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Receptors on the Brush Border Membrane of the Insect Midgut as Determinants of the Specificity of *Bacillus thuringiensis* Delta-Endotoxins

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To investigate the biochemical basis of the differences in the insecticidal spectrum of *Bacillus thuringiensis* insecticidal crystal proteins (ICPs), we performed membrane binding and toxicity assays with three different ICPs and three lepidopteran species. The three ICPs have different toxicity patterns in the three selected target species. Binding studies with these ^{125}I-labeled ICPs revealed high-affinity saturable binding to brush border membrane vesicles of the sensitive species. ICPs with no toxicity against a given species did not bind saturably to vesicles of that species. Together with previous data that showed a correlation between toxicity and ICP binding, our data support the statement that differences in midgut ICP receptors are a major determinant of differences in the insecticidal spectrum of the entire lepidopteran-specific ICP family. Receptor site heterogeneity in the insect midgut occurs frequently and results in sensitivity to more than one type of ICP.

Bacillus thuringiensis has been used as a microbial insecticide for over two decades. Its insecticidal activity resides in proteinaceous crystalline inclusions that are produced during sporulation. Strains containing insecticidal crystal proteins (ICPs) that are toxic to lepidopteran (2), dipteran (5), and coleopteran (18) insects have been identified. Furthermore, within the lepidopteran-specific group, strains with different insecticidal spectra have been identified (2, 16, 17, 20; for a review, see reference 12). Most *B. thuringiensis* strains contain a mixture of structurally different ICPs, and each of these may contribute to the insecticidal spectrum of a strain. ICPs usually are protoxins which require solubilization and proteolytic activation in the insect midgut. The activated toxin binds to a membrane receptor and lyses midgut epithelial cells (3).

In this study we used three distinct pure recombinant anti-lepidopteran ICPs to study the molecular basis of their different toxicities towards three selected target insects. Some authors suggest that the pH (15) and proteases (7) in the insect midgut play a role. By using a binding assay with ^{125}I-labeled toxins (8), we focused on the possible role of specific receptors on the plasma membrane of gut epithelial cells of target insects. A correlation between binding and toxicity for two toxins [a CryIA(b)-type and a CryIB-type ICP according to the classification of Höfte and Whiteley (12)] and two insect species was previously demonstrated (9). Subsequently, a correlation was observed between the receptor site concentration and the toxicity of three ICPs [a CryIA(a)-, a CryIA(b)- and a CryIA(c)-type ICP] in *Heliothis virescens* (23). Our present study, in which we performed toxicity and binding assays with a subset of three other types of ICPs [a CryIA(a)-, a CryIC-, and a CryIE-type ICP] and three insects (*Spodoptera littoralis*, *Manduca sexta*, and *H. virescens*), now allows us to investigate the validity of such a correlation within the family of lepidopteran-specific ICPs in general. We demonstrate that ICPs that are toxic to larvae of a certain insect species bind saturably and with high affinity to brush border membrane vesicles of these insects. In contrast, nonsusceptible insects lack specific binding sites for the toxins. These data strongly suggest that receptors on the brush border membrane of the midgut epithelium are a key factor in determining the specificity of lepidopteran-specific *B. thuringiensis* ICPs in general.

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MATERIALS AND METHODS

Purification and activation of recombinant *B. thuringiensis* delta-endotoxins. From *B. thuringiensis* subsp. *aizawai* HD-68, a delta-endotoxin gene which encodes a protein with a molecular weight of about 133,000 (Bt3 protoxin [11]) was cloned. This protein belongs to the CryIA(a)-type ICP according to the classification of Höfte and Whiteley (12). A gene cloned from *B. thuringiensis* subsp. *entomocidus* HD110 codes for a 135-kilodalton protoxin (Bt15 protoxin; H. Höfte, unpublished data) that belongs to the CryIC-type ICP. The *Bt15* gene differs from the *cryIC* gene isolated by Honée et al. (13) only at the following positions: an Ala codon (GCA) instead of an Arg codon (CGA) is present at position 925 and a sequence of Thr-His (ACGCAT) instead of Thr-Asr (ACCGAT) is present at position 1400. Bt18 protoxin was cloned from *B. thuringiensis* subsp. *darmstadiensis* (unpublished data). The Bt18 protoxin represents a novel type of ICP not previously described. Because of its structural differences from other ICPs and its insecticidal spectrum, the Bt18 protein should be defined as a novel ICP type. Based on the classification method of Höfte and Whiteley (12), we propose the name CryIE. Sequence comparisons with the GENALIGN computer program indicate that Bt18 toxin has 50% amino acid homology with Bt3 toxin and 54% homology with Bt15 toxin. Protoxin purification and activation were performed by the method of Höfte et al. (10). Toxic fragments were purified by the methods described previously (9). The activated and purified Bt3, Bt15, and Bt18 toxins are referred to as CryIA(a), CryIC, and CryIE toxins, respectively, throughout the remainder of this study.

Toxicity tests. Toxicity assays were performed with newly

hatched larvae. Toxin samples were diluted in phosphate-buffered saline (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl [pH 7.4]) with 0.1% bovine serum albumin, layered on artificial diet, and allowed to dry. Larvae were then placed on the diet. Details of the bioassays were described previously (10, 11). Mortality was scored after 5 (*M. sexta*) or 6 (*S. littoralis* and *H. virescens*) days. Mortality data were analyzed by means of probit analysis (4). In some cases, larval weight was recorded at the end of the assay.

Iodination of delta-endotoxins. Iodination of CryIA(a) and CryIE toxins was performed by using the chloramine-T method (14). A 1-mCi amount of $Na^{125}I$ and a 20-μg portion of chloramine-T in phosphate-buffered saline were added to 25 μg of purified toxin. After gentle shaking of the mixture for 20 s, the reaction was stopped by adding 53 μg of potassium metabisulfite in H_2O. This mixture was loaded onto a Bio-Gel P-30 (Bio-Rad Laboratories) column to remove free iodine and possible degradation products. CryIC toxin was labeled by using the Iodogen method, since iodination with the chloramine-T method yielded preparations of labeled toxin for which an unusually high percentage of nonspecific binding to brush border membrane vesicles was observed. CryIC toxin was loaded onto a Superose 12 gel filtration column (Pharmacia) in the presence of dithiothreitol prior to being labeled. Dithiothreitol was then removed from the purified protein by dialysis. This additional purification step was necessary to allow labeling to a high specific activity. Iodogen (Pierce Chemical Co.) was dissolved in chloroform at 0.1 mg/ml. A 100-μl volume of this solution was pipetted into a disposable glass vessel and dried under a stream of nitrogen gas. The vessel was rinsed with Tris buffer (20 mM Tris, 150 mM NaCl [pH 8.65]). A 25-μg amount of toxin (in Tris buffer) was incubated with 1 mCi of $Na^{125}I$ in the tube for 10 min. The reaction was then stopped by the addition of 1 M NaI (one-fourth of the sample volume).

Determination of specific activity of iodinated toxin. The specific activities of iodinated toxin samples were determined by using the "sandwich" enzyme-linked immunosorbent assay technique described by Voller et al. (24). Primary antibody was a polyclonal antiserum raised against CryIA (b), CryIC, or CryIE toxin for enzyme-linked immunosorbent assay of CryIA(a), CryIC, or CryIE toxin, respectively. Second antibody was monoclonal antibody 4D6 [to detect CryIA(a) and CryIE toxins] or 1A10 (to detect CryIC toxin). The complex of primary antibody-antigen-second antibody was detected by the conjugate alkaline phosphatase coupled to anti-mouse immunoglobulin G. The reaction intensities of a standard dilution series of unlabeled toxin and dilutions of the iodinated toxin sample (in phosphate-buffered saline with 0.1% bovine serum albumin) were measured. Linear regression calculations yielded the protein content of the radioactive toxin sample.

Specific activities were 29,700, 463,000, and 45,000 Ci/mol (on reference date) for CryIA(a), CryIC, and CryIE toxins, respectively.

Preparation of brush border membrane vesicles. Brush border membrane vesicles from *M. sexta*, *H. virescens*, and *S. littoralis* were prepared by the method of Wolfersberger et al. (25).

Binding assay. Duplicate samples of ^{125}I-labeled toxin, in combination with increasing amounts of unlabeled toxin, were incubated at room temperature with an appropriate amount of brush border membrane vesicles in a total volume of 100 μl of binding assay buffer (20 mM Tris–150 mM NaCl [pH 7.4] with 0.1% bovine serum albumin). The concentration of vesicles is provided in Table 2. Ultrafiltration through Whatman GF/F glass fiber filters was used to separate bound from free toxin. Each filter was rapidly washed with 5 ml of ice-cold binding assay buffer. The radioactivity of the filter was measured in a gamma counter (1275 Minigamma; LKB Instruments, Inc.). Binding data were analyzed by using the LIGAND computer program (22). This program calculates the concentration of bound ligand as a function of the total concentration of ligand, given initial estimates of the affinity (K_d) and the binding site concentration (R_t). Through an iterative process, the computer adjusts the values of K_d, R_t, and nonspecific binding until the binding curve generated by these parameters approximates the experimental curve as closely as possible. The program uses an exact mathematical model derived from the first-order mass-action law (equation 1) and the conservation-of-mass equation for the receptor sites (equation 2) and the ligands (equation 3) of the system.

$$L_i + R_j \rightleftharpoons L_iR_j, \text{ with } K_{ij} = B_{ij}/(F_iE_j) \quad (1)$$

$$R_j = E_j + \Sigma_i B_{ij} \quad (2)$$

$$L_i = F_i + \Sigma_j B_{ij} \quad (3)$$

where L_i is the total concentration of ligand i, R_j is the total concentration of receptor j, B_{ij} is the concentration of ligand i bound to receptor j, F_i is the free concentration of ligand i, and E_j is the concentration of empty receptor j.

By using this program, it is possible to assess on a statistical basis which model gives the best representation of the experimental data (e.g., one-site versus two-site model).

In homologous competition experiments, the concentrations of the labeled ligands were 0.4, 0.08, and 0.3 nM for CryIA(a), CryIC, and CryIE toxins, respectively. In experiments with labeled CryIA(a) and CryIE toxins, 14 and 20 concentrations of unlabeled CryIA(a) and CryIE toxins were used, respectively. In experiments with labeled CryIC toxin, 24 concentrations (*S. littoralis* and *M. sexta* vesicles) or 15 concentrations (*H. virescens* vesicles) of unlabeled CryIC toxin were used. Incubation time was 90 min, except for experiments with labeled CryIA(a) toxin, when it was 60 min. Concentrations of brush border membrane vesicles were as follow: *S. littoralis*, 100 (for labeled CryIC toxin) or 150 (for labeled CryIE toxin) μg of membrane protein per ml; *M. sexta*, 50 (for labeled CryIC toxin) or 100 [for labeled CryIA(a) and CryIE toxin] μg of membrane protein per ml; *H. virescens*, 150 [for labeled CryIA(a) toxin] or 200 (for labeled CryIC toxin) μg of membrane protein per ml.

To study the dissociation process, an incubation mixture that had reached equilibrium either was 10-fold diluted in binding assay buffer or an excess of unlabeled ligand (1,515 nM) was added.

Autoradiography. Labeled toxin was incubated with *M. sexta*, *S. littoralis*, or *H. virescens* vesicles for 90 min at room temperature. The samples were spun down for 15 min at 14,000 rpm in a Heraeus Sepatech microcentrifuge (Biofuge A). The pellet was suspended in binding assay buffer and again centrifuged. The final pellet, the first supernatant, and the toxin not incubated with vesicles were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) (19). The dried gel was exposed to Fuji RX-Safety film for 1 day.

Determination of protein concentration. Protein concentrations of purified CryIA(a), CryIC, and CryIE toxins were calculated from their optical densities at 280 nm (measured with a Uvikon 810 P spectrophotometer; Kontron Instruments). The protein contents of brush border membrane

TABLE 1. Toxicity of *B. thuringiensis* ICPs

ICP	Toxin LC_{50} (ng/cm^2)[a] against:		
	M. sexta	*S. littoralis*	*H. virescens*
CryIA(a)			
Toxin	20 (15–29)	>1,350	157 (43–574)
Protoxin	5.2 (3.9–7.0)	>1,350	90 (68–118)
CryIC			
Toxin	111 (76–163)	93 (76–114)	>2,700
Protoxin	74 (54–102)	105 (84–131)	>1,875
CryIE			
Toxin	73 (46–115)	88 (68–113)	>2,700
Protoxin	72 (52–101)	62 (48–79)	>1,875

[a] LC_{50}s and 95% confidence intervals (shown in parentheses) were calculated by means of probit analysis. Data are given in nanograms of ICP per square centimeter of artificial diet.

vesicles and protoxins were measured by the method of Bradford (1).

RESULTS

CryIA(a), CryIC, and CryIE toxins were evaluated for toxicity (Table 1). Bioassays on *M. sexta* demonstrated that CryIA(a) toxin is highly toxic to this insect (concentration required to kill 50% of insects tested [LC_{50}] = 20 ng/cm^2). CryIC and CryIE toxins were about 3.5 and 5.5 times less active, respectively. The LC_{50} for CryIA(a) toxin against *H. virescens* larvae was 157 ng/cm^2, whereas the LC_{50}s for CryIC and CryIE toxins were greater than the highest concentration tested (2,700 ng/cm^2). In contrast to control larvae, larvae exposed to CryIC toxin showed a 12-fold decrease in larval weight at the highest concentration tested (data not shown). Larval weight was only slightly reduced by exposure to the same concentration of CryIE toxin. *S. littoralis* larvae were not susceptible to CryIA(a) toxin. CryIC and CryIE toxins were about equally toxic (LC_{50}s = 93 and 88 ng/cm^2, respectively) to this insect. The protoxins of the three ICPs were also tested for toxicity. For all three insects, similar toxicity patterns for protoxins and toxins were observed (Table 1). This observation demonstrates that, in these insects, activation of protoxin to toxin is not a key factor with respect to the differences in insecticidal activity of these ICPs.

^{125}I-labeled CryIA(a), CryIC, and CryIE toxins were detected as one single band by autoradiography (Fig. 1). ^{125}I-labeled ICPs were incubated with vesicles from *M. sexta*, *S. littoralis*, and *H. virescens*, and the bound and free fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No degradation of either fraction was seen by using autoradiography. Figure 1 shows the results of such an experiment with labeled CryIC toxin. The increased mobility of the free toxin (Fig. 1, lanes 4, 6, and 8) was a result of the presence of a relatively high amount of bovine serum albumin in the sample loaded on the gel. Iodination with Na^{127}I did not alter the insecticidal activity of these toxins on *M. sexta* (data not shown).

Labeled ligand was incubated with increasing concentrations of vesicles to determine the appropriate concentrations of brush border membrane vesicles to be used in competition experiments. Binding in the presence of excess unlabeled ligand was subtracted from total binding for each data point (Fig. 2). Maximum binding of labeled CryIA(a) toxin to *M. sexta* and *H. virescens* vesicles was observed at concentrations of 100 and 200 μg of vesicle protein per ml; respectively. In contrast, no saturable binding to *S. littoralis* vesicles was observed, even at 1,000 μg of vesicle protein per ml. About 55% of labeled CryIC toxin was bound to *M. sexta* and *S. littoralis* vesicles at a concentration of 200 μg of vesicle protein per ml. Only 20% was bound to *H. virescens* vesicles, even at concentrations up to 1,000 μg of vesicle protein per ml. Strong binding to *M. sexta* and *S. littoralis* vesicles was also observed for CryIE toxin. In contrast, this toxin showed no saturable binding to *H. virescens* membranes.

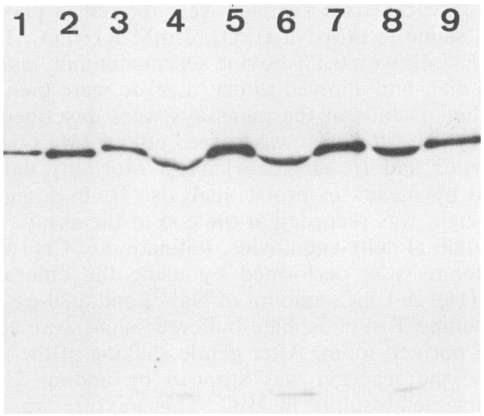


FIG. 1. Autoradiograph of ^{125}I-labeled CryIA(a) (lane 2), CryIC (lanes 3 through 9), and CryIE (lane 1) toxins. Lanes 1, 2, and 3 show the toxins that were not incubated with vesicles; lanes 5, 7, and 9 show bound CryIC toxin; and lanes 4, 6, and 8 show free CryIC toxin after incubation with *M. sexta* (lanes 4 and 5), *S. littoralis* (lanes 6 and 7), or *H. virescens* (lanes 8 and 9) vesicles. CryIC toxin was incubated for 90 min at room temperature with vesicles from *S. littoralis*, *M. sexta*, and *H. virescens* (at 100, 50, and 200 μg, respectively, of membrane protein per ml). Free toxin was separated from bound toxin by centrifugation.

These experiments indicated a qualitative correlation between toxicity and binding. Indeed, toxins with no (CryIE toxin) or only marginal (CryIC toxin) activity against *H. virescens* larvae showed no (CryIE toxin) or weak (CryIC toxin) binding to membranes of this species, whereas CryIA (a) toxin (which is toxic to *H. virescens*) exhibited strong binding. Also, the only ICP with no toxicity to *S. littoralis*, i.e., CryIA(a) toxin, was the only toxin for which no saturable binding to vesicles of this insect could be observed. Finally, all ICPs tested exhibited toxicity to *M. sexta* and all displayed saturable binding.

Homologous competition experiments (competition between labeled ligand and its unlabeled analog) were performed to evaluate binding at a quantitative level. From these studies we calculated the affinity (K_d) and binding site concentration (R_t) for the different toxin-membrane interactions by using the LIGAND computer program (Table 2). CryIA(a) toxin bound with high affinity to membrane vesicles of both *M. sexta* (K_d = 1.48 ± 0.35 nM) and *H. virescens* (K_d = 1.16 ± 0.34 nM). Whereas the affinity of this toxin to both insects was similar, the binding site concentration was about six times higher in *M. sexta* than in *H. virescens*. As demonstrated above, this toxin does not bind to *S. littoralis* membranes. CryIC toxin bound with relatively low affinity (K_d = 22.4 ± 4.53 nM) to *H. virescens* vesicles. In contrast, high-affinity binding to vesicles from insect species which were shown to be much more susceptible to CryIC toxin was demonstrated: *M. sexta* (K_{d1} = 0.41

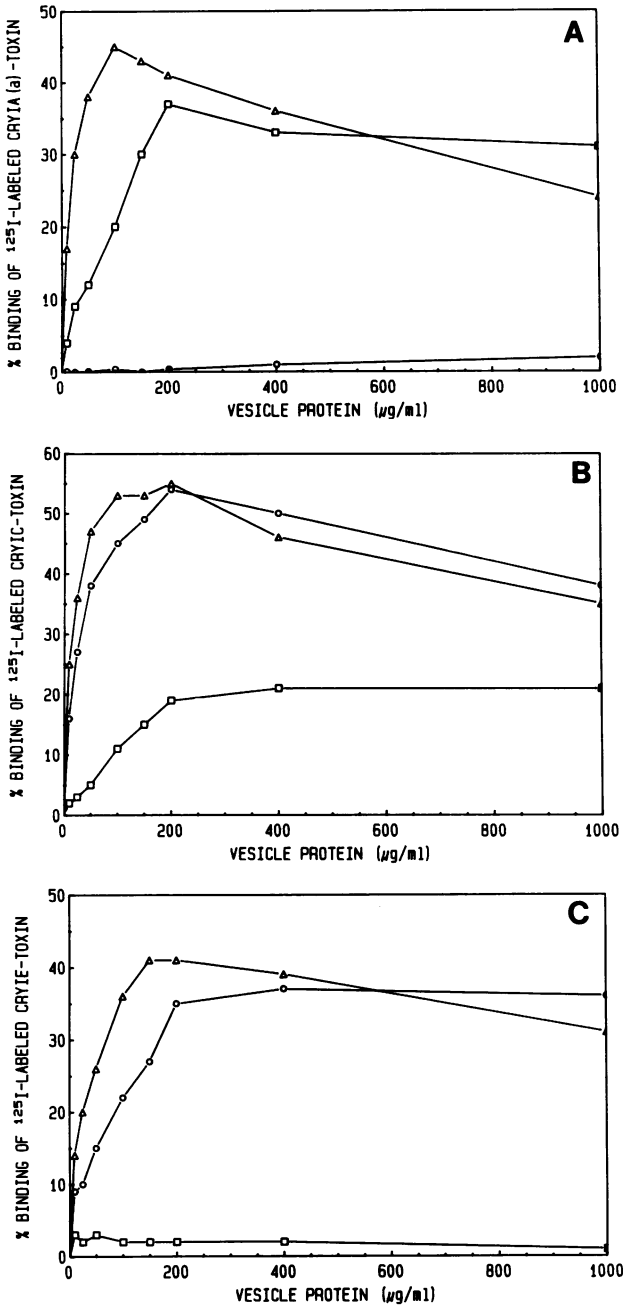


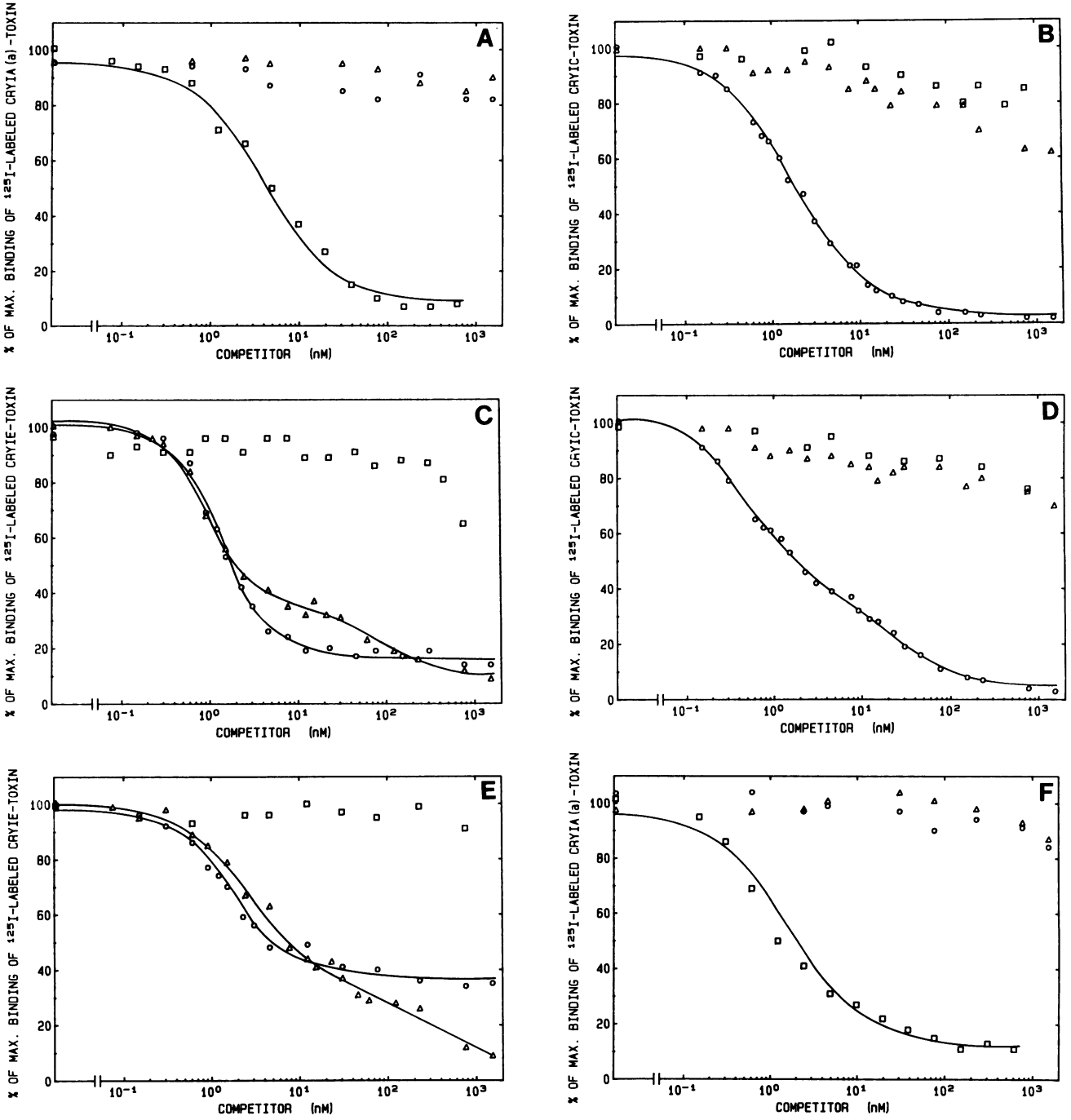
FIG. 2. Specific binding of ^{125}I-labeled CryIA(a) (A), CryIC (B), and CryIE (C) toxins as a function of the concentration of brush border membrane vesicles of *M. sexta* (△), *S. littoralis* (○), or *H. virescens* (□). ^{125}I-labeled toxin [CryIA(a), 0.44 nM; CryIC, 0.05 nM; CryIE, 0.21 nM] was incubated with vesicles in the presence or absence of an excess unlabeled toxin [CryIA(a), 151 nM; CryIC, 1,515 nM; CryIE, 1,515 nM]. Samples were incubated for 60 [CryIA (a) toxin] or 90 (CryIC and CryIE toxins) min. Bound toxin was separated from free toxin by using ultrafiltration through Whatman GF/F filters. Nonspecific binding was subtracted from total binding.

± 0.12 nM) and *S. littoralis* (K_{d1} = 0.18 ± 0.04 nM). In both insects, the toxin also recognized a second site with lower affinity ($K_{d2} \approx$ 10 nM). CryIE toxin displayed high-affinity binding to vesicles of both *M. sexta* (K_{d1} = 0.092 ± 0.032 nM) and *S. littoralis* (K_{d1} = 1.18 ± 0.33 nM). In both insects,

TABLE 2. Binding[a] of *B. thuringiensis* ICPs to brush border membrane vesicles from larval midguts

Insect	Binding characteristics of toxin									
	CryIA(a)		CryIC				CryIE			
	K_d	R_t	K_{d1}	R_{t1}	K_{d2}	R_{t2}	K_{d1}	R_{t1}	K_{d2}	R_{t2}
M. sexta	1.48 ± 0.35	19.9 ± 5.6	0.41 ± 0.12	8.95 ± 2.7	7.05 ± 4.2	9.44 ± 2.4	0.092 ± 0.032	2.97 ± 0.69	216 ± 155	283 ± 202
S. littoralis	NB[b]	NB	0.18 ± 0.04	2.04 ± 0.44	13.9 ± 1.3	38.9 ± 4.5	1.18 ± 0.33	4.73 ± 2.47	352 ± 16	230 ± 96
H. virescens	1.16 ± 0.34	3.47 ± 0.21	22.4 ± 4.53	37.9 ± 7.9			NB	NB	NB	NB

[a] K_d in nanomolar concentration; R_t in picomoles per milligram of vesicle protein. Each value is the mean of three experiments, performed on two independently prepared batches of vesicles, ± standardsicle deviation.
[b] NB, No binding.



CryIE toxin also bound to a second site with much lower affinity. In the previous set of binding experiments, it was demonstrated that this toxin did not bind saturably to membranes of *H. virescens*, reflecting the extremely poor activity towards this insect.

We then performed heterologous competition experiments (competition between a labeled toxin and another unlabeled ligand) to investigate the relationship between the receptor sites for the different ICPs. In *M. sexta*, CryIC and CryIE toxins did not compete for bound ^{125}I-labeled CryIA(a) toxin (Fig. 3A). Also, there was no significant displacement of bound CryIC and CryIE toxins by CryIA(a) toxin in this insect (Fig. 3B and C, respectively). In *H. virescens* vesicles, CryIC and CryIE toxins did not compete for saturably bound ^{125}I-labeled CryIA(a) toxin (Fig. 3F). Also, CryIA(a) toxin competed only with a very low affinity for bound CryIC toxin (Fig. 3G). In *S. littoralis*, CryIA(a) toxin did not compete for saturably bound ^{125}I-labeled CryIC toxin (Fig. 3D) or CryIE toxin (Fig. 3E). These data demonstrate that CryIA(a) toxin recognizes a binding site which is distinct from the binding site(s) of CryIC and CryIE toxins.

On *S. littoralis* vesicles, CryIC toxin competed for part of the binding sites occupied by labeled CryIE toxin (Fig. 3E). Analysis of two such competition experiments revealed that CryIC toxin recognized a binding site with an affinity of 0.30 ± 0.10 nM. This value is very similar to the mean value for

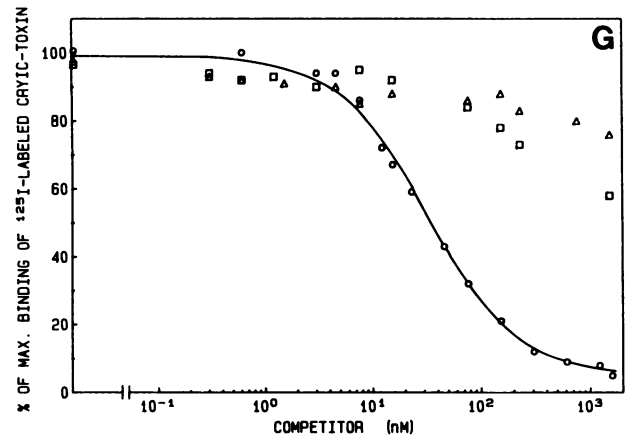


FIG. 3. Binding of ^{125}I-labeled toxins to *M. sexta* (A through C), *S. littoralis* (D and E), and *H. virescens* (F and G) brush border membrane vesicles. Vesicles were incubated with ^{125}I-labeled toxin [A and F, CryIA(a) toxin; B, D, and G, CryIC toxin; C and E, CryIE toxin] in the presence of increasing concentrations of CryIA(a) (□), CryIC (○), or CryIE (△) toxin. Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin alone. Nonspecific binding was not subtracted. Curves are those predicted by the LIGAND computer program. Each point is the mean of a duplicate sample.

K_{d1} of CryIC toxin on this insect (0.18 nM, as calculated from homologous competition experiments). Moreover, the mean R_{t1} values of CryIC and CryIE toxins on *S. littoralis* are not significantly different (Student's *t* test). These observations suggest that the high-affinity sites recognized by CryIC and CryIE toxins in this insect are the same site. Accordingly, CryIE toxin would be expected to compete for the high-affinity site occupied by labeled CryIC toxin. Such competition, however, was not observed (Fig. 3D). In *M. sexta*, CryIC toxin competed for a major part of bound ^{125}I-labeled CryIE toxin (Fig. 3C), with a calculated affinity (K_d) of 0.13 ± 0.02 nM. This value is close to the mean value for K_{d1} of CryIC toxin on this insect (0.41 nM, as calculated from homologous competition experiments). Furthermore, the difference between the mean R_{t1} values for both toxins is only weakly significant ($P = 0.02$, Student's *t* test), suggesting that the high-affinity site of CryIC toxin in *M. sexta* might be identical to that of CryIE toxin. However, no significant competition of CryIE toxin for bound CryIC toxin was observed (Fig. 3B).

DISCUSSION

We have investigated the molecular basis for the specificity of *B. thuringiensis* ICPs by using receptor binding studies. For this study we selected three toxins (Bt3, Bt15, and Bt18) which belong to the CryIA(a); CryIC-, and CryIE-type ICPs, respectively. Bioassays indicated remarkable differences in the insecticidal spectra of these toxins. Interestingly, almost identical toxicity patterns were observed for toxins and protoxins. This similarity demonstrates that the differences in toxicity of the ICPs under study are not a result of differential activation in the insect midgut.

Our first series of rather qualitative binding experiments with ^{125}I-labeled toxins demonstrated a strong correlation between toxicity and binding to brush border membrane vesicles derived from larval midguts. No saturable binding could be demonstrated for toxin-insect combinations for which no toxicity (sensitivity) was observed [CryIA(a)-*S. littoralis* and CryIE-*H. virescens*]. One combination with only marginal toxicity (CryIC-*H. virescens*) exhibited only weak binding. For all other combinations, relatively high levels of toxicity and binding were observed.

A second series of quantitative binding studies (homologous competition experiments) gave further evidence for a correlation between toxicity and binding. For CryIA(a) toxin, for example, it appeared that the affinity of the toxin to *M. sexta* and *H. virescens* vesicles differed only slightly, but the higher binding site concentration of the toxin in *M. sexta* could reflect the higher susceptibility of this insect to the toxin. Moreover, in a previous report we presented evidence that CryIA(a) toxin recognizes two different sites in *M. sexta* (23). The affinities for both sites must be very similar since the competition curve is not biphasic. Evidence for the existence of a second site came from competition studies with other ligands. CryIC toxin recognized two sites in both *M. sexta* and *S. littoralis* and only one site in *H. virescens*. We suspect that binding of ICPs to the insect midgut in vivo is dependent on both the affinity and the concentration of receptor sites. Consequently, since the binding of CryIC toxin, expressed as the product $K_{a1} \times R_{t1}$, is similar for *M. sexta* and *S. littoralis*, this could reflect a similar sensitivity of the two species to this toxin. The affinity of this toxin to *H. virescens* vesicles was about 120 and 50 times lower, respectively, than the affinities for the high-affinity sites in *S. littoralis* and *M. sexta*. The affinity and the binding site concentration of CryIC toxin in *H. virescens* vesicles are similar to the binding parameters for the low-affinity site in the other insects. Since *H. virescens* is only marginally susceptible to this toxin, we suggest that the low-affinity sites in the other insects do not significantly contribute to the toxicity of this ICP against these insects. CryIE toxin binds to both high-affinity and low-affinity sites in *M. sexta* and *S. littoralis*. Although the two insects are about equally susceptible to this toxin, a significant difference in the affinity of the first binding site in the two insects was observed. This may indicate that in this case, other factors in the pathway of toxin action besides binding contribute to determine toxicity levels.

Heterologous competition experiments were performed to investigate whether the binding sites for the different toxins were related. A general conclusion from these studies was that the binding site of CryIA(a) toxin was different from the site(s) of CryIC and cryIE toxins in all three insects. Furthermore, it appeared that CryIC toxin recognized the high-affinity binding site of cryIE toxin in both *M. sexta* and *S. littoralis*. It would therefore be expected that cryIE toxin competes for part of the CryIC, toxin binding sites. However, such competition was not observed. We have no complete explanation for this discrepancy. Kinetic aspects of the binding, however, may provide at least a partial explanation. Indeed, we observed virtually no dissociation of CryIC toxin from vesicles of both insects, whereas about 30% of CryIE toxin could be dissociated from its binding site in both insects either by dilution or addition of excess unlabeled ligand (Fig. 4). Thus, the degree of reversibility of the binding may be somehow correlated with the ability of other ligands to compete for this binding. For two other ICPs [a CryIA(b) type and a CryIB type], it was also shown that a major part of the binding was irreversible (8, 23). Accordingly, partial irreversibility of binding appears as a general feature of lepidopteran-specific ICPs. The functional significance of this phenomenon is not known. It was recently proposed that, after membrane binding, the toxin inserts in

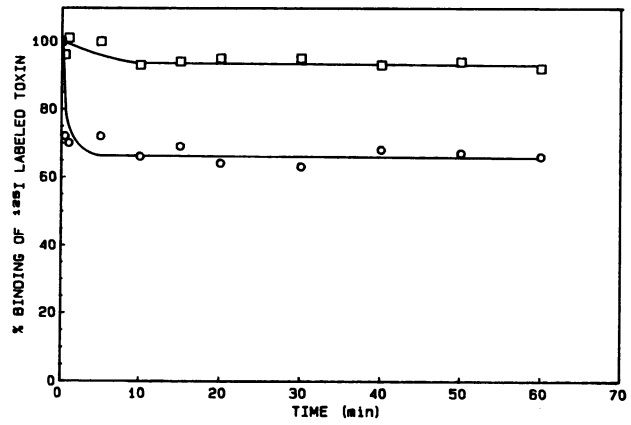


FIG. 4. Dissociation of CryIC (□) and CryIE (○) toxins from *M. sexta* membrane receptors. At 90 min after initiation of the association reaction, the test mixture was diluted 10-fold in binding assay buffer. Nonspecific binding was not subtracted.

the membrane to create a pore or leakage channel which ultimately leads to colloid osmotic lysis (6). The irreversibility we observed is consistent with this hypothesis.

Our data clearly establish a correlation between toxicity and binding to membranes from larval midguts for a subset of three distinct types of *B. thuringiensis* ICPs and three insects. Such a correlation was recently observed for a combination of two other types of ICPs and two insects (9). In a previous report, we demonstrated a correlation between the binding-site concentration and the toxicity of three different CryIA ICPs in *H. virescens* (23). As a result, we have now studied toxicity and binding of all but one known lepidopteran-specific ICP types on a few target insects. From all of these data we can conclude that the presence of receptors on the midgut epithelium is a major factor in determining the differences in insecticidal spectra of the entire family of lepidopteran-specific ICPs. However, this does not exclude the possibility that occasionally other factors, such as proteolytic activity (7), may play a role.

In our recent studies of *B. thuringiensis* toxin binding (9, 23), we found heterogeneity among binding sites. The present data provide new evidence that different ICPs may recognize different binding sites in one insect. A general picture which arises from our studies is that there is a family of distinct (but probably related) insect membrane receptors to which different (but related) toxins, belonging to the family of lepidopteran-specific ICPs, may bind. Insects may have different members of this receptor family. In this context, it should be noted that most naturally occurring *B. thuringiensis* strains contain more than one type of ICP within their parasporal crystals. Some or all of the ICPs present in the crystals of a particular strain may recognize different binding sites in the same insect. The combination of two or more insecticidal factors that have different target sites is considered to diminish the chance of resistance development (21). This might explain why field resistance to *B. thuringiensis* has not yet been observed despite the relatively widespread use of this organism.

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