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Differential Effects of Ionic Strength, Divalent Cations and pH on the Pore-forming Activity of *Bacillus thuringiensis* Insecticidal Toxins

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Abstract. The combined effects of ionic strength, divalent cations, pH and toxin concentration on the pore-forming activity of Cry1Ac and Cry1Ca were studied using membrane potential measurements in isolated midguts of *Manduca sexta* and a brush border membrane vesicle osmotic swelling assay. The effects of ionic strength and divalent cations were more pronounced at pH 10.5 than at pH 7.5. At the higher pH, lowering ionic strength in isolated midguts enhanced Cry1Ac activity but decreased considerably that of Cry1Ca. In vesicles, Cry1Ac had a stronger pore-forming ability than Cry1Ca at a relatively low ionic strength. Increasing ionic strength, however, decreased the rate of pore formation of Cry1Ac relative to that of Cry1Ca. The activity of Cry1Ca, which was small at the higher pH, was greatly increased by adding calcium or by increasing ionic strength. EDTA inhibited Cry1Ac activity at pH 10.5, but not at pH 7.5, indicating that trace amounts of divalent cations are necessary for Cry1Ac activity at the higher pH. These results, which clearly demonstrate a strong effect of ionic strength, divalent cations and pH on the pore-forming activity of Cry1Ac and Cry1Ca, stress the importance of electrostatic interactions in the mechanism of pore formation by *B. thuringiensis* toxins.

Key words: Pore-forming toxins — Membrane potential — Membrane permeability — Electrostatic interactions — *Bacillus thuringiensis* — *Manduca sexta*

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Introduction

During sporulation, the gram-positive bacterium *Bacillus thuringiensis* produces one or more toxic proteins which are highly specific for different species of insects (Höfte & Whiteley, 1989). After ingestion by susceptible insect larvae, these toxins become soluble in the midgut lumen and are activated by partial proteolysis. The activated toxins then bind to specific receptors and form pores in the brush border membrane of the insect midgut epithelial cells. These pores abolish ionic gradients across the membrane and cause the epithelial cells to lyse (Schnepf et al., 1998).

The mechanism of pore formation, which implies that fully soluble toxins are converted to integral membrane proteins, remains poorly understood (Schwartz & Laprade, 2000; Mathur et al., 2004). Electrostatic interactions between toxin molecules and the membrane are nevertheless expected to affect both the binding and insertion of the toxin. A strong potential is present across the luminal membrane of the midgut (Moffet & Koch, 1988a; Dow & Peacock, 1989) and is likely to affect pore formation, as suggested by theoretical (Biggin & Sansom, 1996) and experimental (Lemeshko, Arias & Orduz, 2005) studies on short peptides corresponding to selected α-helices of the pore-forming domain of *B. thuringiensis* toxins. The lepidopteran midgut lumen is also characterized by a highly alkaline pH (Dow, 1984, 1992; Gringorten, Crawford & Harvey, 1993) and a high ionic strength (Dow & Harvey, 1988), two factors that could also modulate electrostatic interactions at the membrane surface.

Earlier studies using a light-scattering assay have indicated that, in the absence of a membrane potential and at low ionic strength, Cry1Ca forms pores considerably more efficiently at pH 7.5 than at pH 10.5 in midgut brush border membrane vesicles

Table 1. Chemical composition of solutions used for electrophysiological and osmotic swelling experiments[a]

Type of experiment	Solution	KCl	$BaCl_2$	$CaCl_2$	$MgCl_2$	Sucrose	HEPES or CAPS[b]
Electrophysiology	0K	–	–	5	–	245	5
	32K	32	–	5	–	181	5
	122K	122	–	5	–	–	5
Osmotic swelling assay[c]	64K/Ba	64	10	–	–	30	10
	64K/Ca	64	–	10	–	30	10
	64K/Mg	64	–	–	10	30	10
	94K	94	–	–	–	–	10
	150K	150	–	–	–	–	10
	244K/Ca	244	–	10	–	30	10
	274K	274	–	–	–	–	10

[a]Values are in mM.
[b]pH was adjusted to 7.5 (HEPES) or 10.5 (CAPS) with KOH.
[c]All solutions used for osmotic swelling experiments also contain 1 mg/ml bovine serum albumin.

isolated from *Manduca sexta* (Tran et al., 2001). In addition, Cry1Ca was much less active than Cry1Ac at the higher pH (Tran et al., 2001). However, Cry1Ca was more active than Cry1Ac at both pH 7.5 and 10.5 in experiments carried out with the same vesicles and a potential-sensitive fluorescent probe, diS-Cs(5) (Kirouac et al., 2003). The main difference between these two techniques resides in the conditions under which the pores are formed. Vesicles are incubated with the toxin at a higher concentration and at a much higher ionic strength for fluorescence assays than for light-scattering experiments. This difference suggests a strong influence of electrostatic interactions on the efficiency with which pores are formed by *B. thuringiensis* toxins. In the present study, the pore-forming activity of Cry1Ac and Cry1Ca was therefore further investigated using membrane potential measurements in isolated midguts of *M. sexta* and osmotic swelling assays with brush border membrane vesicles prepared from the same species. These experiments demonstrate that pore formation by these toxins is indeed modulated by the combined effects of ionic strength, divalent cations, pH and toxin concentration.

Materials and Methods

Insects

Fertilized *M. sexta* eggs were purchased from the Carolina Biological Supply Company (Burlington, NC). Larvae were reared on a standard synthetic medium supplied with the insects.

Toxins

Cry1Ac and Cry1Ca toxins were prepared from *B. thuringiensis* strains producing the appropriate single recombinant toxins, made soluble and trypsin-activated as described elsewhere (Masson et al., 1989, 1994). Activated toxins were purified by fast protein liquid chromatography using a Mono Q ion exchange column (Pharmacia Biotech, Montreal, Qc). Bound toxin was eluted with a 50–500 mM NaCl gradient in a 20 mM sodium carbonate buffer, pH 10.8 (Masson et al., 1994).

Membrane Potential Measurements

Experiments were conducted as described previously (Peyronnet et al., 1997) with minor modifications. The standard 32K solution (Moffet & Koch, 1988b), used in most previous experiments (Peyronnet et al., 1997), was modified as indicated in Table 1 in order to vary ionic strength and pH. Oxygen gas was bubbled vigorously for 30 minutes through the solutions immediately before use. Fresh midguts were isolated from actively feeding third-instar *M. sexta* larvae and rinsed with the solution used for perfusion. The Malpighian tubules and peritrophic membrane, with its food content, were removed with forceps. Isolated midguts were aspirated into a glass pipette until their ends curled over the pipette tip, thus exposing their epithelial cells (Peyronnet et al., 1997). Midgut cells were impaled, through their apical membrane, with a glass microelectrode filled with 1 M KCl. Electrode resistance was between 100 and 200 MΩ. The signal was amplified with a KS-700 microprobe system apparatus (WP Instruments, New Haven, CT) and recorded on a strip chart recorder (Houston Instruments, Austin, TX). The bath was perfused with the appropriate solution (Table 1), as specified below, at approximately 1 ml/min until the membrane potential was stable over 5 min. Perfusion was then stopped and 1 ml of perfusion solution containing the specified toxin concentration (0, 1 or 10 μg/ml) was added directly to the bath. After 5 min, the preparation was rinsed with the toxin-free perfusion solution for 10 min. All experiments were carried out at room temperature (22–24°C). Membrane potential was normalized by dividing experimental values by the average of the initial membrane potentials (V_0) measured over the five first minutes. Electrophysiological data are presented as means ± SEM for 3 to 13 independent experiments.

Osmotic Swelling Assay

Brush border membrane vesicles were purified from isolated fifth-instar *M. sexta* larvae midguts with a magnesium precipitation and differential centrifugation procedure (Wolfersberger et al., 1987). Membrane permeabilizing effects of *B. thuringiensis* toxins were

analyzed with an osmotic swelling assay (Carroll & Ellar, 1993). Vesicles (0.4 mg membrane protein/ml) equilibrated overnight in 10 mM HEPES/KOH (pH 7.5) or CAPS/KOH (pH 10.5) were incubated for 60 min with the appropriate toxin concentration, as specified below. They were then rapidly mixed, directly in a cuvette, with an equal volume of one of the hypertonic solutions listed in Table 1, using a stopped-flow apparatus (Hi-Tech Scientific, Salisbury, England). In response to this hypertonic shock, vesicles rapidly shrink, thereby causing a sharp rise in scattered light intensity. Depending on their permeability to the solutes, the vesicles subsequently recover some of their original volume. Scattered light intensity was monitored at 450 nm at an angle of 90° and at a frequency of 10 Hz in a PTI spectrofluorometer (Photon Technology International, South Brunswick, NJ). Kinetic experiments, designed to monitor changes in membrane permeability as a function of time, were conducted with vesicles that were not incubated with toxin but were instead mixed with the appropriate hypertonic solution (Table 1) containing no toxin or 75 pmol of either Cry1Ac or Cry1Ca/mg membrane protein. In some experiments, the vesicles were incubated for 60 min with the toxin at a 3.7 (3.7×) or 10 (10×) times higher concentration of both, the vesicles and toxin. Independently of the concentration used during the incubation step, the vesicles were always diluted to 0.4 mg membrane protein/ml before the onset of the light-scattering assay. Osmotic swelling experiments, each carried out in quintuplicate, were performed three times with different vesicle preparations. Data are presented as means ± SEM.

Data Analysis

Scattered light intensity values were first normalized between 0 and 1. Percent volume recovery is defined as $[1-I(t)] \cdot 100$ where $I(t)$ is the measured relative scattered light intensity I at a given time t. For kinetic experiments, percent volume recovery was calculated for each experimental point. Control values obtained in the absence of toxin were subtracted from those measured in the presence of toxin. The resulting curves, which illustrate the changes in membrane permeability due to the effect of the toxin, were fitted with a Boltzmann sigmoidal function. The maximal osmotic swelling rate was estimated from the slope of the fitted curves at their inflection point. Statistical analyses were done using two-tailed unpaired Student t-tests and differences were considered significant when $P < 0.05$.

Results and Discussion

Ionic Strength

The effect of ionic strength on the activity of Cry1Ac and Cry1Ca was first examined in isolated *M. sexta* midguts with an electrophysiological procedure that was developed earlier in our laboratory (Peyronnet et al., 1997). Previous electrophysiological experiments have typically been conducted in a standard solution containing 32 mM KCl (Moffet & Koch, 1988b; Peyronnet et al., 1997). However, the potassium concentration in the midgut lumen of *M. sexta* fifth-instar larvae, measured by flame photometry, varies from 190 ± 15 to 211 ± 11 mM, depending on the midgut region (Dow & Harvey, 1988). A substantial fraction of the potassium appears to be bound, however, since the potassium activity, measured with a potassium-specific electrode, varies in the midgut lumen from only 80 ± 0.2 to 84 ± 1 mM (Dow & Harvey, 1988). In the present study, the KCl concentration of some of the solutions was raised to 122 mM, the maximum value that could be used without changing their osmolarity relative to that of the standard 32 mM KCl solution (Moffet & Koch, 1988b; Peyronnet et al., 1997).

As was described earlier (Peyronnet et al., 1997), membrane potential in this tissue is highly sensitive to changes in the KCl concentration of the bathing solution. In preliminary experiments, membrane potentials measured in midguts dissected and perfused with the 32K solution, at pH 7.5 or 10.5, rose rapidly immediately after replacing the bathing solution by the 0K solution. Subsequently, the potential declined gradually over at least 10 min before reaching a stable level (*data not shown*). For each experimental condition, the midguts were therefore dissected in the appropriate solution and perfused with the same solution until the membrane potential was stable for 5 min. At pH 7.5, the initial membrane potentials (V_0) measured during this period in the absence of toxin were −93 ± 5 (n = 16), −73 ± 8 (n = 19) and −70 ± 5 (n = 19) mV when tested in the 0K, 32K and 122K perfusion solutions, respectively. At pH 10.5, the corresponding V_0 values were −93 ± 6 (n = 16), −97 ± 6 (n = 16) and −62 ± 4 (n = 31) mV.

The effect of varying ionic strength in the bathing solution on toxin activity depended strongly on pH as well as on the toxin used and its concentration. At pH 7.5, both toxins depolarized efficiently the membrane in all three KCl concentrations tested. Under these conditions, reducing toxin concentration from 10 to 1 μg/ml only resulted in a relatively slight reduction in the depolarization rate (Fig. 1*A, C* and *E*). At pH 10.5, membrane depolarization was rapid in the presence of 10 μg/ml Cry1Ac at all three KCl concentrations (Fig. 1*B*, *D* and *F*). In contrast, at 10 μg/ml, Cry1Ca was inactive in the absence of KCl (Fig. 1*B*), but its capacity to depolarize the membrane was fairly comparable to that of Cry1Ac in the presence of 32 (Fig. 1*D*) and 122 (Fig. 1*F*) mM KCl. However, at 1 μg/ml, Cry1Ac depolarized efficiently the membrane in the absence of potassium chloride (Fig. 1*B*), but depolarization was substantially slower in the presence of 32 (Fig. 1*D*) and 122 mM KCl (Fig. 1*F*). At 1 μg/ml, Cry1Ca depolarized the membrane at a rate which was comparable to that observed for the same concentration of Cry1Ac in the presence of 32 mM KCl (Fig. 1*D*) but, in contrast to Cry1Ac, completely depolarized the membrane in the presence of 122 mM KCl (Fig. 1*F*).

These results demonstrate a strong pH-dependent effect of ionic strength on the activity of Cry1Ac and Cry1Ca. They also explain apparent discrepancies between previously published results (Tran et al.,

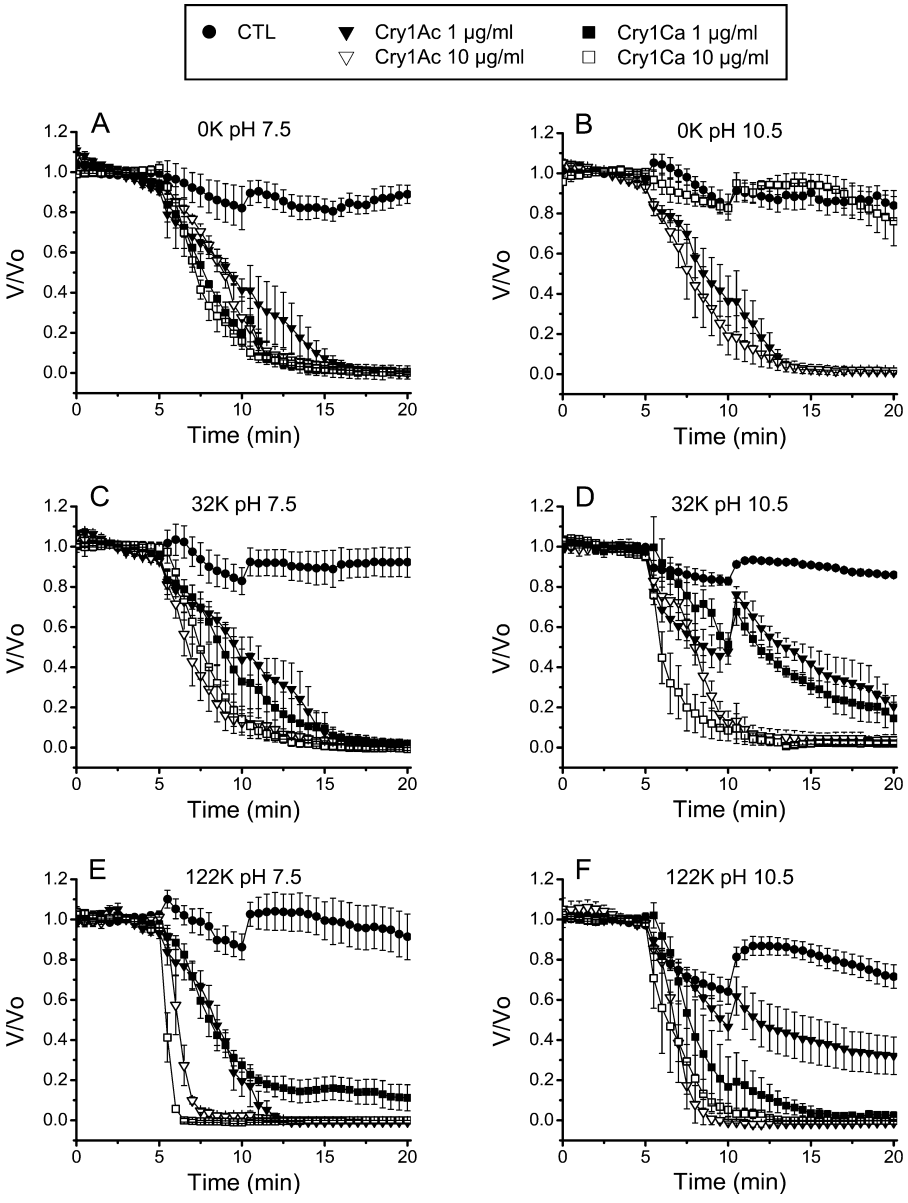

Fig. 1. Effect of ionic strength and pH on the ability of *B. thuringiensis* toxins to depolarize the luminal membrane of isolated *M. sexta* midgut epithelial cells. The bath was perfused with the 0K solution at pH 7.5 (*A*) or 10.5 (*B*), the 32K solution at pH 7.5 (*C*) or 10.5 (*D*), or the 122K solution at pH 7.5 (*E*) or 10.5 (*F*) until the membrane potential was stable over 5 min. Perfusion was then stopped and 1 ml of perfusion solution containing no toxin (●), 1 µg/ml (▼, ■) or 10 µg/ml (∇, □) of either Cry1Ac (▼, ∇) or Cry1Ca (■, □) was added directly to the bath. After 5 min, the preparation was rinsed with perfusion solution for 10 min.

2001; Kirouac et al., 2003). The fact that Cry1Ca was able to depolarize efficiently the membrane in the absence of KCl at pH 7.5, but unable to do so at pH 10.5, correlates well with its much stronger ability to form pores in brush border membrane vesicles at pH 7.5 than at pH 10.5, in the presence of a very low ionic strength, as observed during osmotic swelling experiments (Tran et al., 2001). On the other hand, the fact that Cry1Ac and Cry1Ca were both able to depolarize the membrane at pH 7.5 and 10.5 in the presence of 122 mM KCl agrees well with fluorescence experiments which showed that, following incubation of the vesicles with the toxins in a high ionic strength solution, pH had little effect on the activity of both toxins (Kirouac et al., 2003). Cry1Ac activity is nevertheless pH- and concentration-dependent at this higher ionic strength, as evidenced by the observation that it depolarized completely the membrane when tested at 10 µg/ml at both pH values and at 1 µg/ml at pH 7.5, but considerably slower when tested at the lower concentration at pH 10.5 (Fig. 1*E* and *F*).

Divalent Cations

Ca^{2+} is known to play an important role in the structural stability of animal tissues (Takeichi, 1990). All solutions used in the electrophysiological experiments therefore contained 5 mM $CaCl_2$ (Table 1). Experiments carried out at lower Ca^{2+} concentrations proved to be impracticable because cells tended to detach from the tissue and membrane potential could rarely be measured for sufficiently long time periods. Therefore, the effect of Ca^{2+} on the pore-forming activity of Cry1Ac and Cry1Ca was exam-

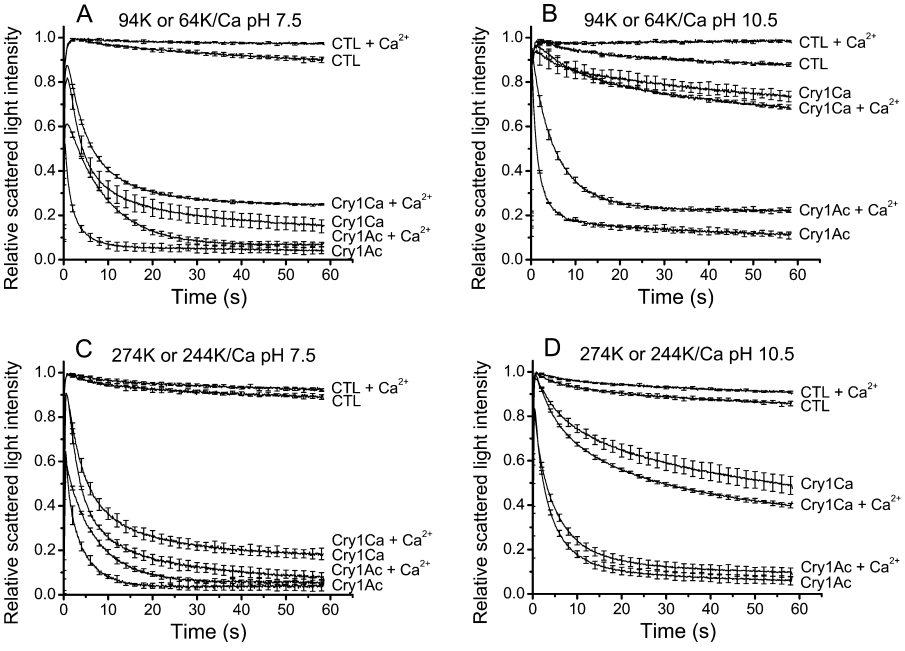

Fig. 2. Effect of ionic strength, calcium chloride and pH on the osmotic swelling of brush border membrane vesicles following incubation with Cry1Ac or Cry1Ca. Vesicles equilibrated overnight in 10 mM HEPES/KOH, pH 7.5 (*A, C*) or CAPS/KOH, pH 10.5 (*B, D*) were incubated for 60 min with no toxin (CTL) or with 75 pmol of Cry1Ac or Cry1Ca/mg membrane protein and rapidly mixed with an equal volume of either the 64K/Ca solution (Ca^{2+}) or the 94K solution (*A, B*) or the 244K/Ca solution (Ca^{2+}) or the 274K solution (*C, D*) at pH 7.5 (*A, C*) or 10.5 (*B, D*). For clarity, error bars are only shown for every 20th experimental point.

ined using *M. sexta* midgut brush border membrane vesicles and an osmotic swelling assay based on light-scattering measurements (Carroll & Ellar, 1993). Because Ca^{2+}, like ionic strength and pH, could possibly influence the properties of the pores and those of the vesicles, osmotic swelling experiments were first performed using vesicles that had been previously incubated with the toxin for 60 min (Fig. 2). In these experiments, for a given pH, incubation with the toxins was therefore carried out under identical conditions, independently of those used for the subsequent osmotic swelling step. Experiments were conducted at pH 7.5 (Fig. 2*A* and *D*) and at pH 10.5 (Fig. 2*B* and *D*) using solutions of relatively low (94K and 64K/Ca) (Fig. 2*A* and *B*) and higher (274K and 244K/Ca) (Fig. 2*C* and *D*) ionic strengths. Thus, the final KCl and Ca^{2+} concentrations, after mixing the 64K/Ca and 244K/Ca solutions with the vesicles in light-scattering experiments, corresponded to those of the 32K and 122K solutions used for the electrophysiological experiments.

Because the pH and osmotic conditions used for these experiments differ for each panel, swelling rates cannot be strictly compared between panels, since changing ionic strength implies changing osmolarity and the amplitude of the solute transmembrane gradient. Within each panel, however, the solutions used were isoosmotic and of identical ionic strength to ensure that all curves were as comparable as possible (Table 1). To adjust osmolarity, some of the KCl had nevertheless to be replaced by sucrose in the solutions containing calcium (Table 1). Although calcium chloride diffuses at a rate comparable to that of KCl (Kirouac et al., 2002), differences in osmotic swelling rates observed in the presence and absence of calcium could result, at least in part, from the fact that sucrose diffuses through the pores formed by the toxins at a smaller rate than KCl (Carroll & Ellar, 1993; Tran et al., 2001). On the other hand, the midgut brush border membrane is known to contain cadherin molecules, which could cause the vesicles to aggregate when exposed specifically to calcium, a phenomenon which would alter scattered light intensity measurements (Griko et al., 2004).

In the absence of toxin, vesicle swelling rates were always somewhat smaller in the presence of Ca^{2+} (Fig. 2), thus confirming the possibility that Ca^{2+}-dependent aggregation of the vesicles and Ca^{2+}-dependent effects on membrane properties could contribute slightly to the observed changes in scattered light intensity. In the presence of toxin, except when vesicles were incubated with Cry1Ca at pH 10.5, vesicle swelling rates were also always somewhat smaller in the presence of Ca^{2+} (Fig. 2). This result confirms that the effect of replacing some of the KCl with a slower diffusing solute, sucrose, on solute entry through already formed pores, is relatively small, but not negligible.

Further experiments were then carried out to evaluate the effect of Ca^{2+} on the rate of pore formation by Cry1Ac and Cry1Ca (Fig. 3), using the same solutions as those used for the experiments illustrated in Fig. 2. Scattered light intensity was monitored following rapid mixing of vesicles, which had not been incubated with toxin, with an equal volume of the appropriate hypertonic solution containing no toxin or 75 pmol of either Cry1Ac or Cry1Ca/mg membrane protein. At pH 7.5, Cry1Ac formed pores more rapidly than Cry1Ca in the 94 K solution although a similar final volume recovery was

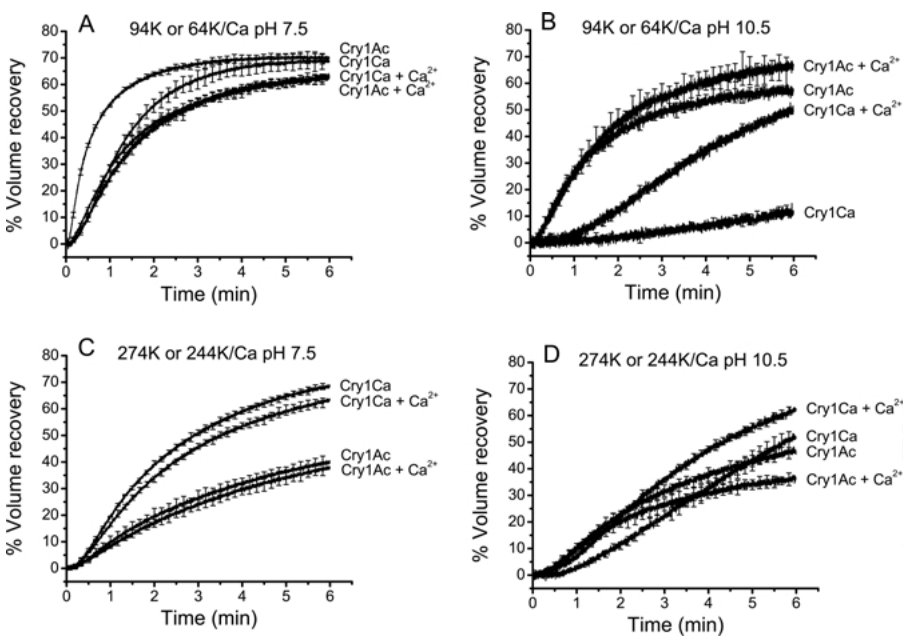

Fig. 3. Effect of ionic strength, calcium chloride and pH on the kinetics of pore formation by Cry1Ac and Cry1Ca in brush border membrane vesicles. Vesicles equilibrated overnight in 10 mM HEPES/KOH, pH 7.5 (*A, C*) or CAPS/KOH, pH 10.5 (*B, D*) were rapidly mixed with 75 pmol of Cry1Ac or Cry1Ca/mg membrane protein in an equal volume of either the 64K/Ca solution (Ca^{2+}) or the 94 K solution (*A, B*), or the 244K/Ca solution (Ca^{2+}) or the 274K solution (*C, D*) at pH 7.5 (*A, C*) or pH 10.5 (*B, D*). Percent volume recovery was calculated as described in the Materials and Methods section. Values obtained for control vesicles, assayed without toxin, were subtracted from experimental values measured in the presence of toxin. For clarity, error bars are only shown for every 100th experimental point.

reached after 6 min in the presence of both toxins (Fig. 3*A*). Under these conditions, the maximal swelling rate observed in the presence of Ca^{2+} was considerably reduced for Cry1Ac but was unaffected for Cry1Ca (Fig. 3*A*). In contrast, at a higher ionic strength, Cry1Ca formed pores considerably more rapidly than Cry1Ac (Fig. 3*C*). Under these conditions, the osmotic swelling rate observed for Cry1Ca was slightly but significantly reduced in the presence of Ca^{2+} (Fig. 3*C*). The rate observed for Cry1Ac was unaffected by the presence of this cation (Fig. 3*C*).

As was demonstrated earlier (Tran et al., 2001), at low ionic strength, Cry1Ca forms pores much more readily at pH 7.5 (Fig. 3*A*) than at pH 10.5 (Fig. 3*B*). In agreement with this observation, at pH 10.5 and at the lower ionic strength, the maximal swelling rate induced by Cry1Ac was much higher than that induced by Cry1Ca (Fig. 3*B*). Under these conditions, the presence of Ca^{2+} strongly stimulated pore formation by Cry1Ca, but had little effect on the activity of Cry1Ac (Fig. 3*B*). At the higher ionic strength and in the absence of Ca^{2+}, maximal swelling rates induced by Cry1Ac were only slightly higher than those induced by Cry1Ca (Fig. 3*D*). As was observed at the lower ionic strength, Ca^{2+} stimulated pore formation by Cry1Ca but had little effect on the activity of Cry1Ac (Fig. 3*D*).

These differential effects of ionic strength on the swelling rates induced by Cry1Ac and Cry1Ca shown in Fig. 3 contrast dramatically with those observed in Fig. 2, where pores were formed before the osmotic swelling experiments. Under the latter conditions, inversions in the relative activity of the two toxins were never observed. These results clearly point to a differential effect of ionic strength on the rate of pore formation, rather than on pore properties. This effect is most likely due to screening of electrical charges at the surface of the toxin and the membrane. Furthermore, the effects of calcium are likely accounted for by the fact that divalent cations have stronger screening effects on membrane surface electrostatic potentials than monovalent cations (Gennis, 1989). Membrane surface potential depends strongly on the concentration of monovalent and divalent cations in the bathing solution. It can be estimated using a basic equation derived from the double-layer theory for electrolytes of unsymmetrical valence types (Grahame, 1953). Assuming a charge density of −0.05 C/m^2, as in a phospholipid membrane composed of 20% anionic lipids (Gennis, 1989), the theoretical membrane surface potential under conditions corresponding to those of the experiments shown in Fig. 3*A* and 3*B* is −69.4 mV in the absence of Ca^{2+} and −57.5 mV in its presence. The corresponding values for the experiments shown in Fig. 3*C* and 3*D* are −49.6 and −46.5 mV. The estimated difference in surface potentials in the presence and absence of calcium is thus considerably lower at the higher ionic

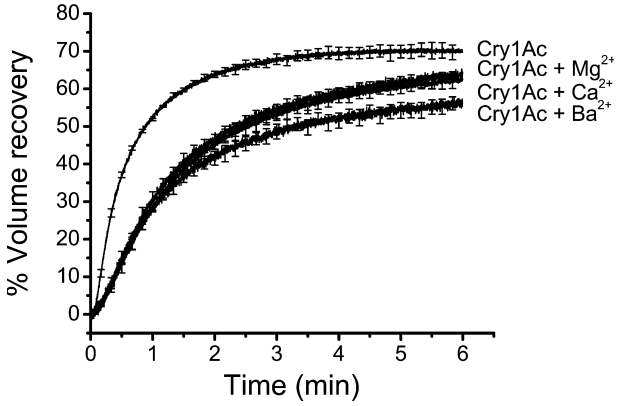

Fig. 4. Effect of divalent ions on the kinetics of pore formation by Cry1Ac in brush border membrane vesicles. Vesicles equilibrated overnight in 10 mM HEPES/KOH, pH 7.5, were rapidly mixed with 75 pmol of Cry1Ac/mg membrane protein in an equal volume of the 64K/Ba (Ba^{2+}), 64K/Ca (Ca^{2+}), 64K/Mg (Mg^{2+}), or 94K solution at pH 7.5. Data were analyzed as described in the legend of Fig. 3.

strength. These observations may explain why Ca^{2+} affects toxin activity much more strongly under the lower ionic strength conditions (Fig. 3). Taking the results shown in Fig. 2*A* into account, the inhibitory effect of calcium on Cry1Ac activity illustrated in Fig. 3*A* cannot be entirely attributed to a reduced rate of pore formation. However, the strong stimulation of Cry1Ca activity by Ca^{2+} illustrated in Fig. 3*B* and *D* appears to result mostly from a genuine increase in the rate of pore formation. In addition, it should also be pointed out that the charge density at the surface of the membrane and toxin is expected to be more strongly negative at pH 10.5 than at pH 7.5. The resulting higher membrane surface potential thus probably contributes to the stronger effects of ionic strength observed at the higher pH (Fig. 1).

If Ca^{2+} alters toxin activity mainly by reducing electrostatic interactions at the membrane surface, other divalent cations should have a similar effect on toxin activity. The effect of Mg^{2+} and Ba^{2+} on the activity of Cry1Ac was therefore compared to that of Ca^{2+} under low ionic strength conditions at pH 7.5 (Fig. 4). The rate of volume recovery of the vesicles was remarkably similar in the presence of either cation (Fig. 4). Divalent cations therefore appear to cause a genuine alteration in the vesicle swelling rates, by modifying electrostatic interactions, rather than causing a cadherin-dependent aggregation of the vesicles. This conclusion is also supported by the observation that increases in the ionic strength of the solutions had similar effects as additions of divalent cations on the activity of Cry1Ac and Cry1Ca (Figs. 1 and 3). Unfortunately, a similar experiment with Cry1Ca at pH 10.5, the other condition under which Ca^{2+} had a markedly strong effect (Fig. 3*B*), could not be performed because magnesium and barium form insoluble hydroxides at high pH.

Vesicle Concentration

As mentioned in the introduction, the effects of pH on the activity of Cry1Ac and Cry1Ca differ depending on whether they are studied with an osmotic swelling assay (Tran et al., 2001) or using a membrane potential-sensitive fluorescent probe (Kirouac et al., 2003). In fluorescence experiments, vesicles were incubated with the toxins, not only at a higher ionic strength, but also under conditions where the concentration of both the vesicles and the toxins was 3.7 times higher than during osmotic swelling experiments. Therefore, the effect of the concentration at which the vesicles were incubated with the toxins was investigated using the osmotic swelling assay. The vesicle concentration, during incubation with the toxins, was 0.4 (1×), 1.48 (3.7×) or 4.0 (10×) mg membrane protein/ml. The toxin concentration was increased proportionally so that the toxin/vesicle ratio was identical regardless of the vesicle concentration during the incubation period. After 1 h, each vesicle suspension was diluted to a final concentration of 0.4 mg membrane protein/ml. Osmotic swelling assays were then carried out by mixing the vesicles with an equal volume of the 150K solution. The activity of Cry1Ac was not affected by increasing the concentration of the vesicles during the incubation step at pH 7.5 (Fig. 5*A*) but was slightly increased at pH 10.5 (Fig. 5*C*). On the other hand, increasing vesicle concentration resulted in an increase in the activity of Cry1Ca at pH 7.5 (Fig. 5*E*) and 10.5 (Fig. 5*G*). This stimulation was particularly strong at pH 10.5 for vesicles concentrated 10-fold during the incubation period.

However, these results (Fig. 5*A, C, E* and *G*) cannot explain the fact that Cry1Ca appears to be more active, in comparison with Cry1Ac, in fluorescence experiments involving incubation at 3.7× (Kirouac et al., 2003), than in the light-scattering experiments in which the vesicles were incubated at 1× (Tran et al., 2001). Consequently, this difference is probably attributable, as mentioned above, to the effect of ionic strength on the activity of these toxins.

The stimulatory effect of incubating the vesicles with toxin at a higher vesicle concentration, which is much more pronounced for Cry1Ca than for Cry1Ac, is somewhat surprising in view of the fact that osmotic swelling rates reach a plateau at the higher toxin concentrations even under standard (1×) incubation conditions. Because divalent cations affect toxin activity, and since vesicle preparation involves the use of relatively high concentrations of magnesium, the effect of increasing vesicle concentration

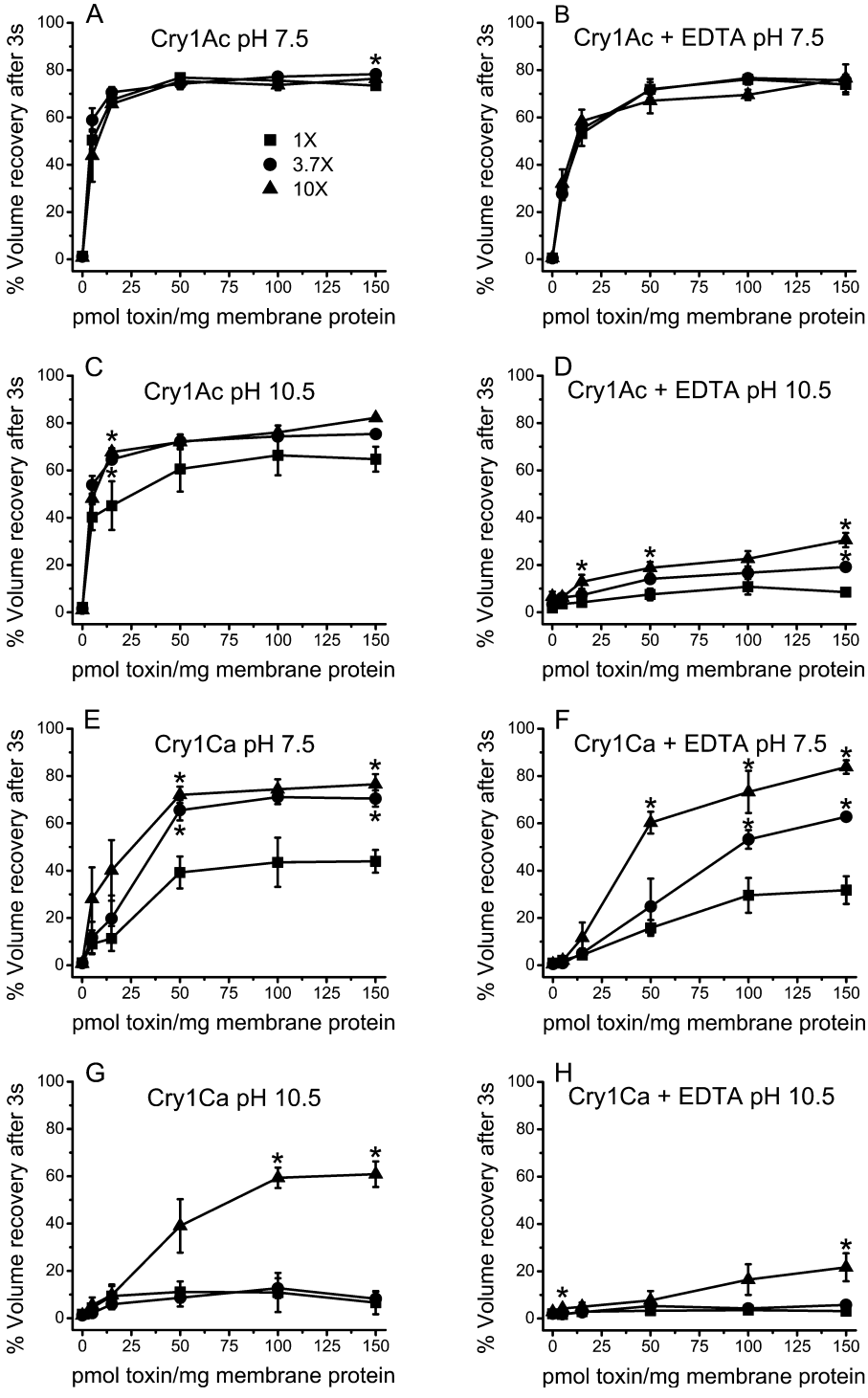

Fig. 5. Effect of brush border membrane vesicle concentration during incubation with Cry1Ac and Cry1Ca on their osmotic swelling. Vesicles (0.4 (1×), 1.48 (3.7×) or 4 (10×) mg membrane protein/ml) were incubated for 60 min with the indicated concentration of Cry1Ac (*A, B, C, D*) or Cry1Ca (*E, F, G, H*) with (*B, D, F, H*) or without (*A, C, E, G*) 2 mM (1×), 7.4 mM (3.7×) or 20 mM (10×) EDTA. After the incubation period, vesicle concentration was adjusted to 0.4 mg membrane protein/ml and rapidly mixed with 150K solution at pH 7.5 (*A, B, E, F*) or 10.5 (*C, D, G, H*). For experiments done in the presence of EDTA, vesicles incubated with 150 pmol Cry1Ac, and for which 2 mM EDTA was added just before the osmotic shock, were used as positive controls (*data not shown*). Asterisks indicate a statistically significant difference ($P < 0.05$) with the corresponding point of the 1× curve.

during incubation on toxin activity could be due to the resulting increase in divalent cation concentration. These experiments were therefore repeated in the presence of ethylenediaminetetraacetic acid (EDTA), a strong chelator of divalent cations. Vesicles were incubated for 60 min with the toxins in the presence of 2 (1×), 7.4 (3.7×) and 20 (10×) mM EDTA. The final EDTA concentration, after dilution, was always 2 mM during the osmotic swelling step. The presence of EDTA had little effect on the activity of Cry 1 Ac at pH 7.5 (Fig. 5*A* and *B*), but decreased it considerably at pH 10.5 (Fig. 5*C* and *D*) for all vesicle concentrations. At pH 7.5, EDTA had only a small effect on the activity of Cry1Ca (Fig. 5*E* and *F*). However, the stimulation of Cry1Ca activity, observed at pH 10.5 for vesicles concentrated 10-fold (Fig. 5*G*), was almost completely abolished in the presence of EDTA (Fig. 5*H*).

The fact that EDTA overcame the concentration-induced stimulation of Cry1Ca activity at pH 10.5 (Fig. 5*G* and *H*) suggests that the enhanced activity could result from a higher divalent cation concentration during the incubation period. However, EDTA had much less effect at pH 7.5 (Fig. 5*E* and *F*), indicating that the stimulation is largely due to a divalent cation-independent mechanism. The absence of effect of EDTA on the activity of Cry1Ac at pH 7.5 (Fig. 5*A* and *B*) contrasts with the inhibition of pore formation by this toxin that was observed in the presence of an increased concentration of divalent cations at this pH (Figs. 3*A* and 4). In addition, given the lack of effect of increased calcium concentration at pH 10.5 (Fig. 3*B*), the strong inhibitory effect of EDTA on the activity of Cry1Ac at this pH (Fig. 5*D*) is quite remarkable. Interestingly, a strong reduction in the rate of pore formation by Cry1Ac in the presence of EDTA at pH 10.5 has been observed recently (Kirouac et al., 2006). In that same study, carried out under standard (1×) conditions, such inhibition was observed for Cry1Aa but did not occur for Cry1Ca. The lack of effect of EDTA at pH 7.5 for Cry1Ac (Fig. 5*A* and *B*), in the present study, is also consistent with the observation that Cry1Aa activity was unaffected by the presence of EDTA during a one hour incubation period at this pH (Kirouac et al., 2006) . Taken together, these observations indicate that the presence of trace amounts of divalent cations promotes the activity of Cry1Ac, at pH 10.5, although higher concentrations are inhibitory at pH 7.5.

Importance of Electrostatic Interactions

The results of the present study underscore the importance of electrostatic interactions in the mechanism of action of *B. thuringiensis* toxins as evidenced by strong effects of ionic strength, divalent cations and pH. As was observed earlier for pH (Tran et al., 2001), ionic strength and divalent cations affect differently the activity of Cry1Ac and Cry1Ca even if these two toxins are relatively closely related (Höfte & Whiteley, 1989; Schnepf et al., 1998). Further experiments will be required in order to identify those amino acid residues responsible for these differences. The importance of electrostatic interactions was already suggested by the observation that Cry1Ca forms pores more efficiently in liposomes composed of a mixture of anionic and neutral lipids than in liposomes composed exclusively of neutral lipids (Butko et al., 1994). These experiments were carried out in the absence of toxin receptors and at pH 4, at which the toxin should be much more positively charged than in the present study (Butko et al., 1994). Under these conditions, increasing the density of negative charges on the vesicle surface strongly favors the interaction of the toxin with the vesicles. On the other hand, the present study was carried out at higher pH values, at which the toxin is more negatively charged. In this case, the interaction of the toxin with the vesicles is favored by increasing ionic strength and the presence of divalent cations, two factors that bring about a screening of the negative charges at the surface of the vesicles and of the toxin.

Several studies have examined the effect of calcium ions on the activity of *B. thuringiensis* toxins with seemingly contradictory results (Crawford & Harvey, 1988; Wolfersbeger, 1989; Hendrickx, De Loof & Van Mellaert, 1990; Lorence et al., 1995; Monette et al., 1997; Potvin et al., 1998; Kirouac et al., 2002). This cation was reported to inhibit (Crawford & Harvey, 1988; Lorence et al., 1995), stimulate (Monette et al., 1997) and have no significant effect (Wolfersberger, 1989; Hendrickx et al., 1990) on the activity of different Cry1 toxins. The results of the present study could contribute, however, to reconcile this apparent contradiction. Indeed, calcium was reported to block the ability of Cry1Ac to inhibit short-circuit current in isolated midgut epithelia (Crawford & Harvey, 1988) and to enhance the toxicity of Cry1Ca for Sf9 cells (Monette et al., 1997). These observations are consistent with the reduction in the rate of pore formation observed for Cry1Ac (Figs. 3*A* and 4) and with the increase in that of Cry1Ca (Fig. 3*B* and *D*) in the presence of divalent cations. On the other hand, addition of calcium to the vesicles after pores were allowed to form had only minor effects on the osmotic swelling rates observed for both toxins (Fig. 2). This observation is in agreement with the results of earlier studies demonstrating that the pores formed by *B. thuringiensis* toxins allow calcium ions to diffuse readily across the membrane (Potvin et al., 1998; Kirouac et al., 2002). It is also consistent with the results of other experiments demonstrating that calcium, added after the pores are allowed to form in insect midgut brush border membrane vesicles, has very little effect on the ability of the toxins to inhibit amino acid uptake (Wolfersberger, 1989; Hendrickx et al., 1990). Taken together, these results indicate that calcium ions reduce the rate of pore formation by Cry1Ac without causing strong alterations in the properties of the pores already formed. This effect is probably due to the ability of calcium ions to shield a negative charge, located either on the toxin molecule or on the surface of the membrane, which somewhat favors pore formation. On the other hand, calcium probably favors pore formation by Cry1Ca by shielding a negative charge which somehow interferes with pore formation. The stimulation by calcium of Cry1Ca activity in Sf9 cells nevertheless appears to be more complex since barium ions were not stimulatory (Monette et al., 1997). High levels of intracellular calcium, resulting from an influx of these ions through the

pores formed by the toxin, probably affect cell viability in a specific manner (Monette et al., 1997; Potvin et al., 1998).

Conclusion

Strong effects of ionic strength, divalent cations and pH indicate that electrostatic interactions between the toxin and the membrane play an important role in the mode of action of *B. thuringiensis* toxins. The present study demonstrates the necessity of taking these effects into account when interpreting the results of in vitro experiments aimed at characterizing the properties and understanding the mode of action of these toxins. This appears to be especially relevant in view of the observation that the apparent activity of a given toxin can vary substantially depending on the experimental conditions used, as illustrated herein for Cry1Ac and Cry1Ca, two fairly closely related toxins.

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