# The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin

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**Abstract The *Bacillus thuringiensis* CryIAa toxin binds a cadherin-like protein (BtR175) on the brush-border membranes of the *Bombyx mori* midgut columnar cells, which are the targets. By introducing the BtR175 gene with a baculovirus, *Spodoptera frugiperda* Sf9 cells expressed BtR175 protein on the cell membrane and became susceptible to the CryIAa toxin. The toxin bound the cadherin repeat adjacent to the membrane and made a pore that passed inorganic ions, causing the cell to swell and burst. This was not observed with a BtR175 variant lacking the toxin-binding site. This in vitro experiment mimicked the specific insecticidal action of the toxin in vivo well.**



*Key words:* Insecticidal protein; Receptor; Cell swelling; Cadherin-like protein; Pore formation; *Bacillus thuringiensis*

## 1. Introduction

Insecticidal crystalline inclusion (called crystal protein, or Cry; also, δ-endotoxin) of *Bacillus thuringiensis* is the most widely used microbial pesticide, harmless to vertebrates or plants. The protein inclusions undergo limited proteolysis in the larval intestinal lumen and release toxic fragments (toxins) [1,2], causing midgut epithelial cells to swell and burst [3–5].

Respective toxins from the various *B. thuringiensis* strains exhibit insecticidal specificities against lepidopteran (CryI), lepidopteran and dipteran (CryII), coleopteran (CryIII) and dipteran (CryIV) insects [6]. Diversities in the partial amino acid sequences and specificities of *B. thuringiensis* toxins were found even among lepidopteran-specific toxins of the CryI group (class). Toxins of the CryIA family (subclass) are highly toxic to *Bombyx mori* (silkworm), *Manduca sexta* (tobacco hornworm) and *Heliothis virescens* (tobacco budworm) but are non-toxic to insects in the *Spodoptera* genus [6]. On the other hand, CryIC toxins are highly toxic to *Spodoptera littoralis*, *Spodoptera exigua* and *Spodoptera frugiperda* [7]. Researchers have tried to explain their specificities in terms of the existences of the corresponding receptors in insect gut brush-bordor membranes [8,9] and several protein candidates [5,10–13] were nominated. However, different species of the receptor molecules were proposed for the same CryIAa toxin [5,13] and also for the CryIAc toxins [10,11,14–16]. Direct evidence of the involvement of the putative receptors in the cell killing action of the toxins is lacking so far.

The effects of the toxin on membrane permeability have been exhaustively examined by various assays using either midgut tissue, brush-border membrane vesicles, cultured cells or artificial lipid layers in search of a plausible explanation for why the cells swell and burst. The following hypotheses for the action of the toxin have been proposed: formation of non-specific pores in the cell membrane [17,18], an inhibitory effect on $K^+$-dependent amino acid transport [19], formation of cation-selective channels [20,21], activation of a calcium channel [22] and effects on $K^+/H^+$ exchange [23]. Which is the primary action of the toxin? Li et al. first determined the three-dimensional structure of a *B. thuringiensis* CryIII toxin [24]. They speculated that the insertion of the domain I of the toxin into cell membrane occurred to make a pore. By using a receptor-free planar lipid bilayer and the CryIAa toxin, cation-selective channels in multiple subconducting states were actually formed [21]. However, the question of whether a similar channel forms in a biological membrane and whether the channels of the small conductance can induce cell swelling remained to be answered.

We felt that there was a need for an in vitro assay system to examine the entire process of toxic action, including specific binding on cell membranes, induction of the membrane permeability change and making the cells swell. Sf9 cells, an established cell line derived from the fall armyworm *S. frugiperda*, are susceptible to CryIC toxins but not to CryIAa, CryIAb and CryIAc toxins [17]. This cell line is famous as an excellent protein factory for external genes introduced with a baculovirus and its susceptibility to *B. thuringiensis* toxins is very similar to that of *Spodoptera* insects in vivo. By introducing BtR175, a CryIAa toxin-binding protein of the *B. mori* midgut brush-border membranes [5], into CryIAa toxin-insusceptible cells, we show that BtR175 protein is an essential factor for the specificity determination and cytotoxic action of *B. thuringiensis* CryIAa toxin.

## 2. Materials and methods

### 2.1. B. thuringiensis toxin

The toxin was prepared by tryptic digestion of CryIAa protein from *B. thuringiensis sotto* T84A1 as described [1]. The accession no. of the sequence data in the GenBank/EMBL/DDBJ databases is AB026261.

### 2.2. Cells and cultivation

*S. frugiperda* Sf9 cells (ATCC 1711-CRL) were grown at 27°C and pH 6.3 in Sf-900 II SFM serum-free medium (Gibco BRL) supplemented with 0.8% fetal bovine serum.

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*Abbreviations:* FITC, fluorescein isothiocyanate; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid

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*2.3. Cloning procedures with Autographa californica nuclear polyhedrosis virus (AcNPV, a baculovirus)*

The 5148 bp coding sequence and the following 140 bp 3′-untranslated region of BtR175 precursor cDNA [25] (accession no. AB026260 in the GenBank/EMBL/DDBJ databases) were cloned into a pFastBac1 plasmid at the cloning sites downstream from the polyhedrin promoter and then, the 5′-untranslated region was shortened to 107 bp. AcNPV bacmid recombined with the plasmid was constructed by using the Bac-to-Bac Baculovirus Expression System (Gibco BRL). C-terminally truncated variants of BtR175 were generated by replacing the downstream segment of the *Sma* I site (see Fig. 3) with the DNA fragments shortened by restriction enzymes and attached with oligonucleotides (5′-CTAATTAATTAA-3′) containing stop codons in three frames. BtRΔCR5-6 and BtRΔCR9 were generated by deleting with *Cla*I and *Sal*I, respectively. N-terminally truncated variants were generated by ligating DNA fragments encoding C-terminal regions in frame with a fragment encoding N-terminal 32 amino acid residues including a signal sequence.

*2.4. Protein expression and in vitro binding assay*

BtR and its variants in the cells were made soluble with phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer, pH 7.4, containing 145 mM NaCl) containing 1% Triton X-100 at 4°C for 30 min. Insoluble substances were removed by centrifugation at 27 000×*g* for 30 min twice and the second supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting assay with anti-BtR175 antibody and in vitro binding assay. For the binding assay, the supernatant was incubated at 4°C with 5 μg of the toxin for 4 h and then, rabbit anti-toxin IgG was added and incubation was continued for an additional 16 h. The immunoprecipitate was collected by centrifugation at 1500×*g* for 10 min. Size markers used were rabbit muscle myosin (205 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa) and hen egg ovalbumin (45 kDa).

*2.5. Immunocytochemistry*

Cells attached to a cover glass were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 30 min and washed with PBS and then, the remaining glutaraldehyde was neutralized in PBS containing 0.5 M $NH_4Cl$. Fixed cells were incubated with rabbit anti-BtR175 IgG purified by a protein G affinity column and then with anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (FITC).

*2.6. Electrical measurements*

Membrane currents of Sf9 cells were examined by the whole-cell patch-clamp method [26]. A patch pipette was filled with an internal solution consisting of 120 mM KCl, 20 mM potassium hydroxide, 2 mM $MgCl_2$, 5 mM ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid and 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (pH 7.5). The experimental chamber was continuously perfused with an external solution consisting of 140 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$ and 5 mM HEPES (pH 7.5). Data were collected using the pCLAMP system (Axon Instruments, USA). To measure steady state membrane currents of the cell, a voltage ramp from −80 to +80 mV (164 mV/s) was applied every 10 s. Between each voltage ramp, the membrane potential was held at −40 mV. The toxin was applied by bath perfusion.

## 3. Results and discussion

BtR175 is a CryIAa toxin-binding glycoprotein on the brush-border membrane of the midgut columnar cell of *B. mori* larva, which is the target of the *B. thuringiensis* toxin [3–5]. It has nine extracellular cadherin repeats (CRs), a mem-

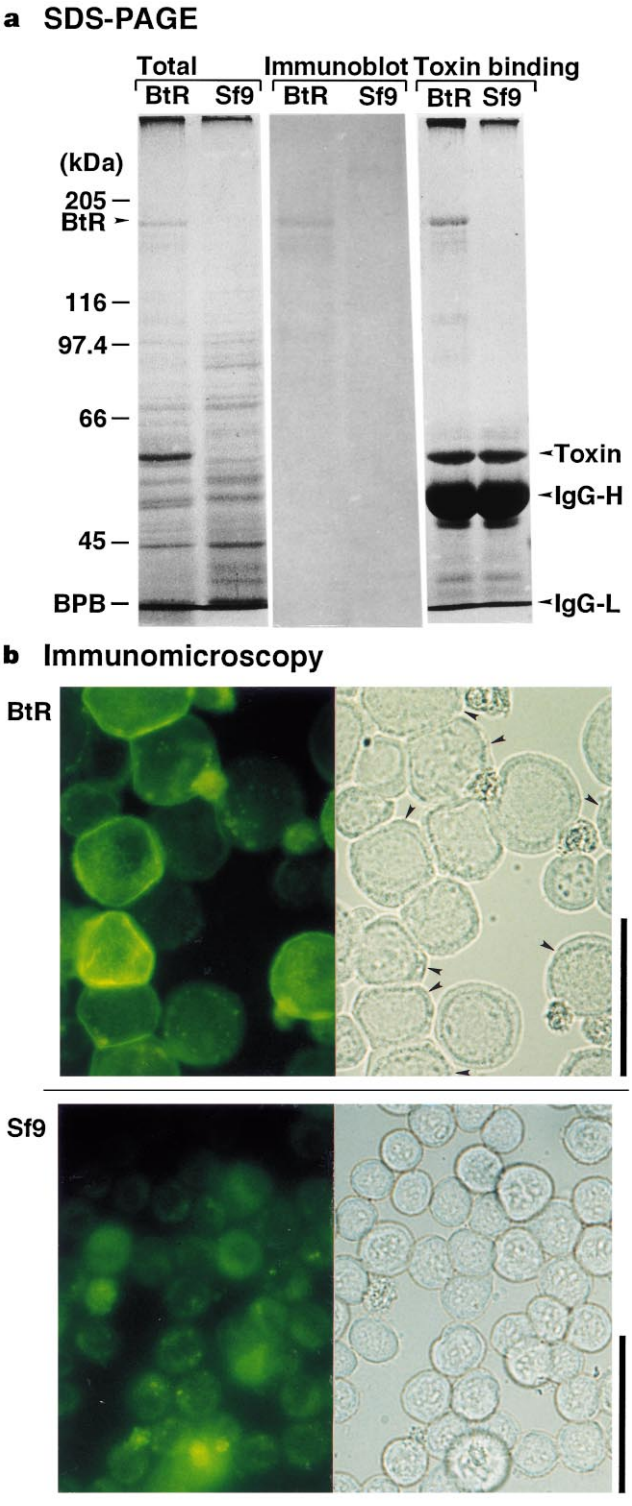


Fig. 1. Expression of *B. mori* BtR175 in *S. frugiperda* Sf9 cells by infection of a recombinant baculovirus, AcNPV-BtR175. (a) SDS-PAGE (7% gels) analyses of proteins made soluble with Triton X-100 from cells infected with AcNPV-BtR175 (BtR) or from control cells (Sf9). Total proteins were stained with Coomassie brilliant blue R-250. Immunoblot analysis was done with rabbit anti-BtR175 IgG and then with anti-rabbit IgG IgG conjugated with horseradish peroxidase. The toxin-binding assay was done by direct immunoprecipitation of toxin-BtR175 complex with polyclonal anti-toxin IgG. BPB indicates the position of BPB (bromophenol blue). IgG-H and IgG-L denote heavy and light chains of IgG, respectively. (b) Immunomicroscopic detection of BtR175 on the surface of cells infected with AcNPV-BtR175. Detection on the fixed cells was done with rabbit anti-BtR175 IgG and then with anti-rabbit IgG IgG conjugated with FITC. Arrowheads indicate the cells whose surfaces were stained. Bars = 50 μm.

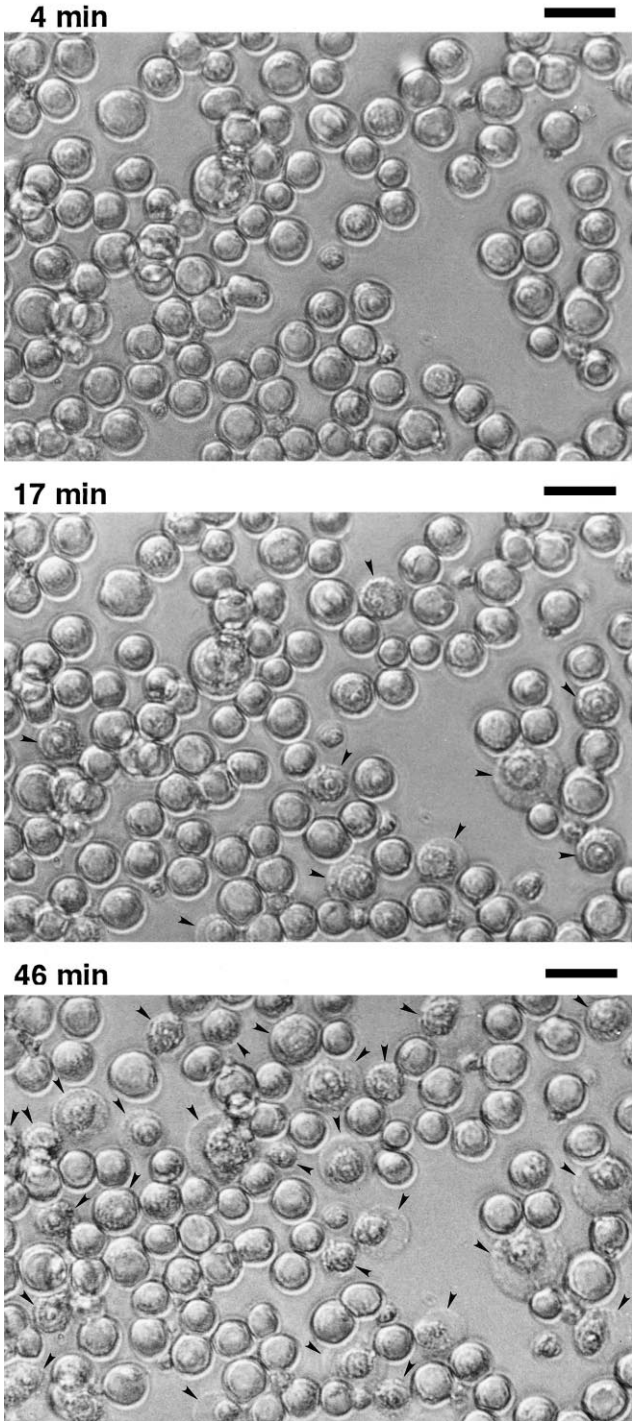


Fig. 2. Osmotic lysis of Sf9 cells infected with AcNPV-BtR175 by the *B. thuringiensis* CryIAa toxin. Sf9 cells infected with AcNPV-BtR175 were grown for 38 h and the cultivation medium was replaced by the medium containing 8 μg/ml of the CryIAa toxin. Times after administration of the toxin are indicated. Swollen or burst cells are indicated by arrowheads. Bars = 50 μm.

brane proximal region, one transmembrane region and a small cytoplasmic domain. Double-stranded DNA complementary to BtR175 mRNA was inserted into a site of AcNPV bacmid downstream from the polyhedrin promoter. The bacmids were used to transfect Sf9 cells with CELLFECTIN lipid reagent and recombinant baculovirus particles were harvested from the culture medium-infected fresh Sf9 cells for subsequent BtR175 expression. Cells infected with the recombinant viruses produced a new protein of an apparent molecular size of 175 kDa (it is invisible in Sf9 cells), which could react with anti-BtR175 antibody and the CryIAa toxin (Fig. 1a). Compared with Sf9 cells, virus-infected cells enlarged about 2-fold together with the nuclei within 36 h of cultivation (Fig. 1b). Virus-infected cells exhibited BtR175 on the cell membranes, while the cell membranes of uninfected Sf9 cells were not reactive with anti-BtR175 antibody (Fig. 1b). The CryIAa toxin caused BtR175-expressing Sf9 cells to swell (Fig. 2). The cytoplasm expanded, whereas its nucleus shrank and apparently lost its clear envelope structure. The morphological changes of the cells were observed within 15 min and the number of swollen cells increased for 45 min after the administration of the toxin. The CryIAa toxin had no detectable effects on either the shape or the growth of uninfected Sf9 cells (data not shown). The swelling of the infected cells in vitro is quite similar to that in the midgut columnar cells of the silkworm fed with the CryIAa toxin [3–5]. These results indicated that BtR175 of *B. mori* midguts gave Sf9 cells susceptibility to the CryIAa toxin and BtR175 was the very true receptor for the CryIAa toxin.

In order to determine CryIAa toxin-binding sites in BtR175, we produced 10 variants of BtR175 by deleting various parts (Fig. 3). The CRs 1–7, the last 103 amino acid residues of the membrane proximal region, the transmembrane region and the cytoplasmic domain of BtR175 were not required for the specific binding with CryIAa toxin. The CR8 domain of BtR175 was not enough for their interaction. As a consequence, the CryIAa toxin-binding site of BtR175 was found to be in a structure formed by the CR9 domain and the first 112 amino acid residues of the membrane proximal region. The CryIAa toxin caused swelling of the cells expressing a BtR variant, CR7-Cyt (Fig. 4). However, the cells expressing BtRΔCR9 were insusceptible to the CryIAa toxin, although the receptor variant was detected on the cell membrane. Thus, CryIAa toxin-susceptibility of Sf9 cells acquired by transfection of AcNPV-BtR genes is due to the receptor function of BtR175. The cells expressing CR8-Cyt were also killed by the CryIAa toxin (summarized in Fig. 3). The molecular size of the toxin is 66 kDa, while the size of the CR9 domain plus the membrane proximal region of the receptor is 36 kDa. A part of the toxin molecule bound to the receptor might be in contact with or inserted into the phospholipid bilayer of the cell membrane, although this is dependent on the orientation of the bound toxin against the cell membrane.

CryIAa toxin-induced swelling of Sf9 cells expressing BtR175 implies some disturbance of the osmoregulation of the cells. One plausible hypothesis is that the toxin forms a pore, which results in an abnormal ionic permeability of the cell. To examine the effects of the toxin on the permeability of the cell membrane, we next measured the membrane currents of Sf9 cells using a whole-cell patch-clamp. Under the present recording conditions, the infected Sf9 cells had a relatively high leakage conductance (the holding current at $-40$ mV was $> -50$ pA). In the cells expressing CR7-Cyt (eight/nine cells), the steady state currents evoked by a voltage ramp as well as the holding currents were greatly increased immediately after the application of the toxin (Fig. 5a). By contrast, the cells expressing BtRΔCR9 (three/three cells) did not show any appreciable responses (Fig. 5a). Fig. 5b shows the cur-

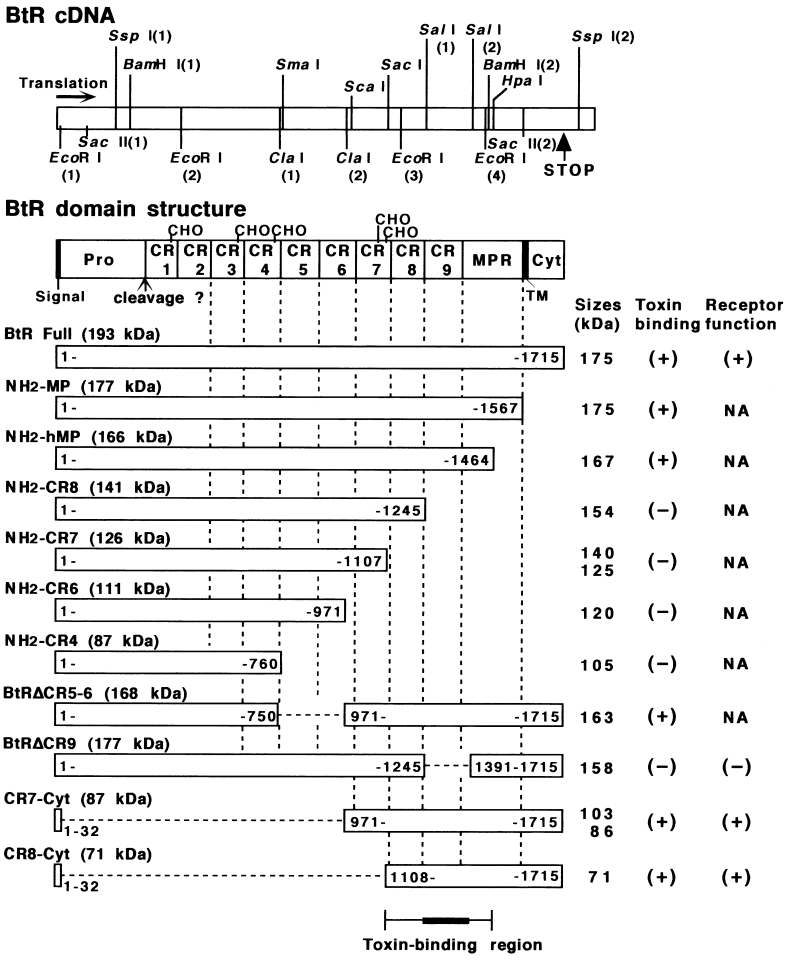


Fig. 3. CryIA toxin-binding and receptor activities of BtR175 variants. BtR175 cDNA encodes a 193.3 kDa preproprotein of BtR175. Its 5288 bp coding and 140 bp 3′-untranslated regions are shown with several restriction enzyme sites. STOP indicates the position of stop codon. BtR175 is a cadherin-like glycoprotein. Putative domains encoded by the cDNA are as follows: signal sequence, proprotein region (Pro), nine CRs, membrane proximal region (MPR), transmembrane region (TM) and cytoplasmic region (Cyt). CHO denotes putative carbohydrate chains from asparagine residue. BtR variants were prepared by deletion of various regions of the cDNA with restriction enzymes. Numbers in the boxes refer to the amino acid residues in the preproprotein. Sizes are apparent molecular weights of the products expressed in Sf9 cells. The toxin-binding assay was done as described in Fig. 1. The receptor function assay was done as in Fig. 1, Fig. 2 and Fig. 4. NA means not applied.

rent-voltage relationship of the cells expressing CR7-Cyt before and after the treatment with the toxin. The toxin-evoked currents were several 100 times larger than the endogenous currents of the cell in the whole voltage range tested. The reversal potential of the evoked current was close to 0 mV. If the currents are due to fluxes of inorganic cations (that is, the influx of extracellular cations in the minus range of the membrane potential), the formed pores do not distinguish $Na^+$, $K^+$ and $Ca^{2+}$ from each other.

By introducing an exogenous receptor into a toxin-insusceptible cell line, we here established an in vitro assay system for *B. thuringiensis* insecticidal toxins. Our experiments showed that the CryIAa toxin made a pore allowing for inorganic ions to pass through the cell membrane whose character was similar to that of the toxin-forming cation-selective channel in the receptor-free planar lipid bilayer [21]. Our results also indicated that the receptor protein was essential to the pore formation in cell membrane and the resulting cell swelling, unlike the case of the channel in the lipid bilayer. Further identification of molecular species of the receptors corresponding to the various *B. thuringiensis* toxins, the molecular mechanism of the pore formation and the following processes leading to the cell swelling remained to be elucidated. This assay system may result in a better understanding of toxins' mode of action.

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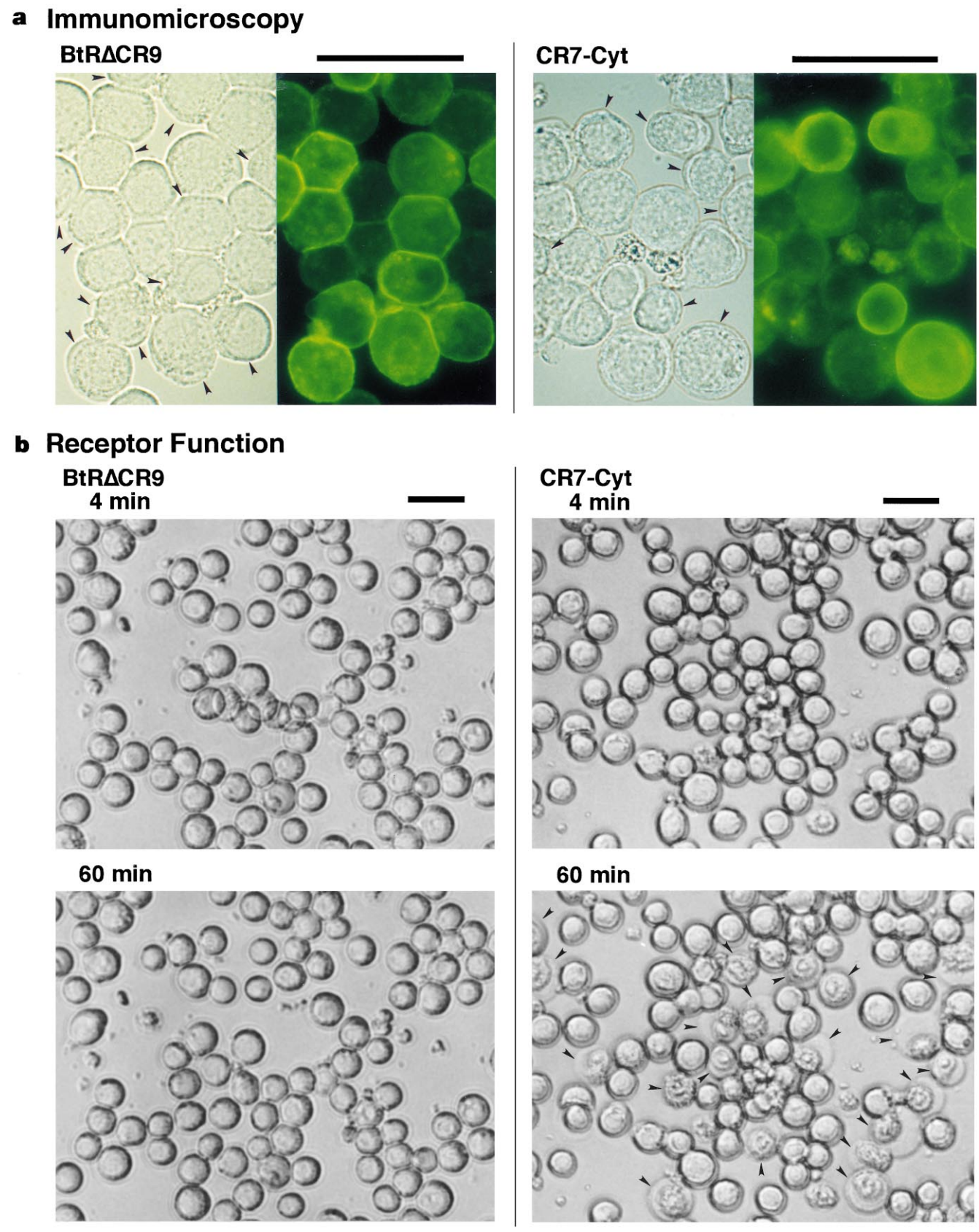


Fig. 4. Receptor activities of BtR variants. (a) Immunomicroscopic detections of BtR variants on the surface of cells infected with AcNPV-BtR variant. See Fig. 3 regarding the variants, CR7-Cyt and BtRΔCR9. Detection methods are the same as in Fig. 1. Positive cells are indicated by arrowheads. (b) Osmotic lysis of Sf9 cells expressing BtR variants. Methods are the same as in Fig. 2. Times after administration of the toxin are indicated. Swollen or burst cells are indicated by arrowheads. Bars = 50 μm.

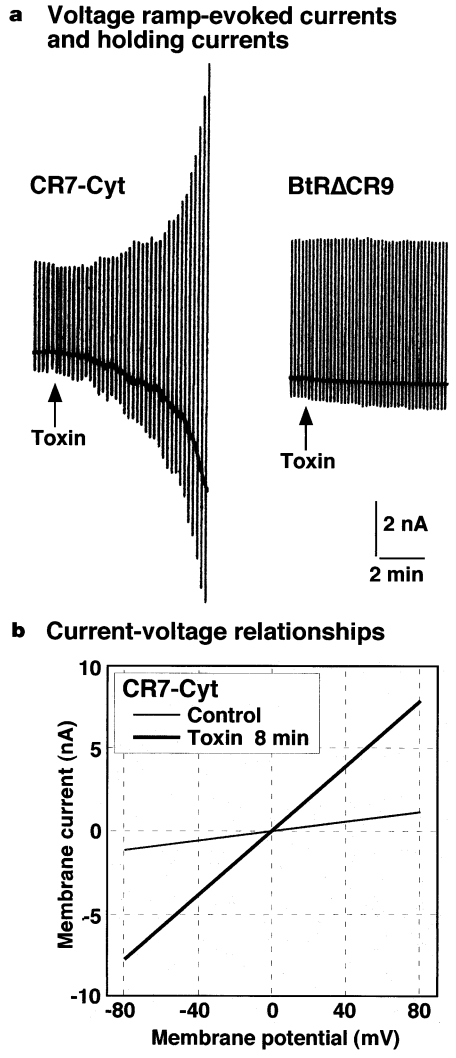


Fig. 5. Effects of the toxin on the membrane currents of cells infected with AcNPV-BtR variant. (a) Effects of the CryIAa toxin (20 μg/ml) on either the CR7-Cyt-expressing cell or the BtRΔCR9-expressing cell. Vertical deflections show membrane currents evoked by voltage ramps. (b) Current-voltage relationships of the cell expressing CR7-Cyt before and 8 min after the application of the CryIAa toxin. The same cell as shown in (a).

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