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Kinetics of pore formation by the *Bacillus thuringiensis* toxin Cry1Ac

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Abstract

After binding to specific receptors, Cry toxins form pores in the midgut apical membrane of susceptible insects. The receptors could form part of the pore structure or simply catalyze pore formation and consequently be recycled. To discriminate between these possibilities, the kinetics of pore formation in brush border membrane vesicles isolated from *Manduca sexta* was studied with an osmotic swelling assay. Pore formation, as deduced from changes in membrane permeability induced by Cry1Ac during a 60-min incubation period, was strongly dose-dependent, but rapidly reached a maximum as toxin concentration was increased. Following exposure of the vesicles to the toxin, the osmotic swelling rate reached a maximum shortly after a delay period. Under these conditions, at relatively high toxin concentrations, the maximal osmotic swelling rate increased linearly with toxin concentration. When vesicles were incubated for a short time with the toxin and then rapidly cooled to prevent the formation of new pores before and during the osmotic swelling experiment, a plateau in the rate of pore formation was observed as toxin concentration was increased. Taken together, these results suggest that the receptors do not act as simple catalysts of pore formation, but remain associated with the pores once they are formed.

Keywords: Pore formation; Insecticidal toxin; Osmotic swelling assay; *Bacillus thuringiensis*; *Manduca sexta*

1. Introduction

Bacillus thuringiensis toxins are widely used as an environment-friendly alternative to chemical insecticides. Because each member of this group of toxins is specifically active against a limited number of insect species, they constitute a very efficient tool for the biological control of targeted insect pests. After ingestion by susceptible insect larvae, these proteins are solubilized and activated by partial proteolysis in the midgut lumen. The activated toxins form pores in the apical brush border membrane of insect midgut epithelial cells after binding to specific receptors [1]. The presence of these pores abolishes the ionic gradients established across the cell membrane and perturbs the osmotic equilibrium of the cells, thus causing deleterious alterations in cellular bioenergetics and disrupting the midgut epithelium [2]. Binding of a toxin to its receptor involves a reversible step [3,4] which is followed by an irreversible binding step [5–7]. Irreversible binding is generally considered to correspond to the insertion of the toxin into the apical membrane [5–7]. However, activated toxins can form pores in the absence of receptors, in liposomes and planar lipid bilayers, although pore formation is considerably more efficient in artificial lipid membranes in which brush border membrane vesicles [8–11] or partially purified receptors [12,13] have been incorporated.

It has been suggested that the receptor may form part of the pore structure, together with the toxin, or may simply facilitate pore formation [7,14]. According to this latter possibility, the receptor should be recycled and become available for the formation of additional pores after the assembly of each pore is completed. In the present study, the likelihood of these two schemes was evaluated on the basis of a detailed analysis of the kinetics of pore formation by Cry1Ac in midgut brush border membrane vesicles isolated from *Manduca sexta* using an osmotic swelling assay. These experiments measured either membrane permeability after a 60-min incubation period of the vesicles with the toxin or estimated the rate of pore formation from the rate of swelling of the vesicles. The results indicate that a maximum number of pores can be formed in a given vesicle

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suspension, suggesting that the receptor remains associated with the pore structure once it is formed.

2. Materials and methods

2.1. Insects

Fertilized *M. sexta* eggs were purchased from the North Carolina State University Entomology Department insectary (Raleigh, NC). Larvae were reared on a standard synthetic diet supplied with the insects.

2.2. Toxins

Cry1Ac and Cry1Ca were prepared from *B. thuringiensis* strains producing the appropriate single recombinant toxin, made soluble and trypsin-activated as described elsewhere [15,16]. The 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 20 mM sodium carbonate buffer (pH 10.8) [16].

2.3. Osmotic swelling assay

Brush border membrane vesicles were purified from isolated midguts of fifth-instar *M. sexta* larvae with a magnesium precipitation and differential centrifugation procedure [17]. The membrane permeabilizing effects of Cry1Ac and Cry1Ca were analyzed with an osmotic swelling assay [18]. 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. They were then rapidly mixed, directly in a cuvette, with an equal volume of 10 mM of the appropriate buffer, 1 mg of bovine serum albumin per ml and either 150 mM KCl or potassium gluconate, or 300 mM sucrose 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 [19]. Kinetic experiments designed to evaluate the rate of pore formation were conducted with vesicles that were not pre-incubated with toxin but were instead mixed with the appropriate hypertonic solution containing the indicated concentration of Cry1Ac. In some experiments, vesicles that were incubated for the indicated time with the toxin at 23 °C were rapidly cooled to 2 °C and mixed with the hypertonic solution at 2 °C. 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) or, for experiments carried out at 2 °C, in a Spex Fluorolog CMIII spectrofluorometer (Jobin Yvon Horiba, Edison, NJ) [20]. Experiments, each carried out in quintuplicate, were performed at least three times with different vesicle preparations. Data are presented as means±standard error of the mean (SEM).

2.4. Data analysis

As described previously [21,22], scattered light intensity measurements were first converted into relative scattered light intensity values for which 1 was attributed to the highest intensity measured in the absence of toxin and 0 was attributed to the lowest intensity measured with 150 pmol Cry1Ac/mg membrane protein. Percent volume recovery is defined as $[1-I(t)]$ 100 where $I(t)$ is the measured relative scattered light intensity 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 Cry1Ac. The resulting curves, which illustrate the changes in membrane permeability due to the effect of the toxin, were fitted with a Boltzmann sigmoidal function with the software Origin (OriginLab Corporation, Northampton, MA). The delay preceding vesicle swelling, a parameter arbitrarily defined as the time required to reach a volume recovery of 1%, was derived directly from these fitted curves. The maximal osmotic swelling rate was estimated from the slope of the fitted curves at their inflection point. Dose–response curves obtained after either a 1-min or a 60-min incubation of the vesicles with the toxin were fitted with the Hill equation using the software Origin. Statistical analyses were done using two-tailed unpaired Student's t tests and differences were considered significant when $p<0.05$.

3. Results

3.1. Toxin-induced permeability

The permeability induced by Cry1Ac in midgut brush border membrane vesicles isolated from *M. sexta,* an index of the number of pores formed, was measured using an osmotic swelling assay based on light scattering measurements [18]. The vesicles were first incubated for 60 min with various concentrations of Cry1Ac and then submitted to a hypertonic shock by mixing them rapidly with an equal volume of 150 mM KCl (Fig. 1A). The osmotic swelling rate of the vesicles increased rapidly to a maximum with increasing toxin concentration (Fig. 1A). On the other hand, when vesicles were exposed simultaneously to the toxin and KCl gradient, their maximal osmotic swelling rate, corresponding to the

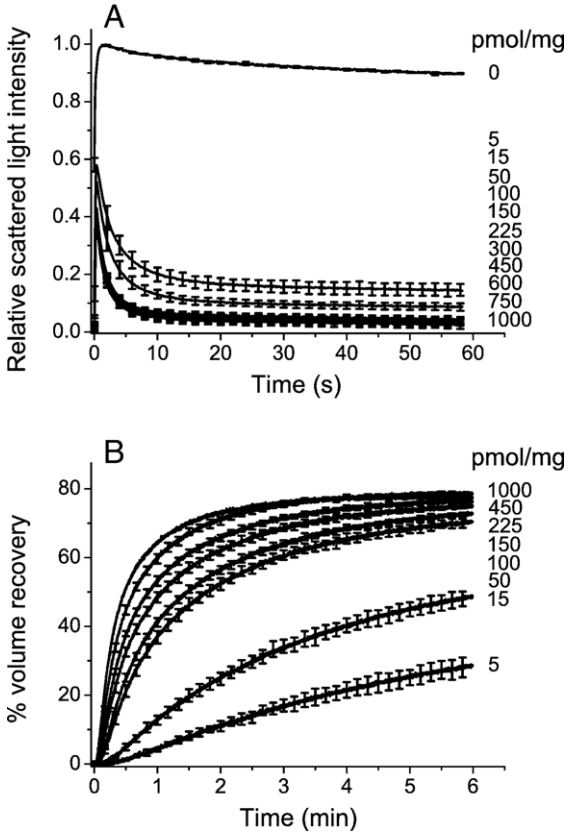

Fig. 1. Osmotic swelling of brush border membrane vesicles induced by Cry1Ac. Vesicles equilibrated overnight in 10 mM HEPES–KOH (pH 7.5) were rapidly mixed with a hypertonic solution composed of 150 mM KCl and 10 mM HEPES–KOH (pH 7.5). The indicated concentrations of Cry1Ac were mixed with the vesicles 60 min before the osmotic shock (A) or with the hypertonic solution used to impose the osmotic shock (B). Percent volume recovery was calculated as described in Materials and methods (B). Values obtained for control vesicles, assayed without toxin, were subtracted from the experimental values measured in the presence of toxin (B). For clarity, error bars are only shown for every 20th (A) or 100th (B) experimental point. Data are means± SEM of 3 independent experiments.

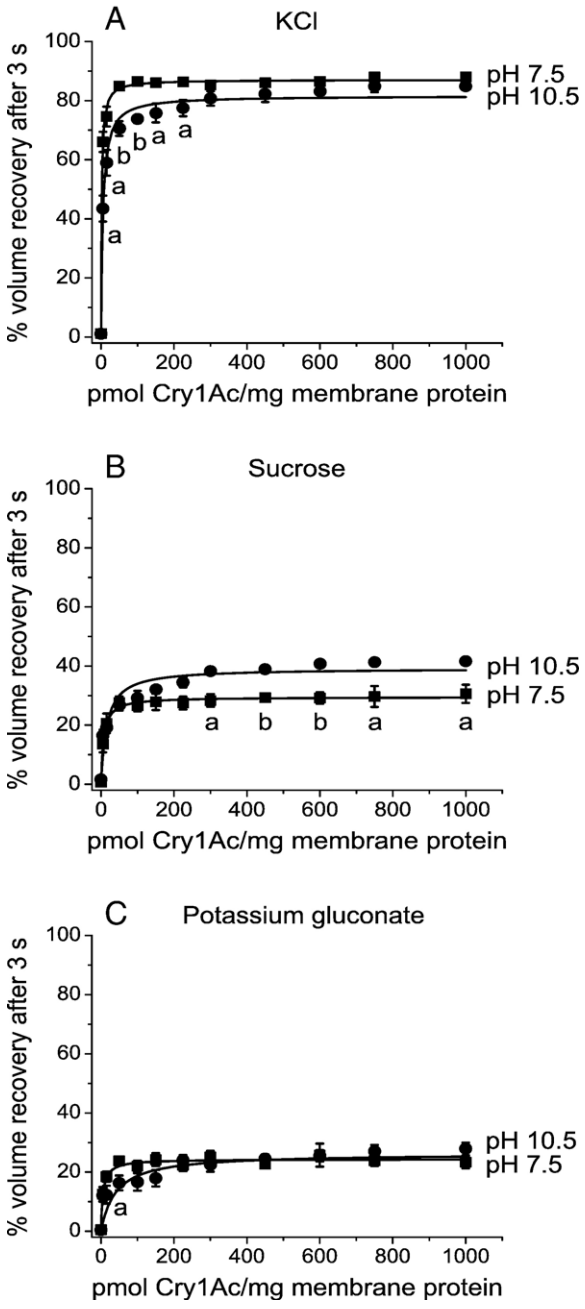

Fig. 2. Cry1Ac-induced permeability of brush border membrane vesicles. Vesicles equilibrated overnight in 10 mM HEPES–KOH (pH 7.5) (■) or CAPS–KOH (pH 10.5) (●) were incubated for 60 min with various concentrations of Cry1Ac and rapidly mixed with 10 mM HEPES–KOH (pH 7.5) (■) or CAPS–KOH (pH 10.5) (●) and either 150 mM KCl (A), 300 mM sucrose (B) or 150 mM potassium gluconate (C). Data points were fitted with the Hill equation. Data are means±SEM of 3 independent experiments. Letters indicate a statistically significant difference between the values measured at pH 7.5 and 10.5 at the same toxin concentration. a, $p<0.05$; b, $p<0.01$.

maximal slopes of the curves, increased gradually with increasing toxin concentration (Fig. 1B).

Toxin activity can be strongly influenced by pH and ionic strength [21,23]. Although these factors affected Cry1Ca considerably more strongly than Cry1Ac, significant differences were observed in the rate of pore formation by Cry1Ac as pH was increased to 10.5 [21,23]. Because these earlier experiments were carried out at a single toxin concentration, the effect of pH and ionic strength was further analyzed, in the present study, by performing most experiments at pH 7.5 and 10.5 in the presence of charged and uncharged solutes. Fig. 2 summarizes a large number of experiments performed after incubation of the vesicles with Cry1Ac. Percent volume recovery after 3 s in the presence of KCl was strongly dose-dependent and reached a plateau (Fig. 2A). The data were best fitted with a Hill equation bearing a Hill coefficient of 1. This value corresponds to that estimated by Hoffmann et al. [3] from the binding of Cry1B to *Pieris brassicae* brush border membrane vesicles and by Guihard et al. [24] on the basis of potassium efflux from Sf9 cells induced by Cry1Ca. The deduced parameters VR_{MAX}, corresponding to the percent volume recovery after 3 s at the plateau, and $K_{0.5}$, the apparent half-saturation constant, are given in Table 1. As the plateau was reached at a volume which approaches that of fully re-swelled vesicles, corresponding to the maximal volume that the vesicles can attain in this type of experiment [19], KCl was replaced by isotonic solutions of two less permeable solutes, sucrose (Fig. 2B) and potassium gluconate (Fig. 2C) [21]. Again, a plateau was reached at both pH values, but at a lower level (Table 1), confirming that the plateau was reached because a maximal number of pores was formed rather than because of a limitation in the osmotic swelling capacity of the vesicles. In agreement with the results of a previous study [21], permeability to sucrose was slightly, but significantly higher at pH 10.5, while permeability to potassium gluconate was similar at both pH values. In all cases, half-saturation levels were attained at a relatively similar toxin concentration (Table 1). The differences in apparent half-saturation constants observed between pH 7.5 and 10.5 were significant ($p<0.05$) in the presence of KCl and sucrose.

It remains possible, however, that pore formation could be limited by a lack of space in the membrane, rather than by the saturation of the receptors. To test this possibility, osmotic swelling experiments were performed with vesicles that had been incubated beforehand for 60 min with 150 pmol Cry1Ac/mg membrane protein and various concentrations of Cry1Ca. While, in *M. sexta*, the receptors for Cry1Ac have been identified as an aminopeptidase N of 120 kDa [12,25] and a cadherin-like protein, BT-R1 [26], Cry1Ca binds to a different receptor, an aminopeptidase N of 106 kDa [27]. These assays

Table 1
Parameters derived from fitting of the data from Fig. 2 with the Hill equation

	pH 7.5		pH 10.5	
	VR_{MAX} [a] (% VR)	$K_{0.5}$ [b] (pmol/mg)	VR_{MAX} [a] (% VR)	$K_{0.5}$ [b] (pmol/mg)
KCl	87.1±0.5	1.8±0.5 (0.7±0.2)	82±3	5.2±0.7 (2.1±0.3)
Sucrose	29±2	7±2 (2.7±0.9)	39±1	15±2 (6.1±0.8)
KGlu [c]	24±2	5±2 (2.1±0.9)	26±2	40±33 (16±13)

[a] Maximum percent volume recovery after 3 s.
[b] Half-saturation constant. Values are in pmol toxin per mg membrane protein. Corresponding values in nM are given in parentheses.
[c] Potassium gluconate.

were only performed at pH 7.5 because, at pH 10.5, Cry1Ca is poorly active in osmotic swelling experiments performed with *M. sexta* vesicles [21]. Increasing the concentration of Cry1Ca clearly caused a dose-dependent increase in the vesicle swelling rate above the level attained in the presence of Cry1Ac alone (Fig. 3). The highest percent volume recovery values reached after 3 s were 55±4 and 41±2, in the presence of sucrose and potassium gluconate, respectively (Fig. 3). As was observed previously [21], the dose–response curves obtained with Cry1Ca were more sigmoidal than those obtained with Cry1Ac. Furthermore, the increases in volume recovery attributable to the presence of 150 pmol of Cry1Ca/mg membrane protein were similar to those previously observed in the absence of Cry1Ac [21]. The concentration of Cry1Ca necessary to reach half of the maximum swelling rate (Fig. 3) was much higher than the apparent half-saturation constants calculated from the experiments using various concentrations of Cry1Ac (Table 1).

3.2. Osmotic swelling rates

Experiments similar to that illustrated in Fig. 1B were also carried out in the presence of the same solutes as those used in the experiments summarized in Fig. 2, at pH 7.5 and 10.5 (Fig. 4). In these experiments, percent volume recovery increased as a sigmoidal function of time. Each curve is therefore characterized by two parameters, defined in Materials and methods, the delay preceding the onset of osmotic swelling (Fig. 4A, C, E) and the maximal osmotic swelling rate (Fig. 4B, D, F). For all three solutes tested, the delay decreased rapidly as toxin concentration was increased, but soon reached a relatively constant value (Fig. 4A, C, E).

As the percent volume recovery values measured in the absence of toxin were subtracted from those obtained in the presence of Cry1Ac, the maximal osmotic swelling rates presented in Fig. 4 are exclusively due to the activity of the toxin. For each solute tested, the maximal osmotic swelling rates of the vesicles increased rapidly, at the lower toxin concentrations, and more or less linearly, at the higher concentrations, without apparently reaching a plateau, even at 1000 pmol Cry1Ac/mg membrane protein (400 nM). Interestingly, the maximal osmotic swelling rates measured in the presence of KCl were considerably higher at pH 7.5 than at pH 10.5 (Fig. 4B), although such a difference was not apparent in the presence of sucrose (Fig. 4D) or potassium gluconate (Fig. 4F). In agreement with the results shown in Fig. 2, the maximal osmotic swelling rates were much higher in the presence of KCl (Fig. 4B) than in the presence of sucrose (Fig. 4D) or potassium gluconate (Fig. 4F).

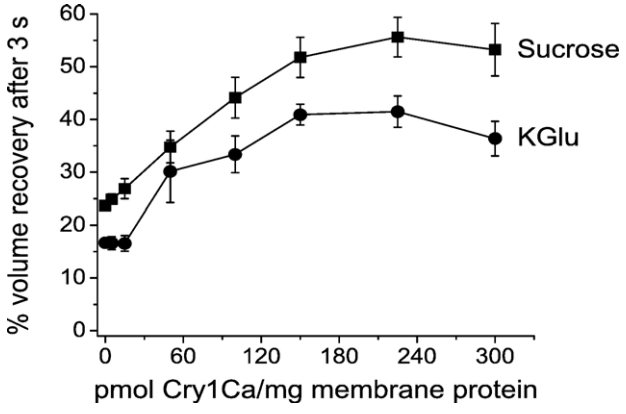

Fig. 3. Combined effect of Cry1Ac and Cry1Ca on the osmotic swelling of brush border membrane vesicles. Vesicles equilibrated overnight in 10 mM HEPES–KOH (pH 7.5) were incubated for 60 min with 150 pmol Cry1Ac/mg membrane protein and the indicated concentrations of Cry1Ca and rapidly mixed with 10 mM HEPES–KOH (pH 7.5) and either 300 mM sucrose (■) or 150 mM potassium gluconate (●). Data are means±SEM of 3 independent experiments.

3.3. A new approach to measure pore formation kinetics

In the previous set of experiments (Fig. 4), the maximal swelling rates occurred at different times and different values of percent volume recovery. The vesicles also have a small, but non-negligible permeability to the different solutes. As a consequence, the maximal osmotic swelling rates were measured under different solute gradient conditions for each toxin concentration. A new approach was therefore developed to measure the rate of pore formation as a function of toxin concentration. Vesicles were first incubated with 50 pmol Cry1Ac/mg membrane protein for 0 to 60 min at 23 °C before being cooled rapidly to 2 °C (Fig. 5A). The osmotic swelling assay was then performed at 2 °C. At this temperature, pore formation by the toxin is prevented, but the vesicles swell readily [20]. Percent volume recovery after 3 s is then a good indicator of the number of pores formed during the incubation period. This value reached a maximum after a shorter incubation period at pH 7.5 than at pH 10.5 (Fig. 5A). After about 10 min at pH 7.5 and 30 min at pH 10.5 no new pores were formed (Fig. 5A). Based on Fig. 5A, vesicles were incubated for 1 min, an incubation time which is short enough to provide a good indicator of the initial rate of pore formation, but long enough to allow accurate measurements of the osmotic swelling rate (Fig. 5B). Once more, the data were best fitted with a Hill equation bearing a Hill coefficient of 1. Osmotic swelling rates reached a maximum value that was not significantly different at pH 7.5 (54±4 percent volume recovery after 3 s) and at pH 10.5 (43±4 percent volume recovery after 3 s). The toxin concentration needed to attain half of this maximal value was also not significantly different at pH 7.5 (9±2 pmol/mg membrane protein) and pH 10.5 (36±15 pmol/mg membrane protein).

4. Discussion

Although *B. thuringiensis* toxin receptors clearly play a critical role in specificity [3–5,28] and greatly facilitate pore formation [8–13], their exact contribution to the mechanism of pore formation remains unclear. In the present study, we examined the hypothesis according to which the receptors act as catalysts for pore formation. As such, they should be expected to not only accelerate pore formation, but also to be continuously recycled for the formation of new pores. By

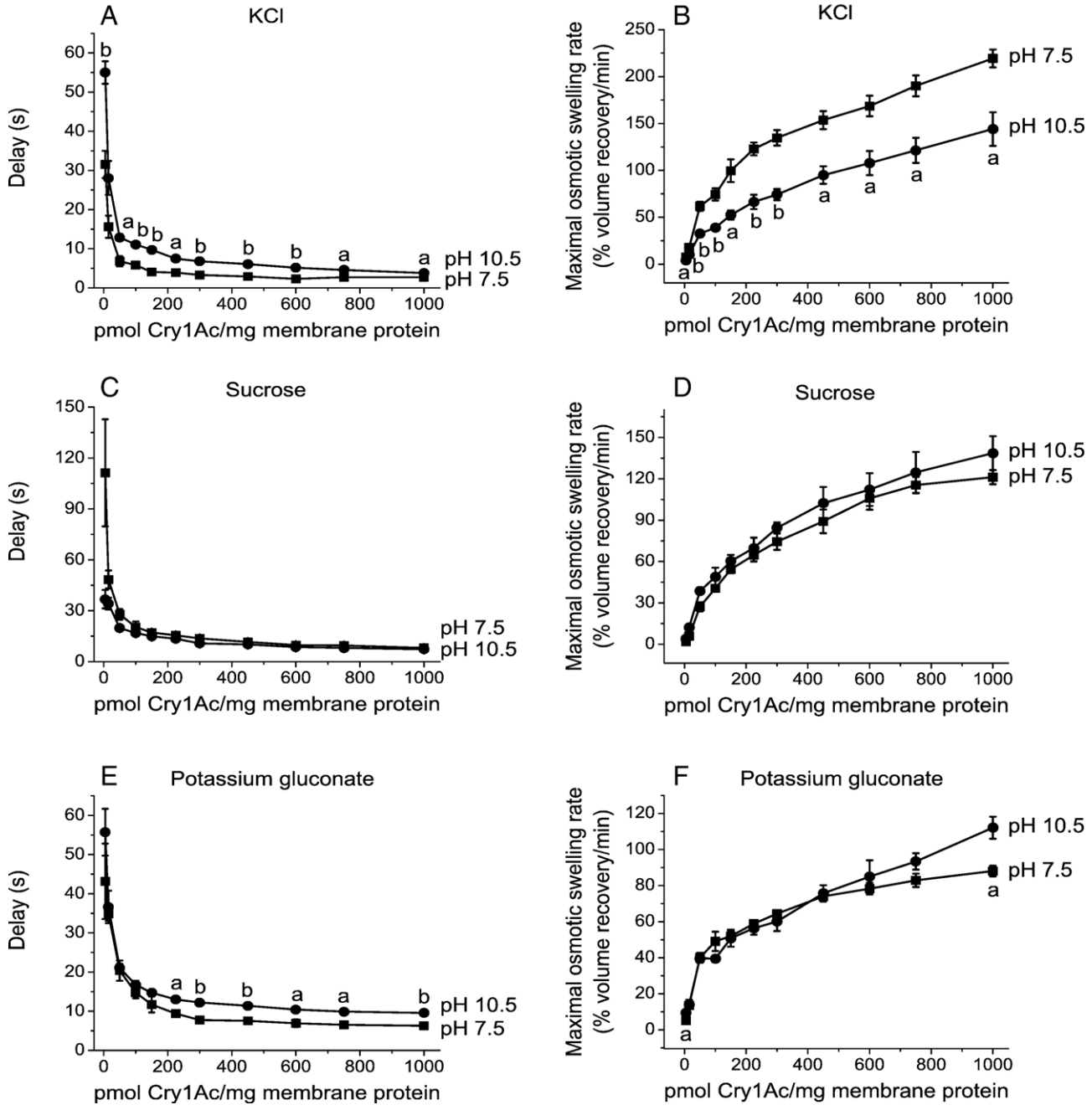

Fig. 4. Osmotic swelling rates induced by Cry1Ac in brush border membrane vesicles. Vesicles equilibrated overnight in 10 mM HEPES–KOH (pH 7.5) (■) or CAPS–KOH (pH 10.5) (●) were rapidly mixed with 10 mM HEPES–KOH (pH 7.5) (■) or CAPS–KOH (pH 10.5) (●), various concentrations of Cry1Ac, and either 150 mM KCl (A, B), 300 mM sucrose (C, D) or 150 mM potassium gluconate (E, F). The time required for volume recovery to reach 1% (delay) (A, C, E) and the maximal osmotic swelling rate (B, D, F) were estimated as described in Materials and methods. Data are means±SEM of 3 independent experiments. Letters indicate a statistically significant difference between the values measured at pH 7.5 and 10.5 at the same toxin concentration. a, $p<0.05$; b, $p<0.01$.

analogy with an enzyme-catalyzed reaction, the cumulative number of pores formed should increase with time as long as a sufficient concentration of toxin is available for the formation of new pores. On the other hand, the rate at which pores are formed should reach a maximum as soon as the toxin concentration is sufficient to ensure that all receptors are actively contributing to the formation of new pores.

In a first series of experiments, membrane permeability was evaluated after brush border membrane vesicles had been incubated with various concentrations of Cry1Ac for 60 min. Under these conditions, the osmotic swelling rate of the vesicles, an indication of the number of pores formed within the membrane, rapidly reached a maximum as the toxin concentration was increased (Fig. 2). In these experiments, membrane permeability did not appear to be limited by the time during which the vesicles were exposed to the toxin since, in the presence of a saturating toxin concentration, increasing the incubation time from one to at least ten h had little effect on the

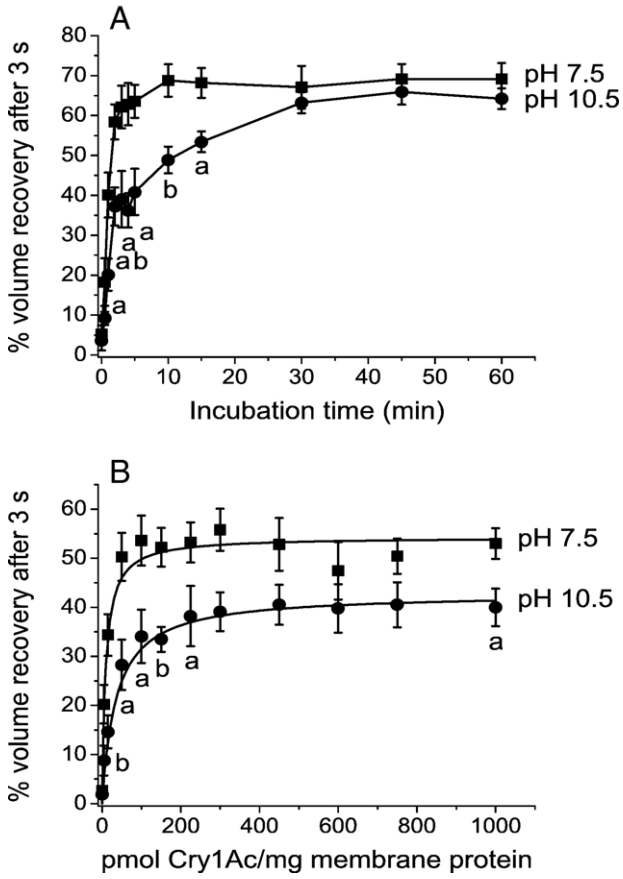

Fig. 5. Kinetics of pore formation by Cry1Ac. Vesicles equilibrated overnight in 10 mM HEPES–KOH (pH 7.5) (■) or CAPS–KOH (pH 10.5) (●) were incubated for various time periods with 50 pmol Cry1Ac/mg membrane protein (A) or during 1 min with various concentrations of Cry1Ac (B) at 23 °C and rapidly cooled to 2 °C. Vesicles were then rapidly mixed with 150 mM KCl and 10 mM HEPES–KOH (pH 7.5) (■) or CAPS–KOH (pH 10.5) (●) at 2 °C. Data points were fitted with the Hill equation (B). Data are means ± SEM of 5 independent experiments. Letters indicate a statistically significant difference between the values measured at pH 7.5 and 10.5 at the same toxin concentration. a, $p<0.05$; b, $p<0.01$.

osmotic swelling rate of the vesicles (data not shown). In addition, experiments in which the vesicles were exposed to a saturating concentration of Cry1Ac for various periods of time revealed that the osmotic swelling rate of the vesicles also reached a maximum much before the end of the 60-min incubation period (Fig. 5A). Furthermore, the maximum osmotic swelling rate was much higher when the vesicles were incubated with both Cry1Ac and Cry1Ca, an indication that the number of pores formed by each of the toxins is limited by the availability of their respective receptors.

These results indicate that, in contradiction with the catalytic hypothesis, the receptors are not recycled following the formation of a new pore. They are consistent however with a large number of studies demonstrating that toxin binding to insect midgut brush border membrane vesicles is saturable e.g., [3–7,28] and that a maximum level of membrane permeability is reached as toxin concentration is increased e.g., [21,29–31]. Since irreversible binding, following incubation of brush border membrane vesicles with toxin, accounts for the majority of total specific toxin binding [7], most membrane-bound toxin appears to be inserted into the membrane and is likely to participate in pore formation. In agreement with this conclusion, the half-saturation constants presented in Table 1 are reasonably similar to published estimates of the dissociation constant for the binding of Cry1Ac to *M. sexta* brush border membrane vesicles (0.2 ± 0.04 and 0.6 ± 0.03 nM [5], and 1.58 ± 0.06 nM [32]) and to the reported value for the concentration of Cry1Ac needed to inhibit by 50% the accumulation of L-phenylalanine in vesicles isolated from the same species (3.24 ± 0.35 pmol/mg membrane protein [33]). On the other hand, estimates of the binding site concentration vary from 4 ± 0.2 and 6.3 ± 1.4 pmol/mg membrane protein [5] to 20.2 ± 0.04 pmol/mg membrane protein [32]. In reasonable agreement, percent volume recovery after 3 s reached a constant value in the presence of approximately 15 to 50 pmol/mg membrane protein, depending on the solute used to test membrane permeability (Fig. 2).

B. thuringiensis Cry toxins have long been known to form pores in artificial lipid bilayers, in the absence of receptors [34,35]. Because pore formation under these conditions is nevertheless relatively inefficient, the concentration of Cry1Ac was increased up to 1000 pmol/mg membrane protein to test whether pores formed by this non-specific pathway could be detected in the brush border membrane once the receptors are saturated with toxin. As shown in Fig. 2, this increase in toxin concentration was not accompanied by an observable increase in membrane permeability. These results clearly show that pore formation is considerably less efficient in the absence of available receptors than in their presence. Its detection in planar lipid bilayers [34,35] is probably only possible because of the very sensitive electronic amplification systems used in this type of experiment which allows the study of individual pores.

In the presence of a finite number of receptors, the rate of pore formation, as a function of toxin concentration, is expected to reach a maximum value when the receptors are saturated with toxin whether or not they are reutilised after each cycle of pore formation. The absence of plateaus in Fig. 4, which summarizes the results of experiments designed to estimate the rate of pore formation by Cry1Ac, could therefore be somewhat surprising. This result is nevertheless consistent with that of a previous study in which the maximal osmotic swelling rate of the vesicles never reached a constant value as the concentration of Cry1Aa was increased up to 600 pmol/mg membrane protein [22]. However, the delay ranged from 2 to 28 s, depending on the solute being tested, for all toxin concentrations tested, except 5 and 15 pmol/mg membrane protein for which it was considerably longer (Fig. 4A, C, E). With the exception of the experiments performed at these lower toxin concentrations, the maximal osmotic swelling rates (Fig. 4B, D, F) were measured between about 2 to 33 s. This time period therefore corresponds to the very beginning of the ascending part of the curves.

This observation and the fact that the maximal osmotic swelling rates did not reach a plateau suggest that, in experiments such as that illustrated in Fig. 1B, it was measured before the reversible binding reaction had reached equilibrium. The maximal osmotic swelling rate therefore does not necessarily correspond to the maximal rate of pore formation.

This possibility is supported by the observation that the osmotic swelling rate reached a plateau when measured after 1 min, under conditions in which the vesicles are prevented from swelling before the end of the incubation period and in which additional pores are prevented from forming after this period (Fig. 5B). It therefore appears that the osmotic swelling rates measured in the experiments summarized in Fig. 5 are more representative of the maximal rate of pore formation than the maximal osmotic swelling rates measured under the conditions used in the experiments summarized in Fig. 4. In the latter experimental approach, the vesicles begin to swell as soon as pores begin to form at a time where the trans-membrane solute gradient is maximal. Thereafter, the osmotic swelling rate decreases as this gradient becomes smaller even if additional pores continue to accumulate within the membrane. Consequently, during the early phase of pore formation, membrane permeability appears to be limited by the rate at which toxin molecules can react with the receptors at the surface of the membrane. Afterwards, equilibrium is reached at the level of the receptors, the rate of pore formation reaches a maximum and membrane permeability increases with toxin concentration in a saturable manner (Fig. 5).

A protocol similar to that used in the experiments illustrated in Fig. 1B has been followed extensively to estimate the rate at which different toxins increase the permeability of insect midgut brush border membrane vesicles [18,20–23,29–31]. In these studies, the activity of different toxins [29–31], or the effect of various factors including pH [20,21,23], ionic strength [23], protease inhibitors [22] and temperature [20], were compared rather than the effect of toxin concentration. Under these conditions, the comparisons remain valid despite the fact that the maximal osmotic swelling rate is measured rather than the actual maximum rate of pore formation.

In the present study, the apparent effect of pH on the kinetics of pore formation observed with this protocol, in the presence of KCl (Fig. 4B), was not reflected by a significant difference in the maximum osmotic swelling rates or in the apparent half-saturation constants estimated with the new protocol (Fig. 5B). Significant differences were nevertheless observed, using the latter experimental approach, between the swelling rates measured at both pH values for all toxin concentrations up to 225 pmol/mg membrane protein (Fig. 5B). The fact that the new protocol involves a greater number of manipulations probably accounts for the larger variability in the results shown in Fig. 5. A slower rate of osmotic swelling at pH 10.5, in the presence of 150 pmol Cry1Ac/mg membrane protein and KCl, was observed previously [21]. However, the absence of a pH effect in the presence of sucrose (Fig. 4D) and potassium gluconate (Fig. 4F) indicates that this difference cannot be explained by a simple combined effect of ionic strength and pH [23]. It suggests that this effect of pH depends mainly on the size of the anion being tested. Taken together, these results are probably better explained by a specific electrostatic interaction which affects mostly an early step in the mechanism leading to pore formation.

In summary, the results of the present study strongly suggest that the receptors remain associated with the pore-forming structure once it is formed, rather than becoming available for the formation of new pores in the membrane. Although the possibility cannot be excluded that, once a limited number of pores are formed by a given receptor, new pore-forming structures cannot be inserted in the membrane because of lack of available space in the vicinity of the receptor, our results are consistent with the hypothesis according to which the receptors could form part of the pore structure [14]. In agreement with this possibility, the pores formed by Cry1Ac in lipid bilayers into which a partially purified receptor preparation was reconstituted [13], and those formed by Cry1Aa in lipid bilayers into which brush border membrane material had been previously incorporated [11], displayed altered biophysical properties, including conductance, ionic selectivity and current rectification, when compared with those formed in receptor-free lipid bilayers.

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