µg/ml SLO (right panel). Parasporin-1 or SLO was added at the time indicated by the black arrow. The red arrow indicates the time at which valinomycin and nigericin were added. The data represent three independent experiments.

Fig. 3. Inhibition of cellular protein synthesis and DNA synthesis in HeLa cells by parasporin-1. (A) The effect of parasporin-1 on cellular protein synthesis. HeLa cells were incubated in the presence of 2.5 µg/ml parasporin-1 for the indicated times, followed by incubation with [3H]-leucine for 15 min. The cells were then washed and the amount of TCA-insoluble radioactivity incorporated into the cells was measured. (B) The effect of parasporin-1 on DNA synthesis. HeLa cells were incubated in the presence of 2.5 µg/ml parasporin-1 for the indicated times, followed by incubation with [3H]-thymidine for 15 min. The cells were then washed and the amount of TCA-insoluble radioactivity incorporated into the cells was measured.

Fig. 4. The parasporin-1-induced increase in the [Ca2+]i concentration resulted from an influx of extracellular Ca2+. HeLa cells were loaded with the intracellular Ca2+-indicator Fura-2/AM. The [Ca2+]i concentration was calculated as described in the “Materials and methods” section. Changes in the [Ca2+]i concentration induced by parasporin-1 were measured in cells in extracellular buffer containing 1.2 mM CaCl2 (A) or 0 mM CaCl2 (B). The data represent three
independent experiments.

**Fig. 5.** The cytotoxic activity and parasporin-1-induced elevation of the [Ca$^{2+}$]_{i} concentration in different cell lines. (A) The cytotoxicity of parasporin-1 against different cell lines. Each cell was incubated with various concentrations of parasporin-1 for 20 h. Cell viability was measured using an MTS assay. Cytotoxicity was calculated by subtracting the cell viability from 100%. Data represent the mean values ± S.D. from three independent experiments. Changes in the [Ca$^{2+}$]_{i} concentrations in Caco-2 cells (B), Sawano cells (C), and UtSMCs (D) induced by parasporin-1 treatment. Fura-2/AM was loaded into each cell. Fluorescence intensity was measured in the presence of 1.2 mM CaCl$_2$. Parasporin-1 was added at the time indicated by the arrow and fluorescence intensity was measured every 30 sec. The [Ca$^{2+}$]_{i} concentration was calculated as described in the “Materials and methods” section. Similar results were obtained in three independent experiments.

**Fig. 6.** Involvement of extracellular Ca$^{2+}$ in parasporin-1 toxicity and the elevation of the [Ca$^{2+}$]_{i} concentration in HeLa cells. (A) HeLa cells were treated with various concentrations of parasporin-1 for 20 h. Cell viability was estimated using an MTS assay and values are given as described in the “Materials and methods” section. The Ca$^{2+}$ concentrations of the low- and normal-Ca$^{2+}$ medium were approximately 0.3 and 2.1 (mM) respectively. (O), treatment in low Ca$^{2+}$-medium; (●), treatment in normal-Ca$^{2+}$ medium. Similar results were obtained in three independent experiments and representative data are shown. (B) After loading the cells with Fura-2, HeLa cells were treated with parasporin-1 in Ca$^{2+}$-free Hanks’-HEPES buffer that contained 2.1 mM CaCl$_2$ (left panel) or 0.3 mM CaCl$_2$ (right panel). The [Ca$^{2+}$]$_{i}$ concentration was calculated from the fluorescence intensity as described in the “Materials and methods” section. The arrow indicates the time at which parasporin-1 was added. (C) Parasporin-1 toxicity against HeLa cells in low-Ca$^{2+}$ medium was restored by adding Ca$^{2+}$. Similar results were obtained in three independent experiments and representative data are shown.

**Fig. 7.** Inhibition of parasporin-1-induced Ca$^{2+}$ influx and cytotoxicity by suramin. Similar results were obtained in three independent experiments and representative data are shown. (A) Inhibition of parasporin-1-induced Ca$^{2+}$ influx by suramin. After loading the HeLa cells with Fura-2, the cells were treated with 10 µg/ml parasporin-1 in the presence (right panel) or absence (left panel) of suramin. The [Ca$^{2+}$]$_{i}$ concentrations were determined from the fluorescence intensity as described in the “Materials and methods” section. The arrow indicates the time at which parasporin-1 was added to the HeLa cells. These experiments were carried out in
extracellular buffer containing 2.1 mM CaCl₂. (B) Suppression of parasporin-1 toxicity by suramin. HeLa cells were treated with various concentrations of suramin prior to the application of parasporin-1. After treatment with parasporin-1 for 20 h, cell viability was estimated using an MTS assay.

Fig. 8. Ca²⁺ ionophores mimic parasporin-1 toxicity. (A) The dose-dependent cytotoxic effects of parasporin-1 (○), ionomycin (●), and A23187 (△). HeLa cells were treated with parasporin-1, A23187, or ionomycin at 37°C for 20 h. Cell viability was determined using an MTS assay. The molar concentration of 1 µg/ml parasporin-1 is 0.014 µM. (B) Morphological changes of HeLa cells treated with parasporin-1 or Ca²⁺ ionophores. HeLa cells were treated with parasporin-1 (0.014 µM), ionomycin (100 µM), or A23187 (100 µM). After 20-h incubations at 37°C, cells were observed under a phase-contrast microscope. Bar, 10 µm.

Fig. 9. Parasporin-1 treatment induces apoptosis in HeLa cells. (A) Formation of active caspase-3 and cleavage of the caspase substrate PARP in parasporin-1-treated HeLa cells. HeLa cells were treated with parasporin-1 for the indicated times and total protein was extracted. Cleavage of pro-caspase-3 and PARP was assessed by western blot analysis using an anti-caspase-3 and anti-PARP monoclonal antibodies, respectively. Cisplatin (cis-diamminedichloroplatinum) was used as positive control. (B) Inhibition of parasporin-1 toxicity by caspase inhibitors. HeLa cells were incubated with parasporin-1 (10 µg/ml) in the presence or absence of a caspase inhibitor for 20 h at 37°C. Cell viability was estimated using an MTS assay as described in the “Materials and Methods” section.
Figure 3

A  Protein synthesis

$[^3]H$ leucine incorporation (% control)

Time (min)

B  DNA synthesis

$[^3]H$ thymidine incorporation (% control)

Time (min)
Figure 5

A

Cell viability (% control) vs. Parasporin-1 (μg/ml)

Cell line:
- Caco-2
- UISMC
- Sawano
- HeLa

B

Caco-2

Parasporin-1 (μg/ml)

- 10
- 1
- 0

Time (min)

Increase of [Ca^{2+}]_i (nM)

C

Sawano

Parasporin-1 (μg/ml)

- 10
- 1
- 0

Time (min)

Increase of [Ca^{2+}]_i (nM)

D

UISMC

Parasporin-1 (μg/ml)

- 10
- 1
- 0

Time (min)

Increase of [Ca^{2+}]_i (nM)
Figure 6

A

Cell viability (% control)

0 25 50 75 100

Parasporin-1 (μg/ml)

0 0.01 0.1 1 10

Low Ca²⁺ medium
Normal Ca²⁺ medium

B

[Ca²⁺]_{ex} = 2.1 (mM)

[Ca²⁺]_{ex} = 0.3 (mM)

Parasporin-1 (μg/ml)

0 10 1 0

Increase of [Ca²⁺]_{i}

0 250 500 750 1000

Time (min)

0 10 20 30

C

Ca²⁺ addition

Mg²⁺ addition

Cell viability (% control)

0 25 50 75 100

Parasporin-1 (μg/ml)

0 0.01 0.1 1 10

+0 (mM)
+1.8 (mM)
+0.9 (mM)
+0.45 (mM)

+0 (mM)
+1.8 (mM)
+0.9 (mM)
+0.45 (mM)
Figure 7

A

Absence of suramin

Present of suramin

Increase of $[Ca^{2+}]_i$ (nM)

Time (min)

Parasporin-1

Suramin (mM)

0.6

0.3

0.15

0.075

0.6

Parasporin-1

B

Cell viability (% control)

Parasporin-1 (μg/ml)

Suramin (mM)

0.6

0.3

0.15

0.075

0

0
Figure 8

A

Cell viability (% control)

Log[μM]

-5 -4 -3 -2 -1 0 1 2

○ Parasporin-1
△ A23187
● Ionomycin

B

Parasporin-1 or Ca2+ ionophore

Control (No treatment)

Parasporin-1 (1 μg/ml)

A

B

Ionomycin (100 μM)

C

D

A23187 (100 μM)

E

F
Figure 9

A

Parasporin-1
(100 µg/ml)

Untreated

Cisplatin (50 µM)

Untreated

116 kDa
85 kDa

Pro-Caspase-3
(32 kDa)

Active Caspase-3
(17 kDa)

0 2 4 6 8 10 12 14 16 18 20 20 20
Time (h)

PARP

Caspase-3

B

Z-DEVD-FMK

Cell viability (% control)

0 25 50 75 100

0 0.1 1 10 100
Inhibitor (µM)

Z-VAD-FMK

0 25 50 75 100

0 0.1 1 10 100
Inhibitor (µM)

PS-1 (-)

PS-1 (+)

PS-1 (-)

PS-1 (+)
Parasporin-1, a novel cytotoxic protein from Bacillus thuringiensis, induces Ca\(^{2+}\) influx and a sustained elevation of the cytoplasmic Ca\(^{2+}\) concentration in toxin-sensitive cells

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