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A comparison and analysis of the toxicity and receptor binding properties of *Bacillus thuringiensis* CryIC ∂-endotoxin on *Spodoptera littoralis* and *Bombyx mori*

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Abstract The binding of L-[^{35}S]methionine in vivo labelled CryIC toxin to its receptor in brush border membrane vesicle (BBMV's) prepared from *Spodoptera littoralis* and *Bombyx mori* was studied. Both insect species were highly susceptible to the CryIC toxin in bioassays, *B. mori* being 7-fold more sensitive to CryIC than *S. littoralis* (LC50's of 10 ng/cm^2 and 70 ng/cm^2, respectively). Competition and direct binding experiments revealed saturable high-affinity binding sites on BBMV's from both insects which had similar binding characteristics for the CryIC toxin (K_d = 10 nM, B_{max} = 8 to 9 pmol/mg BBMV's and IC_{50} = 37 nM for both insect species). Thus a specific receptor for the CryIC toxin is present in both insect species and the 7-fold greater potency of CryIC towards *B. mori* is not due to qualitative or quantitative differences in binding affinity or receptor site concentration. Dissociation experiments also indicated that the binding of [^{35}S]CryIC to *B. mori* BBMV's is partially reversible.

Key words: ∂-Endotoxin; Toxicity; Binding affinity; *Bacillus thuringiensis*; *Spodoptera littoralis*; *Bombyx mori*

1. Introduction

During sporulation *Bacillus thuringiensis* synthesizes crystalline inclusions composed of one or several insecticidal proteins (known as ∂-endotoxins or Cry proteins) that form a large family of related proteins. The target of these toxins is the brush border membrane of the larval midgut epithelial cells [1] and it is now generally accepted that the ∂-endotoxins act by opening or by forming new specific ion-selective channels [2–5] or non selective pores [6,7] in the midgut cells of susceptible insects. Thereby, the toxins destroy the osmotic balance across the cell membrane causing swelling and eventual lysis of midgut epithelial cells.

The ∂-endotoxins have been classified into five major classes according to their insecticidal properties and molecular structures (CryI, II, III, IV and V). They are active against Lepidoptera (CryI), Lepidoptera and Diptera (CryII), Coleoptera (CryIII), Diptera (CryIV) and Lepidoptera and Coleoptera (CryV) [8,9]. Individual toxins are highly specific for particular insects and the high insect specificity of these toxins has been correlated with the presence of specific toxin receptors in BBMV preparations from the gut of susceptible insect species [10–12]. Recently, several authors have indicated that both the affinity and the number of binding sites for the toxin appear to be important factors in the insecticidal specificity and potency of the ∂-endotoxins [11,13,14]. It has also been proposed that there is a general correlation between larvicidal potency and the product of receptor site concentration and affinity [14]. However, Wolfersberger [15] found that the dissociation constants for CryIA(b) and CryIA(c) toxin binding to *Lymantria dispar* larvae were inversely correlated with their potency towards this insect species. Also, Garczynski et al. [16] found that *Spodoptera frugiperda* BBMV had saturable high affinity binding sites for CryIA(c) but the larvae were not killed by this toxin.

Therefore, an important step towards the understanding of the molecular basis of the specificity of these toxins would be a comparative analysis of the in vivo toxicity and properties of the interaction of each toxin with its specific receptors. In this paper, we report the preparation and purification of ^{35}S in vivo labelled CryIC toxin. We then studied and compared the toxicity and biochemical properties of the interaction between [^{35}S]CryIC and *S. littoralis* and *B. mori* BBMV's. The parameters of the interaction (maximal binding capacity, dissociation constants and IC_{50}'s) and the in vivo larvicidal activity of the CryIC towards the two insects species were determined. The relationship between the binding parameters of the interaction of the CryIC toxin with its specific receptors and the specificity and larvicidal potency of the ∂-endotoxin is discussed.

2. Materials and methods

2.1. Bioassays

Biological assays were conducted using 2nd instar larvae of the two insect species *S. littoralis* and *B. mori* by free ingestion techniques. For *S. littoralis*, larval artificial diet was dispensed into 50-well plates, each well having a surface area of 165 mm^2. Twenty-five μl of several successive dilutions of the CryIC crystal preparation were applied to each well. One 2nd instar larvae was placed in each of the 50 wells. Bioassays on *B. mori* were performed by surface contamination of mulberry leaf disks using a calibrated sprayer that delivers uniformly a known amount of toxin per square centimeter of leaf surface. Leaf disks (2.5 cm diameter) were cut out, treated, placed into individual cups and six 2nd instar larvae were added in each cup. The plates and cups were incubated at 25°C (photoperiod 16 h light, 8 h dark) for 5 days. At least 50 larvae were challenged with each of five dilutions of the toxin preparation. Larval death was monitored after five days. The results of the assays were analyzed by probit analysis [17] and expressed in terms of 50% lethal concentration (LC50) (nanograms of protein/cm^2).

2.2. Chemicals

L-[^{35}S]Methionine in vivo cell labelling grade (specific activity >37 TBq/ml) was purchased from Amersham France, Sephadex G-75 was from Pharmacia, France. All other reagents were of analytical grade.

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2.3. Brush border membrane vesicle (BBMV) preparation

S. littoralis and *B. mori* BBMV's were prepared according to the protocol described by Wolfersberger et al. [18] except that the following protease inhibitor cocktail was included: 0.1 mM 1.10 phenanthroline, 0.1 mM 3,4 dichloroisocoumarin and 0.05 mM E64 (*N*-[*N*-(L-3-*trans*-carboxyrane-2-c arbonyl)-L-leucyl]-agmatine). The protein content of the BBMV's was determined by the Bio-Rad protein assay with bovine serum albumin as a standard.

2.4. In vivo ^{35}S-labelling of the CryIC ∂-endotoxin

A 5 kb *Hin*dIII–*Eco*RI DNA fragment from *Bacillus thuringiensis* subsp. *aizawai* 7–29 that contains the entire coding sequence, the promoter and putative terminator of the *cryIC* gene ([19]; and J. Muller-Cohn and V. Sanchis, unpublished results) was cloned into the *Hin*dIII and *Eco*RI sites of the plasmid vector pHT315 [20]. The resulting plasmid was designated pHTIC. An acrystalliferous derivative of *B. thuringiensis* 407 (H1 serotype) isolated by O. Arantes [21] was transformed by electroporation as described by Lereclus et al. [21]. *B. thuringiensis* 407 *cry*$^-$ (pHTIC) cells were grown with shaking at 30°C in HCT liquid medium supplemented with erythromycin at 10 μg/ml as previously described by Lecadet et al. [22]. One hour before the end of the exponential phase (t −1 of sporulation) 185 MBq of L-[^{35}S]methionine was added to the culture. The culture was continued until the liberation of spores and crystals was complete. The sporulated and lyzed culture was washed twice with 0.15 M NaCl and rinsed twice with distilled water. The CryIC crystals were then solubilized by resuspending them at 1 mg/ml in 50 mM sodium carbonate-bicarbonate buffer pH 10.2 containing 10 mM dithiothreitol and incubation at 37°C for 1 h. Spores and undissolved crystals were eliminated by centrifugation at room temperature for 15 min at 14,000 × g and the soluble supernatant was then incubated at 37°C for 2 h with bovine pancreatic trypsin (enzyme/substrate protein concentration ratio of 1/50). Cleaved toxin was then purified by a passage through a column (1.7 × 45 cm) loaded with Sephadex G-75 equilibrated with 50 mM sodium carbonate-bicarbonate buffer pH 10.2 containing 10 mM dithiothreitol. Aliquots from gel filtration were subjected to SDS-PAGE and the gel was autoradiographed overnight. Fractions containing pure radiolabelled toxin were dialyzed for 16 h at 4°C against large volumes of 20 mM Tris, 150 mM NaCl (pH 7.4) containing 0.1% bovine serum albumin, the protein concentration determined as described above and aliquots were stored at −20°C until use. From the predicted molecular mass of the CryIC activated toxin [23] (1 μg of CryIC activated toxin corresponds to 15.4 pmol) the specific activity of the ^{35}S-labelled CryIC preparations were 3 to 7 · 10^4 cpm/pmol depending on the batch. Non-labelled CryIC toxin was prepared and purified as described above except that L-[^{35}S]methionine was not added to the culture.

2.5. Binding experiments

Binding experiments were performed for 90 min at room temperature in a total volume of 0.15 ml containing 250 μg BBMV's protein/ml and 15 nM ^{35}S-labelled CryIC toxin. The standard incubation buffer consisted of 20 mM Tris, 150 mM NaCl (pH 7.4) with 0.1% bovine serum albumin. After incubation bound ligand was separated from free ligand by centrifugation at 14,000 × g for 10 min at 4°C or by filtration through Whatman GF/F glass fibre filters. Washed pellets (or filters) were placed in counting vials containing 3 ml of Ready Safe liquid scintillation cocktail for liquid samples (Beckman) and counted in a Beckman LS6000SC counter.

2.5.1. Competition binding experiments. *S. littoralis* or *B. mori* BBMV's at 250 μg BBMV's protein/ml were incubated for 90 min at room temperature with various concentrations of unlabelled CryIC toxin (from 0 to 1540 nM). ^{35}S-labelled CryIC toxin was then added to a final concentration of 15 nM and the samples were incubated at room temperature for another 90 min. Association of ^{35}S-labelled CryIC to BBMV's in the presence of unlabelled toxin was estimated as described above.

2.5.2. Saturation binding experiments. Saturation binding experiments were performed using *B. mori* BBMV's at 250 μg BBMV's protein/ml and a range of ^{35}S-labelled CryIC toxin (0 to 40 nM) in the absence (total binding) or the presence (nonspecific binding) of 1 μM unlabelled CryIC. Bound ligand was separated from free ligand and the radioactivity measured as described above.

2.5.3. Kinetics of association and dissociation of [^{35}S]CryIC to B. mori *BBMV's.* *B. mori* BBMV's at 250 μg BBMV protein/ml were incubated in the standard incubation buffer at 26°C for 20 min. Association of [^{35}S]CryIC to membranes was measured by filtering 150 μl aliquots of the incubation mixture at various times after the addition of the radioligand. Filters were washed and the radioactivity on the filters was measured. Once the amount of specifically bound [^{35}S]CryIC reached a plateau value (steady state) dissociation was measured by adding 500 nM final concentration of unlabelled ligand to the incubation mixture. The displacement of the labelled toxin from BBMV's was followed by measuring the amount of bound [^{35}S]CryIC to BBMV's at different times after the addition of the excess of unlabelled CryIC.

2.6. Binding data analysis

Binding data were analyzed by using the EBDA/LIGAND computer program [24] adapted for Apple-Macintosh (Biosoft/Elsevier).This program calculates the bound concentration of ligand as a function of the total concentration of ligand, given initial estimates of the affinity (K_d) and binding site concentration (B_{max}). Through an iterative process, the program adjusts the values of K_d, B_{max} and non specific binding until the curve generated by these parameters approximates the experimental curve as closely as possible. This program also assesses which model (e.g. a one-site versus a two-site model) is compatible with the experimental data.

3. Results

3.1. Insect bioassays

The activity of the CryIC toxin to *S. littoralis* and *B. mori* was determined (Table 1). The CryIC toxin is highly specific towards *S. littoralis* and other insects of the Noctuidae family that are poorly susceptible to the other types of ∂-endotoxins [19]. It was also 7-fold more toxic for *B. mori* larvae (LC50 of 10 ng/cm^2). Binding studies with other lepidopteran insects have shown that the presence of receptors specific for a given crystal protein is essential for toxicity [13]. We therefore decided to further investigate and compare the properties of the interaction of CryIC with its receptors on larval *S. littoralis* and *B. mori* BBMV's.

3.2. Homologous competition experiments

The CryIC toxin was labelled using [^{35}S]methionine and an in vivo labelling technique which has the advantage that the toxin is not structurally affected by the labelling. This is important (particularly in competition experiments) for the precise determination and comparison of the toxicity and binding parameters of the interaction between the CryIC toxin and the two different insect systems. Preliminary experiments were then performed to establish the optimal BBMV concentrations to be used (the concentration of 15 nM of labelled CryIC was fixed as it gave a minimum of 70,000 cpm of total radioligand per experimental point). A final concentration of 250 μg BBMV/ml

Table 1
In vivo toxicity and binding characteristics of *B. thuringiensis* CryIC ∂-endotoxin to brush border membrane vesicles from *S. littoralis* and *B. mori*

Insect	LC_{50} in ng of protein/cm^2	K_d (nM)	B_{max} (pmol/mg BBMV)	IC_{50} (nM)
S. littoralis	73 (60–86)	10.5 ± 3.8	9 ± 1.44	37
B. mori	10 (7–14)	10.3 ± 4.5	8 ± 2	37

Fifty percent lethal concentrations (LC_{50}) on second instar larvae after 5 days are expressed in nanograms of protein per cm^2 of diet. LC_{50}'s were calculated by probit analysis and 95% confidence intervals are given in parentheses.

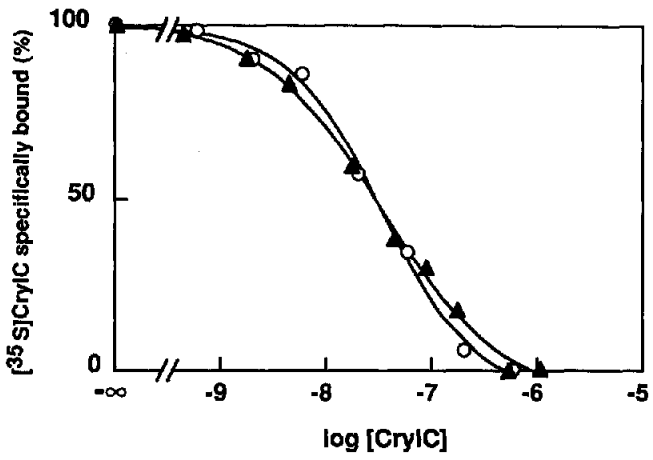

Fig. 1. Homologous competition experiments between [^{35}S]CryIC toxin and non-labelled toxin for binding to *S. littoralis* and *B. mori* BBMV's. Samples containing BBMV's (250 μg/ml) were incubated in duplicate for 90 min at room temperature with various concentrations of unlabelled CryIC toxin in 20 mM Tris, 150 mM NaCl (pH7.5) with 0.1% bovine serum albumin. Then, 15 nM of [^{35}S]CryIC was added to each sample and the BBMV's were incubated for another 90 min. Bound and free ligands were separated as described in section 2. Binding is expressed as a percentage of the maximal binding obtained in the presence of labelled toxin alone. Maximal [^{35}S]CryIC binding on *S. littoralis* and *B. mori* was 1.4 nM and 1.2 nM, respectively. (▲), *S. littoralis*; (○), *B. mori*.

was chosen as it gave the lowest non-specific binding (always less than 30% of the total binding, and generally less than 10%). The appropriate incubation time needed for equilibrium in saturation and inhibition experiments was then determined. For 250 μg BBMV/ml incubated with 15 nM radiolabelled CryIC, binding was constant between 60 and 120 min (results not shown). Therefore, an incubation time of 90 min was chosen.

The results of typical competition binding experiments using this [^{35}S]CryIC and unlabelled CryIC are presented in Fig. 1. In the absence of competitor, between 10 to 14% of the ^{35}S-labelled CryIC toxin bound to *S. littoralis* or *B. mori* midgut BBMV's depending on the experiment. The non-specific binding was less than 10% of the total binding for *S. littoralis* BBMV's and between 20% to 30% of the total binding for *B. mori* BBMV's. The results clearly indicate that CryIC binds with high affinity to both *S. littoralis* and *B. mori* BBMV's and that in both insect BBMV's the CryIC toxin displays monophasic, superimposable competition curves. The equilibrium dissociation constants (K_d), the concentrations of binding sites (B_{max}) and the concentrations of unlabelled toxin that inhibit half of the [^{35}S]CryIC binding (IC50's) are similar for both insect BBMV preparations (Table 1). In addition, each Hill coefficient is close to 1 (data not shown), which indicates the presence of a single class of binding sites. LIGAND analysis also indicated that, for BBMV preparations from both insect species, the binding data were only compatible with the one site model. This comparative analysis shows that binding of purified CryIC ∂-endotoxin to membranes preparations of two different lepidopteran insects that have different sensitivities to CryIC is comparable. As *B. mori* appeared to be 7-fold more sensitive to CryIC and is 10 times bigger than *S. littoralis*, we decided to choose the CryIC /*B. mori* system as a model for further investigates of the properties of the interaction of CryIC with its receptors.

3.3. *Direct binding of [^{35}S]CryIC to* B. mori *BBMV's*

A series of concentrations of [^{35}S]CryIC were added to a fixed concentration (250 μg protein/ml) of *B. mori* BBMV's either in the presence (non-specific binding) or the absence (total binding) of a large excess of unlabelled toxin (Fig. 2). Between 16 to 18% of the added radiolabelled toxin was bound to the BBMV's in the absence of competitor and about 80% of the total binding was specific. The incubation of *B. mori* BBMV's with increasing concentrations of [^{35}S]CryIC resulted in specific and saturable binding that reached a plateau at 12 nM of radiolabelled toxin added. Scatchard transformation of the specific binding (Fig. 2, inset) revealed a single class of high-affinity binding sites. The calculated dissociation constant of the complex formed between [^{35}S]CryIC and *B. mori* BBMV's (K_d) was 9 ± 1.56 nM and the maximal binding capacity (B_{max}) was 8.9 ± 1.52 pmol/mg BBMV proteins. These values are not significantly different from the values determined by homologous competition for the unlabelled CryIC toxin (Table 1).

3.4. *Association and dissociation kinetics of the interaction between [^{35}S]CryIC and* B. mori *BBMV's*

Kinetics of association between [^{35}S]CryIC and *B. mori* BBMV's at 26° C are shown in Fig. 3. The maximal concentration of specifically bound [^{35}S]CryIC was 1.9 nM that corresponds to the binding of 7.1 pmol of labelled CryIC toxin per mg of BBMV. This value represents 13% of the radiolabelled toxin added (total binding was 18% of the radiolabelled toxin added and non-specific binding represented 30% of the total binding) and is similar to the maximum binding capacity (B_{max}) of 8 pmol/mg *B. mori* BBMV previously determined. The binding was rapidly reaching a steady state at 60 min. Ninety percent of the maximum specific binding of [^{35}S]CryIC to *B. mori* BBMV's was achieved in 10 min and the remaining 10% bound over the following 50 min. Once the binding was maximal, the dissociation was initiated by addition of an excess of unlabelled toxin (indicated with an arrow). After addition of the competitor, the final concentration of labelled CryIC was 12 nM and

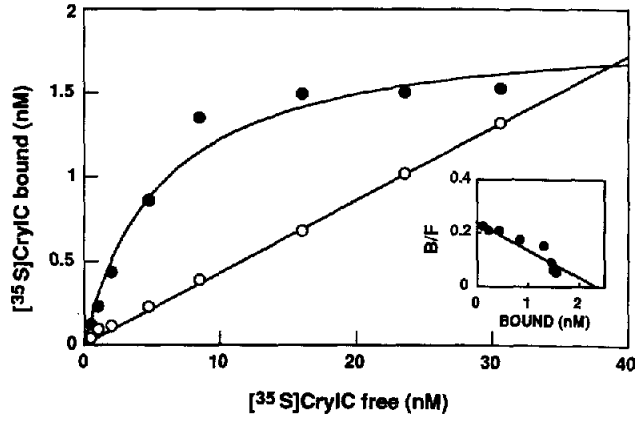

Fig. 2. Saturation binding of [^{35}S]CryIC to *B. mori* BBMV's. BBMV's (250 μg protein per ml) were incubated for 90 min at room temperature in binding buffer with one of a series of concentrations of [^{35}S]CryIC. The samples were then centrifuged, the pellets collected and washed twice with incubation buffer and the radioactivity bound to BBMV's determined. Assays were performed in duplicate. Non-specific binding (○) was determined in the presence of 1000 nM of unlabelled toxin. Specific binding (●) is the difference between total binding (not shown) and non-specific binding. (inset) Scatchard transformation of the specific binding. B, bound; F, free.

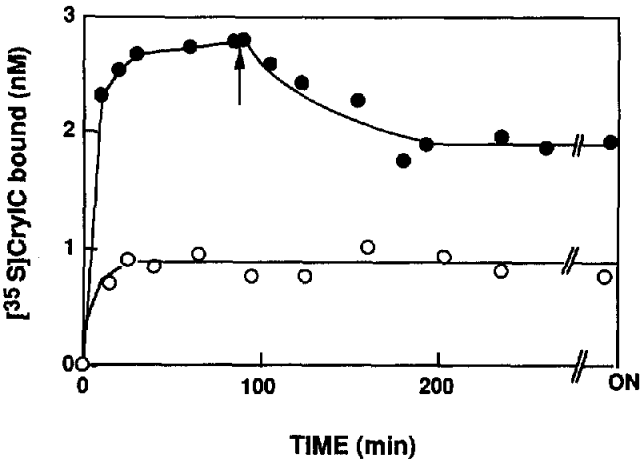

Fig. 3. Association and dissociation of [^{35}S]CryIC binding to *B. mori* BBMV's. Membranes were incubated with 15 nM [^{35}S]CryIC for various times to study the association. After 90 min incubation (steady state), dissociation was initiated by adding an excess of unlabelled CryIC toxin (indicated by the arrow) to the incubation mixture and the binding was determined at different times. Non-specific binding was determined in a parallel experiment in the presence of an excess of unlabelled ligand. (●), total binding ; (○), non-specific binding; ON, over night.

the final concentration of unlabelled ligand was 500 nM. Addition of 500 nM of unlabelled toxin resulted in the dissociation of about 45% of the radiolabelled CryIC toxin bound to *B. mori* BBMV's.

4. Discussion

In this report we have investigated the relationship between the specificity, larval potency and binding characteristics of the CryIC ∂-endotoxin with its receptors in two different insect species. The toxicity data presented in Table 1 indicate that the CryIC toxin is 7-fold more toxic for *B. mori* than for *S. littoralis*. However, the binding experiments show that CryIC binds with high affinity to both *S. littoralis* and *B. mori* BBMV's and that the binding parameters are identical for both insect BBMV preparations. These values of K_d and B_{max} are also similar to the values obtained by Ferré et al. [25] for the interaction of CryIC with *Plutella xylostella* BBMV's or to the values observed by Van Rie et al. [13] for the lower affinity site of the CryIC toxin on *S. littoralis* or *Manduca sexta*. However, we did not find evidence for a second binding site of higher affinity for the CryIC toxin on *S. littoralis* BBMV's (K_d = 0.18 nM and B_{max} = 2.04 pmol/mg BBMV) as described by Van Rie et al. [13]. Therefore, our binding data with CryIC and *S. littoralis* and *B. mori* BBMV's demonstrate that the 7-fold greater potency of CryIC towards *B. mori* is not correlated with a qualitative or quantitative difference in binding affinity or receptor site concentration. Therefore, larvicidal activity and receptor concentration or affinity of the receptor sites for the ∂-endotoxins are not necessarily closely correlated as previously reported for other ∂-endotoxins. It is likely that other physiological factors are involved in the susceptibility of insects to a given toxin and/or their ability to survive sublethal doses of toxin as it is the case for chemical insecticides.

Time course experiments of association and dissociation indicated that the binding of [^{35}S]CryIC to *B. mori* BBMV's was partially reversible. Hofmann et al. [11,12] and Van Rie et al. [12,13] previously showed that the binding of CryIB, CryIA(b) and CryIC to BBMV's from different insect species was irreversible. More recently, Lee et al. [14], also observed no dissociation of CryIA(a) from membrane vesicles of *B. mori* within 2 h. These studies therefore suggested that irreversibility of binding was a general feature of lepidopteran ∂-endotoxins. However, Van Rie et al. [13], also observed that 30% of the CryIE toxin bound to *M. sexta* or *S. littoralis* BBMV's could be dissociated from its binding site in both insects. Our binding data on the BBMV's of *B. mori* is thus the second report indicating that, in some cases, the binding of a ∂-endotoxin can be partially reversed. Therefore, these data indicate that there may be some insect species or ∂-endotoxin type differences in the reversible binding characteristics of the ∂-endotoxins. Moreover, Schwartz et al. [5] have recently reported that the CryIC ∂-endotoxin was able to partition in planar lipid bilayers and SF9 lepidopteran cells to form ionic channels. Therefore, the insertion of this toxin into the lipid bilayer can occur even in the absence of receptor and it is possible that the receptors may only help increase the concentration of toxin at the membrane surface. The presumed post-binding event, the self-insertion of the toxin into the membrane to form a pore, may therefore occur slowly, be dependent on the nature of the phospholipids and/or fluidity of the membrane and be reversible until the toxin has assembled into a channel in the membrane. Our results with the CryIC/*B. mori* model are consistent with such hypothesis. Alternatively, receptors may bind the toxins and trigger or catalyze channel formation. Should this be the case, the final step of pore formation could proceed much more rapidly (as the receptor would be actively involved and assist partioning of the ∂-endotoxin into the membrane) and thus explain the differences in toxicity, selectivity, pore size or binding irreversibility that have been reported for the lesions made by different ∂-endotoxins.

In conclusion, *Bacillus thuringiensis* CryIC ∂-endotoxin binds similarly to both *S. littoralis* and *B. mori* BBMV's but is 7-fold more toxic for *B. mori* indicating that factors other than the binding affinity and receptor site concentration are involved in ∂-endotoxin insecticidal activity. Moreover, the binding of CryIC to *B. mori* BBMV's is partially reversible suggesting that the toxin-receptor interaction is much more rapid than the final step of insertion of the toxin in the membrane. These data on the CryIC/*B. mori* system provide new evidence of the heterogeneity and complexity of the interaction of the ∂-endotoxins with their receptors. It is now generally accepted that all Cry ∂-endotoxins have a similar general conformation [26]. It is also reasonable to assume that the two-step mechanism of receptor binding and pore formation correctly describes the mechanism of action by which *Bacillus thuringiensis* ∂-endotoxins kill the insects. However, the elucidation of the mode of action of these toxins at the molecular level may well reveal that the nature and contribution of the receptors to toxicity differs from toxin to toxin or insect species.

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