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A cadherin-like protein functions as a receptor for *Bacillus thuringiensis* Cry1Aa and Cry1Ac toxins on midgut epithelial cells of *Bombyx mori* larvae

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Abstract **Aminopeptidase N (APN) and cadherin-like protein (BtR175) from *Bombyx mori* larvae were examined for their roles in Cry1Aa- and Cry1Ac-induced lysis of *B. mori* midgut epithelial cells (MECs). APNs and BtR175 were present in all areas of the midgut, were particularly abundant in the posterior region, and were found only on columnar cell microvilli and not on the lateral membrane that makes cell–cell contacts. This distribution was in accordance with the distribution of Cry1A-susceptible MECs in the midgut. The lytic activity of Cry1Aa and Cry1Ac on collagenase-dissociated MECs was linearly dependent on toxin concentration. Although pre-treatment of MECs with anti-BtR175 antibody was observed to partially inhibit the lytic activity exerted by 0.1–1 nM Cry1Aa toxin or 5 nM Cry1Ac toxin, no significant inhibition was observed when MECs were pre-treated with anti-APN antibody. These results suggest that BtR175 functions as a major receptor for Cry1A toxins in the midgut of *B. mori* larvae.**

Key words: Aminopeptidase N; BtR175; Cry1Aa; Cry1Ac; *Bacillus thuringiensis*; *Bombyx mori*

1. Introduction

Bacillus thuringiensis, a Gram-positive bacterium, produces various proteinaceous crystal inclusions during sporulation. Because these inclusions display insecticidal activity [1], certain *B. thuringiensis* strains have been utilized throughout the world as organic insecticides. The strains selected for insecticidal use are those that produce inclusions exhibiting appropriate insecticidal specificity and safety profiles.

When susceptible insects ingest *B. thuringiensis*, the inclusions, which are composed of protoxins, are solubilized in the insect midgut. The protoxins are subsequently processed proteolytically to yield smaller, active Cry toxins [2] that bind specifically to receptor molecules of the midgut epithelial cells (MECs) of host insects [3,4]. This binding causes a change in the ion permeability characteristics of the midgut cell membranes [5] that results in a net influx of ions and an accompanying influx of water. As a result, the cells lyse [6,7], the midgut disintegrates, and the insect dies.

In initial studies, various aminopeptidase N (APN) isoforms and two cadherin-like proteins from several insect species were identified as candidate receptors for *B. thuringiensis* Cry toxins on the basis of their ability to bind these toxins. Among these candidates are three APN isoforms found in *Bombyx mori* [8–11], *Manduca sexta* [12,13], and *Plutella xylostella* [13,14]; two APN isoforms found in both *Heliothis virescens* [15,16] and *Lymantria dispar* [17,18]; and one cadherin-like protein found in both *M. sexta* and *B. mori* [19,20,21]. APN molecules can be divided into at least four phylogenetic classes based on their amino acid sequences [16,22], and various APN isoforms have differential affinities for the various classes of Cry toxins. For example, in *M. sexta*, Cry1C binds to a 106-kDa APN but not to a 115-kDa APN, and Cry1Ac binds to a 115-kDa APN but not to a 106-kDa APN [23]. In *L. dispar*, Cry1Ac binds to the 100-kDa APN1 molecule, but Cry1Aa and Cry1Ab bind to a different molecule [24,25].

In phospholipid membrane reconstitution experiments, APN appeared to promote irreversible binding of Cry1A toxins to the membrane and/or formation of ion channels [26–29]. A cadherin-like protein (BtR175) from *B. mori* promotes Cry1Aa-induced cell lysis in a baculovirus gene expression system [30]. Thus, both APN and cadherin-like protein have been shown to function as Cry toxin receptors in artificial contexts, but it is unknown whether these molecules function to promote epithelial cell lysis in the environment of the insect midgut. In addition, whether a particular receptor is bound by the same class of Cry toxin across different insect species is unknown. On the other hand, disruption of a cadherin-like protein gene by retrotransposon-mediated insertion has been linked to high levels of resistance to Cry1Ac toxin in *H. virescens* [31]. This finding suggests that cadherin-like protein plays an important role in Cry toxin susceptibility, although it does not constitute direct evidence that cadherin-like protein is the physiological Cry toxin receptor in the insect midgut.

In this study, we investigated the role of the APN and BtR175 of the *B. mori* midgut epithelium. We report here that BtR175, which specifically binds Cry1Aa and Cry1Ac toxins [20,21], is distributed on the microvilli in all areas of the larval midgut, consistent with the distribution of suscep-

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tible MECs. In addition, it plays a major role in the susceptibility of columnar cells to low concentrations of Cry1Aa and Cry1Ac. In contrast, APN was found on the midgut microvilli at a 100-fold greater concentration than was BtR175, but no evidence for a role in Cry toxin binding was found.

2. Materials and methods

2.1. Insects

Kinshu×Showa, a hybrid race of the silkworm *B. mori*, was purchased from Ueda-Sanshu Co. and reared on an artificial diet (Silkmate, Nihon-Nosanko Co.) at 25°C.

2.2. Preparation of Cry1Aa and Cry1Ac toxins

Cry1Aa and Cry1Ac crystals were purified with the biphasic system of Goodman et al. [32] from single toxin producers, *B. thuringiensis* serovar *sotto* T84A1 and *B. thuringiensis* HD-73, respectively. The crystals were solubilized and trypsinized, and then activated toxins were purified as described elsewhere [8]. Protein concentrations were determined by the Bradford method [33] using bovine serum albumin (BSA) as a standard.

2.3. Immunostaining of whole midgut

Midguts were dissected from fifth instar larvae, turned inside out, and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. After washing, the midguts were incubated for 1 h with PBS-buffered 3% hydrogen peroxide, washed, incubated in PBS-buffered 2% BSA for 1.5 h, incubated with anti-APN antibody or anti-BtR175 antibody for 1.5 h at room temperature, washed, incubated with anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad), washed, and then stained with Vector® VIP Substrate Kit (Vector Laboratories).

2.4. Preparation of paraffin section

After starvation for 10 h, third instar larvae were exposed to artificial diet contaminated with or without Cry1Aa protoxin for 1 h. Head and feet were removed and the larvae were fixed with PBS-buffered 4% paraformaldehyde for 1 h, dehydrated, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (Wako Pure Chemical Industries) or immunostained in the same way as the whole midgut.

2.5. Immunoblotting of brush border membrane vesicles (BBMV)

BBMV from the midgut of fifth instar larvae of *B. mori* were prepared according to the method described by Wolfersberger et al. [34]. BBMV proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 2% BSA in TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween), incubated with mouse anti-APN or anti-BtR175 antibodies, followed by incubation with goat anti-mouse IgG-HRP conjugate (Bio-Rad). The bound antibody was detected using the ECL Western blotting detection system (Amersham Pharmacia Biotech). To quantitate the amount of APN in a 5-μg BBMV sample by comparing band intensities, 25, 50, 100, 200, 400 ng APNs (BmAPN1 and BmAPN4) that were prepared by the method of Yaoi et al. [8] were used as standards and bands detected by immunoblotting were compared by densitometry.

2.6. Expression of partial fragment of APN and BtR175 and preparation of anti-APN and anti-BtR175 antibodies

A partial fragment of BmAPN1 containing the Cry1Aa binding region of Ile135 to Pro198 was expressed in *Escherichia coli* cells as a GST fusion protein as described previously [35]. Total RNA was extracted from the midgut of fifth instar larvae of *B. mori* using QuickPrep® Total RNA Extraction Kit (Amersham Biosciences), treated with DNase, and reverse-transcribed with oligo(dT) primer. The Cry1Aa binding region [30] containing cDNA of BtR175 (nt positions 3322–4392 encoding Glu1108–Val1464) was amplified by polymerase chain reaction using primers BtR175-3322-Sma (5′-TCCCCCGGGAGCTCGTGATTCGTCCTTAC) and BtR175-4392AS (5′-TTAAACAAACAAGAAGAAGACGCG), cloned into the T-overhang vector p123T (MoBi Tec), excised with *Sma*I and *Not*I, subcloned into GST-tagged expression vector pGEX-4T-3 (Amersham Pharmacia Biotech), and transfected into *E. coli* BL21. The transfected cells were cultured and gene expression was induced with 1 mM IPTG for 4 h at 37°C. Each GST fusion protein was produced as inclusion bodies, purified as single bands sequentially by sonication, centrifugation, and preparative SDS–PAGE, and used to raise anti-APN or anti-BtR175 antisera in mice. IgGs were purified from antisera using HiTrap® protein G columns (Amersham Biosciences).

2.7. MEC isolation

MECs were isolated according to the method of Wang and McCarthy [36]. Briefly, midguts from fourth instar larvae were excised to remove the peritrophic membrane and food contents were washed with sterile saline (154 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2$, 0.7 mM $NaHCO_3$, and 11.1 mM D-glucose, pH 7.0). Midguts were placed in a Petri dish containing the saline with 190 U/ml collagenase (Wako Pure Chemical Industries). After stirring for 1.5 h at 25°C, dissociated MECs were centrifuged for 3 min at 500×*g* and the resulting pellet was resuspended in the saline. The cells were washed by centrifugation several times until the supernatant was clear.

2.8. Lactate dehydrogenase (LDH) assay

Cell lysis was assessed by measuring the amount of cytosolic LDH according to the method of Wang and McCarthy [36] with minor modifications. Briefly, MECs were incubated with or without 1 μM antibodies for 1 h at 25°C at a concentration of 2.8×10^6 cells/50 μl saline in a microplate well. Cry1Aa or Cry1Ac toxins were added to the well and cells were incubated for an additional 1.5 h at 25°C. For complete lysis, Triton X-100 was added to a concentration of 1%. Remnant cells were precipitated by centrifugation at 250×*g* for 10 min and supernatants were transferred to a different microplate into which 50 μl of substrate of the LDH Cytotoxicity Detection Kit (Takara) was added. After incubation for 30 min at 24°C, the enzymatic reaction was stopped and OD was read by a microplate reader (Model 550, Bio-Rad) at 450 nm for measuring and at 595 nm for reference. The extent of the toxin-mediated cytolytic response is defined as the absorbance ratio of $(A_{tox}-A_0)/(A_{max}-A_0)\times100\%$, where A_{tox} is absorbance of the formazan product derived from the coupled assay to the LDH released from the supernatant of the cell–toxin mixture, A_{max} is absorbance of the formazan products derived from the coupled assay to the LDH released from the supernatant of a cell–Triton mixture (100% maximum lysis), and A_0 is absorbance of the formazan products derived from the coupled assay to the LDH released from the supernatant of a cell–buffer mixture which was composed of the residual LDH present in a MEC preparation and LDH released from spontaneous cell lysis during the incubation time. The extent of the antibody-mediated inhibition of cell lysis is defined as the absorbance ratio of $100-(A_{anti}-A_0)/(A_{tox}-A_0)\times100\%$, where A_{anti} is absorbance of the formazan product derived from the coupled assay to the LDH released from the supernatant of the antibody-pretreated cell–toxin mixture. Significant differences were analyzed by Student's *t*-test between sample treated with antibody and Cry1A toxin and sample treated with Cry1A toxin alone.

2.9. Assessment of antibody inhibition ability

Partial fragments of APN (Ile135 to Pro198) and BtR175 (Glu1108 to Val1464) containing the Cry1Aa binding regions were produced and purified as inclusion bodies of GST fusion proteins, solubilized in 0.1 M NaOH, and dialyzed in 0.1 M PBS. Their purity was confirmed by SDS–PAGE to exceed 90%. EIA plates (Costar) were coated with 0.18 μM of each recombinant protein for 2 h at 37°C and blocked with 2% BSA. After washing with PBS, 100 nM biotin-labeled Cry1Aa was added with or without 1 μM antibodies to each well and incubated for 1.5 h at 37°C. After washing with PBS, HRP-labeled streptavidin (Vector Laboratories) was added and incubated for 1.5 h at 37°C. After washing, ABTS (2,2′-azino-bis(3-ethylbenzthiazokine-6-sulfonic acid)) solution (0.04% ABTS, 0.01% H_2O_2 in 100 mM citrate-HNa_2PO_4 buffer, pH 4.0) was added and the absorbance at 415 nm was quantified using a microtiter plate reader.

3. Results

3.1. Distribution of Cry toxin-sensitive MECs

Functional receptors for Cry toxins are most likely to be present in the epithelial cell regions that undergo disruption

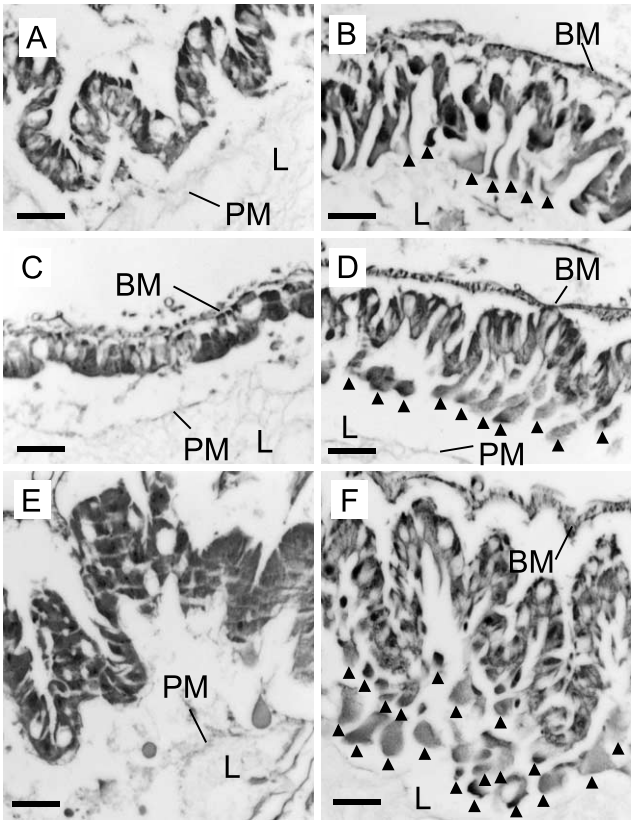

Fig. 1. Histopathological effects of Cry1Aa toxin on the midgut of *B. mori* larvae. Longitudinal sections of Cry1Aa-treated (B, D and F) or non-treated larvae (A, C and E) were stained with hematoxylin and eosin, and the anterior (A and B), middle (C and D), and posterior (E and F) parts of the midgut were observed by light microscopy. Arrowheads indicate disintegrated MECs. BM, basement membrane; L, lumen; PM, peritrophic membrane. Bar = 50 μm.

upon ingestion of Cry toxin. Therefore, we examined the distribution of Cry1Aa toxin-sensitive MECs in the *B. mori* midgut. After starvation for 10 h, third instar larvae were presented with an artificial diet contaminated with Cry1Aa protoxin (40 μg/g diet). The larvae stopped feeding after 15–25 min and died within 4 h. One hour after feeding, larvae were fixed and sections of the midgut were examined by microscope. Columnar cell enlargement, elongation, and extrusion into the gut lumen and disintegration of the MEC layer were observed in all regions of the midgut (anterior, middle, and posterior) (Fig. 1).

3.2. Distribution of APNs and BtR175

The distribution of APNs and BtR175 in the MEC layer was initially examined by immunoblotting (Fig. 2A,B). A 115-kDa band tentatively identified as BmAPN1 [22] was detected with 2 nM anti-APN antibody in every part of the midgut, especially in the posterior region (Fig. 2A). A 175-kDa band (presumably BtR175) with a similar distribution was detected with 3 nM anti-BtR175 antibody (Fig. 2B). In the posterior region, 100-kDa and 90-kDa bands that have been identified as BmAPN4 and BmAPN2, respectively [22], were also observed (Fig. 2A, lane 3). In the middle and posterior midgut regions, a 195-kDa band that has been reported to comprise part of the purified BtR175 fraction [37] was also observed (Fig. 2B, lanes 2, 3). The bands in Fig. 2A,B with molecular weights lower than 90 and 175 kDa, respectively, were thought to be APN and BtR175 degradation products, since the ladder pattern differed from that with Coomassie brilliant blue staining. When an entire midgut from a fifth instar larva was inverted and probed immunocytochemically using an anti-APN or anti-BtR175 antibody, all microvilli-containing areas of the midgut surface were stained (data not shown). In addition, when the midgut section of a third instar larva was probed with the same concentration of antibodies only the columnar cell brush border was stained (Fig. 2C,D).

Electrophoresis and immunoblotting were used to estimate the amount of APN content in the brush border microvilli by comparing the intensities of the sample bands with those from known quantities of APNs (25, 50, 100, 200, 400 ng purified BmAPN1 and BmAPN4). In 5 μg of BBMV protein, approximately 80 μg of 115-kDa APN (BmAPN1) and 20 μg of 100-kDa APN (BmAPN4) were present (Fig. 2E), indicating that APNs (BmAPN1 and BmAPN4) account for about 2% of the total BBMV protein.

3.3. Role of APNs and BtR175 in MEC sensitivity to Cry1Aa and Cry1Ac toxins

To assess the sensitivity of MECs to Cry1A toxins, the amount of LDH activity released as a result of Cry1A-induced cell lysis was measured. For Cry1Aa or Cry1Ac concentrations up to 100 nM, LDH activity was proportional to

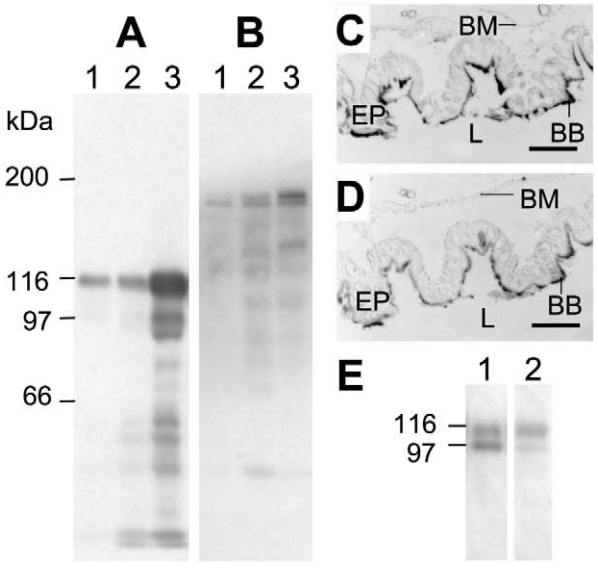

Fig. 2. Expression of APNs and BtR175 in the midgut of *B. mori* larvae. A,B: Immunoblotting of BBMV proteins using 2 nM anti-APN (A) or 3 nM anti-BtR175 (B) antibody. BBMV containing 20 μg total protein were prepared from the anterior (lane 1), middle (lane 2), or posterior regions (lane 3) of a fifth instar larva midgut, separated by SDS–PAGE, and immunoblotted as described in Section 2. C,D: Immunostaining of tissue sections from a third instar larva midgut. Posterior midgut sections were incubated with 2 nM anti-APN (C) or 3 nM anti-BtR175 (D) antibody and then treated as described in Section 2. BB, brush border; BM, basement membrane; EP, midgut epithelia; L, lumen. Bar = 80 μm. E: Quantitation of APNs in midgut BBMV. BBMV were subjected to SDS–PAGE and immunoblotting as described in Section 2. A control sample (1) containing 80 ng of purified 115-kDa APN (BmAPN1) and 120 ng of purified 100-kDa APN (BmAPN4) was used to quantitate the amount of APNs in a 5-μg BBMV sample (2) by comparing band intensities.

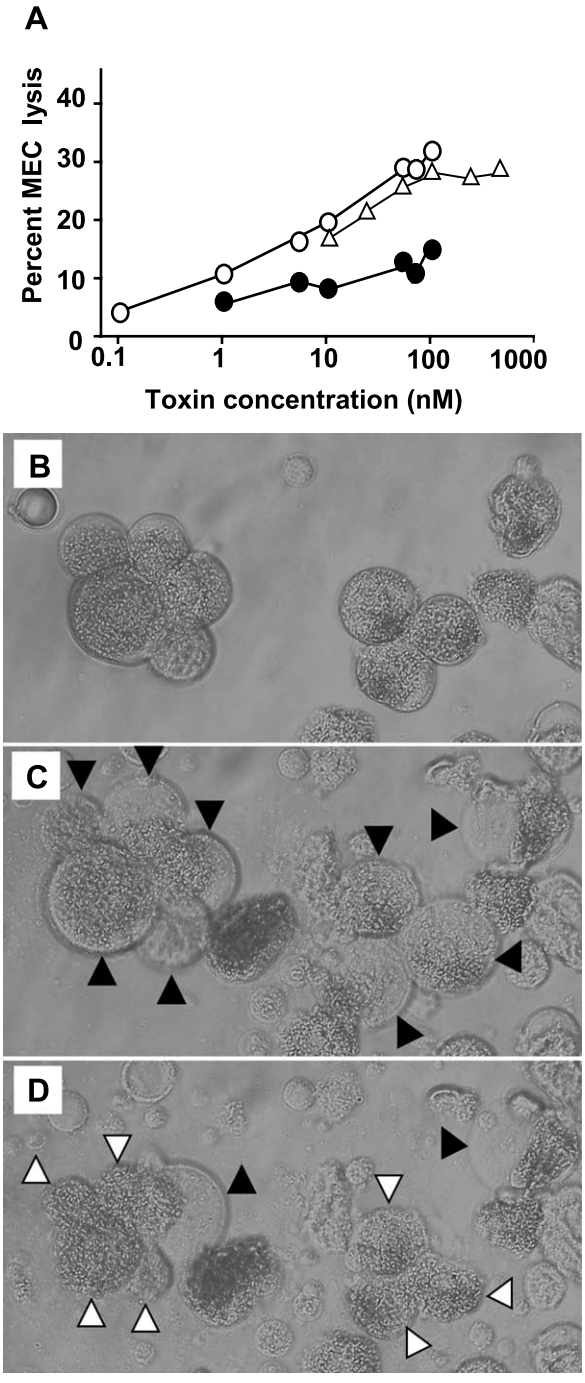

Fig. 3. Sensitivity of MECs to Cry1Aa and Cry1Ac and microscopic observation of Cry1Aa-treated MECs. A: Sensitivity of MECs to Cry1Aa and Cry1Ac. MECs from fourth instar *B. mori* larvae were incubated with Cry1Aa (open circles and open triangles) or Cry1Ac (filled circles), and the relative LDH release compared to a Triton X-treated control was calculated, as described in Section 2. Each data point shown is the average of four replicates. B–D: Microscopic observation of Cry1Aa-treated MECs. MECs were observed before (B), 40 min after (C), and 90 min after (D) Cry1Aa (100 nM) treatment. Black arrowheads indicate swollen cells and white arrowheads indicate ruptured cells.

the toxin concentration. For Cry1Aa concentrations from 100 to 500 nM, LDH activity did not increase with Cry1Aa concentration (Fig. 3A). At 100 nM Cry1Aa, LDH activity was 35% of the potential maximum, defined as the amount of LDH activity released upon addition of Triton X-100 (Fig. 3A). At 100 nM Cry1Ac, the LDH activity released from MECs was 13% of the potential maximum (Fig. 3A). By microscopic examination, columnar cells were estimated to comprise 57% of all MECs in fourth instar larvae. On exposure to 100 nM Cry1Aa, most of the columnar cells of the MECs became swollen within 40 min and burst within 90 min (Fig. 3C,D).

The roles of APNs and BtR175 in MEC sensitivity to Cry1Aa and Cry1Ac were assessed by treating MECs with anti-APNs and anti-BtR175 antibodies prior to toxin exposure. Pre-treatment of MECs with 1 μM anti-BtR175 antibody reduced the amount of LDH released by 1 nM Cry1Aa toxin by 60–73% ($P < 0.01$) (Fig. 4A,B). Pre-treatment with anti-BtR175 antibody reduced Cry1Aa-induced LDH release by a similar amount when Cry1Aa was at a low concentration (0.1 nM) (data not shown), but when high concentrations of Cry1Aa were used (100 nM), the anti-BtR175 antibody had no effect (Fig. 4C). On the other hand, pre-treatment of MECs with 1 μM anti-APN antibody had no noticeable effect on LDH release resulting from 1 (Fig. 4A) or 0.1 nM Cry1Aa (data not shown). Higher concentrations (up to 3 μM) of anti-APN antibody also had no significant effect on LDH release, and concurrent addition of anti-APN antibody with anti-BtR175 did not show any additive effect (Fig. 4A–D). When 5 nM Cry1Ac was used, MEC pre-treatment with 1 μM anti-BtR175 antibody reduced LDH release by 67% ($P < 0.01$), but pre-treatment with 1 μM anti-APNs antibody had no effect (Fig. 4D).

To assess the ability of the antibodies to inhibit Cry1Aa binding, Cry1Aa was added to EIA plates coated with the partial fragment of APN or BtR175 containing the Cry1Aa binding region with or without anti-APN or anti-BtR175 antibodies. Anti-APN and anti-BtR175 inhibited Cry1Aa binding by 95.4 and 99.0%, respectively (Fig. 4E,F).

4. Discussion

It has been suggested that in collagenase-dissociated MECs, only columnar cells are sensitive to Cry toxins, and that the relative susceptibilities of different insect species as determined by LDH assays using MECs are similar to, but not the same as, those determined previously by bioassays [36]. Consistent with this proposition, only columnar cells became swollen upon exposure to Cry1A toxins in our study (Fig. 3B–D). The similarity in relative susceptibility results determined by the two methods is further demonstrated here by the finding that the LC_{50} values of Cry1Aa and Cry1Ac for third instar larvae of the hybrid strain Kinshu × Showa were 1.9 and 174.5 μg/g diet, respectively (data not shown). Consequently, LDH assays using collagenase-dissociated MECs appear to be a physiologically relevant system for analyzing Cry toxin receptor function in the insect midgut.

The kinetics of the response of *B. mori* MECs to Cry1Aa and Cry1Ac (Fig. 3A) were similar to those of *Spodoptera exigua* and *S. frugiperda* MECs [36], but the extent of cell lysis was much lower. Only about 60% of the columnar cells in the fourth instar larvae of *B. mori* were lysed by 100 nM Cry1Aa. The extent of cell lysis did not increase when the Cry1Aa toxin concentration was raised to 500 nM. This seemingly low sensitivity to Cry1A toxins is most likely due to incomplete lysis of swollen cells during the time frame of

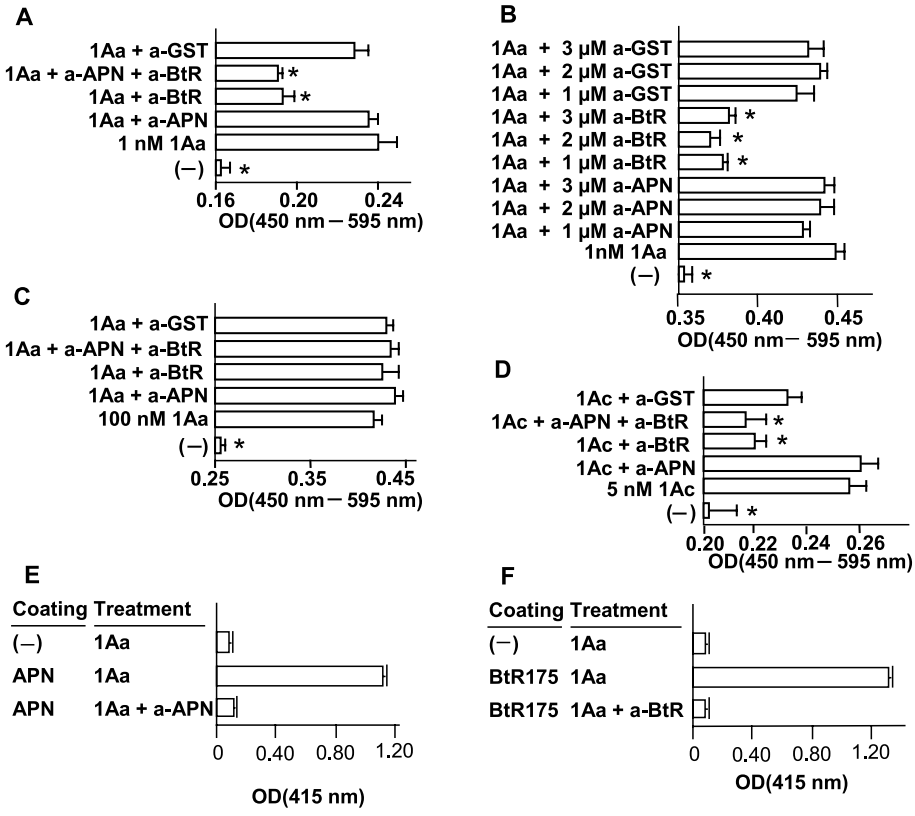

Fig. 4. Effects of anti-APN and anti-BtR175 antibodies on the sensitivity of *B. mori* MECs to Cry1Aa and Cry1Ac toxins. A–D: Effects of anti-APN and anti-BtR175 antibodies on MEC sensitivity to Cry1Aa (A–C) and Cry1Ac (D). MECs from fourth instar larvae were pre-incubated for 1 h with 1 μM (A,C,D) or 1–3 μM (B) anti-APN (a-APN), 1 μM anti-BtR175 (a-BtR), or 1 μM anti-GST (a-GST) antibodies. The cells were then incubated with 1 nM Cry1Aa (A,B), 100 nM Cry1Aa (C), or 5 nM Cry1Ac (D), and LDH release was measured as described in Section 2. Values shown are the mean (+S.E.M.) of four to six replicates. Asterisks denote a significant difference ($P < 0.01$) between the negative control (Cry1A toxin in the absence of pre-treatment with antibody) and experimental samples, as determined by Student's *t*-test. E,F: Assessment of the ability of antibodies to inhibit Cry1Aa binding. Biotin-labeled Cry1Aa (100 nM) was added to EIA plates coated with partial fragments of APN or BtR175 containing the Cry1Aa binding region with or without 1 μM anti-APN (E) or 1 μM anti-BtR175 (F) antibodies. After washing, bound Cry1Aa was detected with HRP-labeled streptavidin, as described in Section 2. Values shown are the mean (+S.E.M.) of six replicates.

the experiment, which is implied by our observation that the degree of swelling of the columnar cells varied from cell to cell under our experimental conditions (Fig. 3B–D).

Pre-treatment with anti-BtR175 antibody inhibited MEC lysis induced by 1 nM Cry1Aa (Fig. 4A,B) and 5 nM Cry1Ac (Fig. 4D) toxins by 60–73% and 67%, respectively. In addition, BtR175 was detected in all areas of the midgut (Fig. 2B,D) and Cry1Aa-sensitive MECs were distributed in almost all areas of the midgut (Fig. 1). Furthermore, BtR175 was not detected in the lateral cell–cell adhesion region of the MECs but rather in the apical microvilli (Fig. 2D). These results indicate that cadherin-like protein functions as a major functional receptor for Cry1Aa or Cry1Ac in *B. mori* larvae. HPT-1, a member of the cadherin superfamily, is expressed also in the epithelial microvilli in the human small intestine [38]. In *H. virescens*, disruption of the gene for cadherin-like protein (BtR-4) is a major reason for the resistance of strain YHD2 to Cry1Ac toxin [31]. Cadherin-like protein may act as a Cry1A toxin receptor in many lepidopteran insects including *H. virescens*.

Our findings would appear to be contradicted by a study in which cadherin-like protein purified from *B. mori* larvae was not confirmed to bind to Cry1Ac toxin [39]. However, larvae from different strains of *B. mori* may have cadherin-like proteins that differ in Cry1Ac toxin binding affinity, since the Cry1Ab sensitivity of different *B. mori* strains differs by up to 10 000-fold (K. Miyamoto, personal communication) and Cry1Ac is thought to have a loop 2 structure quite similar to that of Cry1Ab. Recently, the loop 2 regions of Cry1Aa and Cry1Ab were shown to bind to the *M. sexta* cadherin-like protein Bt-R1 [40]. This finding may support the possibility that both Cry1Aa and Cry1Ac bind to BtR175 in the *B. mori* midgut, since loop 2 in Cry1Ab and Cry1Ac toxins has the same amino acid sequence.

APN has previously been detected in the posterior region of the midgut of *M. sexta* [41], whereas we have detected APNs in microvilli from all areas of the *B. mori* midgut (Fig. 2A). BtR175 has been reported to comprise 0.01–0.02% of the total protein of the BBMV [37]. In contrast, we find that APNs comprise about 2% of the total BBMV protein. This amount of APN may be sufficient for it to function as a Cry toxin receptor. The 170-kDa APN from *H. virescens* can act as a functional receptor for Cry1Aa, Ab, and Ac on artificial phospholipid vesicles [27]. In addition, Cry1Ac binds to a 170-kDa protein, tentatively identified as APN, from the resistant *H. virescens* strain YHD2 [42]. This strain is 10 000 times less susceptible to Cry1Ac than a normally susceptible strain but still retains detectable sensitivity to Cry1Ac. These find-

ings support the possibility that APN is a minor physiological receptor for Cry1Ac toxin in the strain.

In our study, 1–3 μM anti-APN antibody did not inhibit the effect of Cry1Aa or Cry1Ac on collagenase-dissociated MECs (Fig. 4B,C,E), although 1 μM anti-APN antibody reduced Cry1Aa binding to an APN-coated EIA plate by 95% (Fig. 4F). The reported K_d values for binding of Cry1Aa toxin to APN and BtR175 from *B. mori* larvae are 75 and 2.6 nM, respectively [39]. Any demonstration of the possible function of APN in MECs may be hindered by the fact that MECs also have BtR175 on their surface. Even in the presence of anti-BtR175 antibodies, Cry1Aa binding to BtR175 may not be completely inhibited and may overshadow any effect of APN binding. Otherwise, it is possible that anti-APN antibody does not hide every APN molecule at the concentrations used (1–3 μM), since more APNs were present on the brush border than BtR175 (Fig. 2F). Consequently, our data fail to support, but do not preclude, a role for APN as a physiological receptor for Cry1Aa and Cry1Ac toxins in the midgut of *B. mori* larvae.

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