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Midgut juice components affect pore formation by the *Bacillus thuringiensis* insecticidal toxin Cry9Ca

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ABSTRACT

The pore-forming ability of the *Bacillus thuringiensis* toxin Cry9Ca, its two single-site mutants R164A and R164K, and the 55-kDa fragment resulting from its proteolytic cleavage at R164 was evaluated under a variety of experimental conditions using an electrophysiological assay. All four toxin preparations depolarized the apical membrane of freshly isolated third-instar *Manduca sexta* midguts bathing in a solution containing 122 mM KCl at pH 10.5, but the 55-kDa fragment was considerably more active than Cry9Ca and its mutants. The activity of the latter toxins was greatly enhanced, however, when the experiments were conducted in the presence of fifth-instar *M. sexta* midgut juice. This effect was also observed after midgut juice proteins had been denatured by heating at 95 °C or after inorganic ions and small molecules had been removed from the midgut juice by extensive dialysis. A similar stimulation of toxin activity was also observed when the experiments were carried out in the presence of the lipids extracted from an equivalent volume of midgut juice. Depolarization of the cell membrane was also greatly enhanced, in the absence of midgut juice, by the addition of a cocktail of water-soluble protease inhibitors. These results indicate that, depending on the cleavage site and on the experimental conditions used, further proteolysis of the activated Cry9Ca toxin can either stimulate or be detrimental to its activity and that *M. sexta* midgut juice probably contains protease inhibitors that could play a major role in the activity of *B. thuringiensis* toxins in the insect midgut.

1. Introduction

The mechanism of action of the insecticidal crystal toxins produced by the gram-positive bacterium *Bacillus thuringiensis* during sporulation has been the focus of intensive research (Schnepf et al., 1998; Gringorten, 2001; Bravo et al., 2007). Toxicity depends on the ability of these proteins to form pores in the luminal membrane of midgut epithelial columnar cells after having bound to specific receptors located at the surface of this membrane (Gómez et al., 2007; Pigott and Ellar, 2007). The rate of pore formation may vary considerably depending on the toxin which is being studied and the experimental conditions under which it is measured. In particular, pore formation has been found to depend on pH (Tran et al., 2001; Vachon et al., 2006), on the combined effect of pH and ionic strength (Kirouac et al., 2003; Fortier et al., 2005) and on the activity of midgut proteases. In fact, *B. thuringiensis* crystal proteins are first synthesized as inactive protoxins that are later converted into activated toxins by proteases in the insect midgut (Oppert, 1999; Rukmini et al., 2000). Further proteolysis, in the midgut lumen or once the toxin has bound to its receptor, has variously been suggested to be essential (Gómez et al., 2002; Bravo et al., 2004; Rausell et al., 2004), unnecessary (Kirouac et al., 2006; Lebel et al., 2009), stimulatory (Lightwood et al., 2000) and detrimental (Forcada et al., 1996; Keller et al., 1996; Pang and Gringorten, 1998; Shao et al., 1998; Fortier et al., 2007; Rausell et al., 2007) to toxin activity.

Among the crystal toxins studied so far, Cry9Ca is particularly sensitive to proteolysis at a trypsin cleavage site corresponding to R164 and predicted to be located within the α3–α4 loop, in the pore-forming domain of the activated toxin (Lambert et al., 1996). In addition, the pore-forming activity of this toxin, and of its single-site mutants R164A and R164K, has recently been shown to depend strongly on the pH of the solutions used in osmotic swelling experiments carried out with *Manduca sexta* brush border membrane vesicles (Brunet et al., in press). Although neither mutation had significant effects on toxicity, measured by bioassays on insect larvae, the isolated 55-kDa fragment resulting from cleavage

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at R164 was considerably more active at high pH than the other toxin preparations in the vesicles. Because these results demonstrate that the activity of Cry9Ca can be strongly influenced by the experimental conditions under which it is measured, the ability of this toxin, its two mutants, and its 55-kDa fragment to depolarize the apical membrane of *M. sexta* midgut epithelial cells was further analyzed, in the present study, with intracellular microelectrodes. Our results indicate that membrane-bound proteases considerably reduce toxin activity and suggest that midgut juice probably contains protease inhibitors that could, along with the intestinal proteases, play an important role in *B. thuringiensis* toxin function.

2. Materials and methods

2.1. Toxin activation and purification

The various forms of Cry9Ca were prepared using *Escherichia coli* W6K strains producing the appropriate single recombinant proteins as described earlier (Lambert et al., 1996). Protoxins were activated 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 NaCl gradient as described previously (Masson et al., 1990, 1994). The conversion of Cry9Ca and its R164K mutant to the 55-kDa species was minimized by stopping the reaction with 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, in dimethyl sulfoxide, and added at a final 100-fold dilution. On the other hand, the 55-kDa fragment was isolated by repeatedly adding fresh trypsin to the wild-type protoxin preparation, until the conversion was complete.

2.2. Midgut juice and protease inhibitors

M. sexta fertilized eggs were obtained from the North Carolina State University Department of Entomology insectary (Raleigh, NC) and larvae were reared on the artificial diet obtained from the same source. Intestinal juice was collected from whole midguts isolated from fifth-instar larvae as described earlier (Fortier et al., 2007) and stored at −80 °C in small aliquots. For some experiments, the juice was heated at 95 °C for 5 min and centrifuged for another 5 min at 16,000*g* to remove insoluble material. Alternatively, heated midgut juice was dialyzed overnight in Spectra/Por® molecularporous membrane tubing (molecular mass cutoff: 6–8 kDa; Spectrum Laboratories, Rancho Dominguez, CA) against a 1000-fold excess volume of 122 mM KCl, 5 mM $CaCl_2$ and 5 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)–KOH (pH 10.5) (122 K solution). Lipids were extracted from *M. sexta* midgut juice using the method of Bligh and Dyer (1959) as modified by Kates (1972). Because dimethyl sulfoxide had detrimental effects on membrane potential, the protease inhibitor cocktail used in some electrophysiological experiments was composed of an aqueous solution of 50 mM AEBSF, 1 mg/ml antipain, 0.015 mM aprotinin, 0.1 mM E64 and 0.1 mM leupeptin and diluted 100-fold. The inhibitors were obtained from Sigma Aldrich Canada (Oakville, Ont.).

2.3. Membrane potential measurements

Electrical potential across the apical membrane of *M. sexta* midgut epithelial cells was measured as described earlier (Peyronnet et al., 1997). This approach takes advantage of the fact that, when a freshly isolated larval midgut is cut transversely, both of its ends curl back onto themselves. A short segment of midgut was aspirated into a glass pipette from one end until its other end curled around the pipette tip, thus exposing the apical surface of the epithelial cells. Experiments were carried out with midguts freshly isolated from third-instar larvae and bathed in the 122 K solution. Midgut cells were impaled with a glass microelectrode filled with 1 M KCl. Electrode resistance was between 50 and 250 MΩ. The impalements were considered successful when a sharp and sustained change in potential (negative inside) was observed. The signal was amplified with an M-707 microprobe system (WP Instruments, Hamden, CT), monitored with an oscilloscope (Kikusui Electronics, Yokohama, Japan) and a strip chart recorder (BBC Goerz Metrawatt, Wiener Neudorf, Austria). The bath was perfused continuously with the 122 K solution until the membrane potential was stable for 5 min. Perfusion was then stopped and 1.2 ml of perfusion solution containing 10 μg/ml of the appropriate toxin, in the presence or absence of 10% (v/v) midgut juice, the lipids extracted from the equivalent of 10% (v/v) midgut juice, or 1% (v/v) protease inhibitor cocktail, was added directly to the bath. After 5 min, the preparation was rinsed for another 10 min by resuming the perfusion. All experiments were carried out at room temperature.

Because midgut juice contains a high concentration of potassium ions (Fortier et al., 2007), the solutions used for experiments in which the tissue was exposed to either untreated or heated midgut juice were prepared by first diluting these materials sufficiently in a solution composed of 5 mM $CaCl_2$ and 5 mM CAPS–KOH (pH 10.5) to reach a potassium concentration of approximately 122 mM and adjusting the final volume with the appropriate amount of the 122 K solution.

2.4. Data analysis

Measured voltages, V, were normalized relative to the voltage measured just before the perfusion was stopped, V_0. The percentage of depolarization with respect to the control is defined as: $[1-(V/V_0)_{EXP}/(V/V_0)_{CTL}]\ 100$, where $(V/V_0)_{EXP}$ is the normalized experimental result and $(V/V_0)_{CTL}$ is the normalized result measured in the absence of toxin. All statistical comparisons were made with unpaired Student's t tests, using the Welch correction when the standard deviations were too different, with the InStat software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Membrane potential measurements

Pore formation by Cry9Ca, its 55-kDa fragment and its two single-site mutants, R164A and R164K, was studied by measuring membrane potential across the luminal membrane of midgut epithelial cells of isolated *M. sexta* midguts under a variety of experimental conditions (Fig. 1). As pointed out earlier (Peyronnet et al., 1997), membrane potential dropped somewhat during the 5 min during which the perfusion was stopped, even in the absence of toxin. The level attained during this period was especially low when the added material included unmodified (Fig. 1B) or heated midgut juice (Fig. 1C). Because the potassium concentration was adjusted as described under Section 2, this effect is probably due to a higher concentration of other ions in midgut juice than in the 122 K bathing solution. In any case, in the absence of toxin, membrane potential always increased rapidly, as soon as the perfusion was resumed, approaching the value that was measured before the addition was made.

The wild-type toxin and its two mutants depolarized the cell membrane of midguts bathing in the 122 K solution (Fig. 1A). The rate of depolarization was nevertheless rather slow when com-