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Pore-forming properties of the *Bacillus thuringiensis* toxin Cry9Ca in *Manduca sexta* brush border membrane vesicles

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ABSTRACT

The toxicity and pore-forming ability of the *Bacillus thuringiensis* Cry9Ca insecticidal toxin, its single-site mutants, R164A and R164K, and the 55-kDa fragment resulting from its proteolytic cleavage at residue 164 were investigated using *Manduca sexta* neonate larvae and fifth-instar larval midgut brush border membrane vesicles, respectively. Neither the mutations nor the proteolytic cleavage altered Cry9Ca toxicity. Compared with Cry1Ac, Cry9Ca and its mutants formed large poorly selective pores in the vesicles. Pore formation was highly dependent on pH, however, especially for wild-type Cry9Ca and both mutants. Increasing pH from 6.5 to 10.5 resulted in an irregular step-wise decrease in membrane permeabilization that was not related to a change in the ionic selectivity of the pores. Pore formation was much slower with Cry9Ca and its derivatives, including the 55-kDa fragment, than with Cry1Ac and its rate was not influenced by the presence of protease inhibitors or a reducing agent.

1. Introduction

Among the insecticidal toxins of *Bacillus thuringiensis* [1], the three-domain Cry proteins have been most extensively used for pest control [2] and their mode of action has been studied in most detail [3,4]. They are synthesized as protoxins that accumulate within crystalline inclusions, usually during sporulation. Once ingested by susceptible insects, the protoxins are solubilized in the midgut and hydrolyzed to their active form by intestinal proteases [5,6]. The activated toxins then bind to specific receptors located on the surface of the luminal membrane of midgut epithelial columnar cells [7,8]. Finally, the toxins insert into the membrane and form pores that abolish transmembrane gradients and disrupt cellular functions, leading to cell lysis and death of the insect [3,9].

Although the activated toxins are thought to be resistant to further proteolysis, many cases of cleavage within their pore-forming domain (domain I) have been reported (summarized in [10]). However, the position of these cleavage sites differs widely depending on the toxin being studied and the consequences of such modifications remain somewhat controversial. For instance, it has been suggested that exposure of proteolytic sites following binding of the toxins to their receptors could play an important role in their mode of action by either facilitating [11] or allowing [12] toxin oligomerization and membrane insertion. The importance of such a cleavage step has been questioned, however, as the rate of pore formation by Cry1Aa in midgut brush border membrane vesicles was not altered in the presence of a wide variety of protease inhibitors [10]. Furthermore, protease inhibitors stimulated pore formation by Cry1Ab in freshly isolated midguts, suggesting that proteolysis within the activated toxin hinders, rather than stimulates, its activity [13]. On the other hand, elimination of a major trypsin cleavage site located within the putative α3–α4 loop of domain I in Cry9Ca, by replacing Arginine 164 by an alanine residue using in vitro site-directed mutagenesis, did not alter significantly its toxicity [14].

In the present study, the possible role of proteolysis within the pore-forming domain of the activated toxins was further investigated by examining the ability of wild-type Cry9Ca, its R164A and R164K mutants, as well as the 55-kDa fragment resulting from cleavage at Arginine 164, to permeabilize brush border membrane vesicles isolated from *Manduca sexta*, using a light scattering assay. Although in vitro the purified 55-kDa fragment was more active than the other toxin preparations, especially at alkaline pH, its toxicity, measured by in vivo bioassays, was similar to that of Cry9Ca and both of its mutants. This study constitutes the first characterization of the pores

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formed by Cry9Ca, its kinetics of pore formation, and the influence of micro-environmental factors on its pore-forming ability.

2. Materials and methods

2.1. Toxin activation and purification

Wild-type Cry9Ca, and its mutants R164A and R164K were prepared from *Escherichia coli* W6K strains producing the appropriate single recombinant toxins as described previously [14]. The bacteria were grown in TB medium containing 100 μg/ml ampicillin and, when the optical density at 600 nm reached 0.6 ± 0.2, gene expression was induced at 28 °C by addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. Cry1Ac was produced from *B. thuringiensis* HD73 grown in YT medium containing 100 μg/ml ampicillin as described earlier [15]. Protoxins were activated in vitro with porcine trypsin (Gibco, Grand Island, NY) and the resulting toxins were purified by fast protein liquid chromatography using a mono-Q ion exchange column (Pharmacia Biotech, Montreal, Qc) and eluting bound toxin with a 50 to 500 mM NaCl gradient as described elsewhere [16,17]. To minimize conversion of Cry9Ca and its R164K mutant to the 55-kDa species, activation was terminated by adding a cocktail of protease inhibitors (Calbiochem, San Diego, CA) composed of 100 mM 4-(aminoethyl)benzenesulfonyl fluoride (AEBSF), 80 μM aprotinin, 5 mM bestatin, 1.5 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), 2 mM leupeptin and 1 mM pepstatin A at a 100-fold dilution. However, to isolate the 55-kDa fragment, fresh trypsin was repeatedly added to the wild-type protoxin preparation until the conversion was complete.

2.2. Polyacrylamide gel electrophoresis

Toxin preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [18] using either standard 10% polyacrylamide gels or 4–20% polyacrylamide density gradient gels (Pierce, Rockford, IL). These were stained with GelCode Blue Stain reagent (Pierce) following the manufacturer's recommendations.

2.3. Bioassays

Fertilized eggs of *M. sexta* were obtained from the insectary of the North Carolina State University Department of Entomology (Raleigh, NC). Larvae were fed an artificial diet supplied with the insects. Toxicity assays were carried out by raising neonate larvae on artificial diet contaminated with either protoxin or activated toxin, as described elsewhere [15]. These were applied as 100-μl samples that were layered onto 1.8-cm^2 wells and allowed to absorb into the medium. The tests were done with six concentrations ranging from 1.2 to 10 μg/ml (66.7 to 555.6 ng/cm^2) for the protoxins and 2 to 15 μg/ml (111.1 to 833.3 ng/cm^2) for the activated toxins and the 55-kDa fragment, using 10 groups of 25 larvae for each toxin concentration. Mortality and, for surviving insects, weight gain were recorded after 7 days. Values were adjusted for mortality of control larvae reared in the absence of toxin. The toxin concentrations required to kill 50% and 90% of the larvae (LC_{50} and LC_{90}) or to inhibit weight gain by 50% (EC_{50}) were calculated by probit analysis [19]. Data were evaluated by one-way analysis of variance tests with the InStat software (GraphPad Software, San Diego, CA).

2.4. Brush border membrane vesicle preparation

Whole midguts were isolated from fifth-instar *M. sexta* larvae as described earlier [15] and used to prepare brush border membrane vesicles with an Mg^{2+} precipitation and differential centrifugation method [20].

2.5. Osmotic swelling assay

Brush border membrane vesicle permeability was analyzed with an osmotic swelling technique based on light-scattering measurements [21]. Prior to the experiments, vesicles were resuspended to about 90% of the desired final volume in 10 mM of either MES (morpholineethanesulfonic acid)-KOH (pH 6.5), HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)-KOH (pH 7.5), Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol)-HCl (pH 8.5), CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid)-KOH (pH 9.5), or CAPS (3-[cyclohexylamino]-1-propanesulfonic acid)-KOH (pH 10.5) and incubated overnight at 4 °C. At least 1 h before the beginning of the experiments, the vesicle suspensions were further diluted to a final concentration of 0.4 mg of membrane protein per ml with the appropriate buffer supplemented with enough bovine serum albumin to reach a final concentration of 1 mg/ml. The vesicles were preincubated at 23 °C for about an hour with 0 to 150 pmol of activated toxin per mg of membrane protein (0 to 60 nM), except for kinetic experiments where preincubation was omitted. The assay was initiated by rapidly mixing the vesicles with an equal volume of 10 mM of the appropriate buffer, 1 mg/ml bovine serum albumin, and either 150 mM KCl, KSCN, *N*-methyl-D-glucamine-HCl or potassium gluconate, or 300 mM sucrose or raffinose with a Hi-Tech Scientific (Salisbury, UK) stopped flow rapid kinetics apparatus. For kinetic experiments, 150 pmol of toxin per mg of membrane protein was added to the 150 mM KCl solution before mixing with the vesicles. Scattered light intensity was monitored at the rate of 10 Hz and a wavelength of 450 nm, with a photomultiplier tube located at an angle of 90° from the incident beam, at 23 °C in a PTI spectrofluorometer (Photon Technology International, South Brunswick, NJ).

2.6. Light-scattering data analysis

Percent volume recovery was defined as $100(1 - I_t)$ where I_t is the scattered light intensity measured at time t after rapid mixing relative to the maximum attained in the absence of toxin. Data are given as means ± SEM (standard error of the mean) of three experiments carried out with different vesicle preparations, each performed in quintuplicate. For kinetic experiments, percent volume recovery was calculated for each experimental point and control values obtained in the absence of toxin were subtracted from those measured in the presence of toxin.

3. Results

3.1. Toxin activation and further proteolysis

Because the activated Cry9Ca and R164K toxins are subject to further proteolysis [14], the composition of all toxin preparations was analyzed routinely by polyacrylamide gel electrophoresis. As described earlier in more detail [14], the solubilized protoxins of wild-type Cry9Ca and its mutants, R164A and R164K, migrated as 130-kDa proteins. After activation, mutant R164A was transformed into a toxin of 67–69-kDa that did not undergo further proteolysis. Activated preparations of wild-type Cry9Ca and mutant R164K were composed of mixtures of a 67–69-kDa toxin and a 55-kDa fragment. The relative abundance of these two protein species remained remarkably stable for months when the samples were kept refrigerated (data not shown). On the other hand, preparations in which the trypsinization was deliberately prolonged and repeated contained mostly the 55-kDa fragment.

3.2. Toxicity

There was no significant difference between the in vivo toxicities, estimated for neonate larvae of *M. sexta*, of wild-type Cry9Ca, its