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Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors

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Abstract A purified, GPI-linked receptor complex isolated from *Manduca sexta* midgut epithelial cells was reconstituted in planar lipid bilayers. CryIAa, CryIAc and CryIC, three *Bacillus thuringiensis* insecticidal proteins, formed channels at much lower doses (0.33–1.7 nM) than in receptor-free membranes. The non-toxic protein CryIB also formed channels, but at doses exceeding 80 nM. The channels of CryIAc, the most potent toxin against *M. sexta*, rectified the passage of cations. All other toxin channels displayed linear current–voltage relationships. Therefore, reconstituted Cry receptors catalyzed channel formation in phospholipid membranes and, in two cases, were involved in altering their biophysical properties.

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Key words: Ion channel; Receptor; Bacterial toxin; Planar lipid bilayer; Surface plasmon resonance; *Bacillus thuringiensis*

1. Introduction

During sporulation, *Bacillus thuringiensis* (*Bt*), a Gram-positive soil bacterium, produces a large crystalline inclusion made of one or more insecticidal proteins highly specific towards agricultural and forestry pests, and several human and animal disease vectors [1–4]. Recently discovered *Bt* strains demonstrate activity against other invertebrates like protozoan pathogens, parasitic liver flukes and acarians [5]. Over 50 *Bt* genes have been sequenced and grouped into at least 12 different classes and subclasses based primarily on structural homology and insecticidal spectrum [6,7].

It is generally accepted that in target insects, following solubilization and activation of *Bt* crystals in the gut, the toxin disrupts the ion balance of midgut cells after interacting with specific cell surface docking molecules. The cell membrane is made permeable to small ions through the pore formation allowing a net uptake of ions into cells, followed by water, which results in cell swelling and eventual lysis (see [8,9] for reviews). Recent advances have been made which will significantly contribute to the elucidation of the molecular mode of action of *Bt* toxins. On the one hand, several groups have purified toxin-binding proteins from brush border membrane vesicles (BBMVs) of insect midgut cells. In *Manduca sexta*, a 120-kDa glycosylphosphatidyl inositol-anchored (GPI) aminopeptidase N (APN) and a 210-kDa cadherin-like protein were identified, which bound CryIAc and CryIAb, respectively [10–14]. More recently, a 106-kDa aminopeptidase was identified as a CryIC receptor in the same insect [15]. CryIAc-binding APN proteins have also been purified from *Lymantria dispar* [16], *Heliothis virescens* [17] and *Plutella xylostella* [18] BBMVs, but a recent report suggested that not all APN surface enzymes act as toxin-binding proteins [19]. On the other hand, the atomic structures of CryIIIA, a coleopteran-specific toxin, and CryIAa, a lepidopteran toxin, have been elucidated [20,21]. However, several important questions remain unanswered about (i) the nature of the interaction between activated toxins and their receptor(s), (ii) the way toxins partition into cell membranes and (iii) the architecture and the functional components of the pores they form in target cells.

CryIC triggers ion channel activity in live cells [22,23]. This and several other Cry toxins also form ion-selective pores in receptor-free planar lipid bilayers (PLB) at high doses (over 80 nM) [21,24–28]. Ion permeation properties of CryIAc, CryIC and CryID toxins are dramatically altered in PLBs to which membrane vesicles bearing appropriate receptors have been fused [29,30]. Under these conditions, toxin doses 100 to 1000-fold lower were sufficient to induce channel activity.

While BBMVs provide an enriched population of toxin receptors, they are a source of various other proteins and lipids that are naturally found in midgut apical membranes, which constitutes a complicating factor in interpretating results of channel reconstitution studies. When purified under non-denaturating conditions, the 120-kDa CryIAc-binding APN forms a tight aggregate with four to five other GPI-anchored proteins and possibly lipid-containing molecules [31]. This complex, upon treatment with phosphatidylinositol phospholipase C, loses its lipid moieties and yields the soluble 115-kDa APN receptor for CryIAc [32]. When reconstituted in proteoliposomes the complex catalyzed CryIAc-induced permeability to rubidium ions over 1000-fold, demonstrating that toxin binding promotes its partition into the membrane [11]. Therefore, this aggregate provides an excellent tool for investigating postbinding receptor-toxin interaction in planar lipid bilayers.

In the present study, using a proteoliposome fusion procedure, we reconstituted the *M. sexta* receptor complex in PLBs to (i) investigate its role on *Bt* toxin channel formation and biophysical properties, and (ii) determine if toxin recognition correlates with a decreased dose requirement for channel formation. We demonstrate that receptors reconstituted in phospholipid membranes have a profound influence on both protein insertion efficacy and channel characteristics of *Bt* toxins active against *M. sexta* in vivo.

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2. Materials and methods

2.1. Receptor purification

M. sexta eggs were purchased from Carolina Biological Supply (Burlington, NC, USA) and larvae reared to 5th instar on artificial diet (Southland Products, Lake Village, AK, USA). BBMVs were prepared as reported [31] using the $MgCl_2$ precipitation method of Wolfersberger et al. [33]. BBMVs (10 mg protein) were solubilized with 2 ml 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) in Buffer A (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF) on ice for 30 min. The sample was centrifuged at $100000 \times g$ for 1 h at 4°C. The supernatant was chromatographed in Buffer A containing 0.2% CHAPS on a Sephacryl 300 column (1.5×45 cm Pharmacia). Fractions showing absorbance were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein amounts were measured by Bio-Rad or BCA protein assay (for samples containing detergent) using bovine serum albumin as a standard. SDS-PAGE was performed according to Laemmli [34]. ^{125}I-CryIAc ligand blotting was performed as described in Lu and Adang [31]. After gel electrophoresis, proteins were electrophoretically transferred to nitrocellulose filters. Filters were blocked with 5% dry milk in TBS-T (20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 0.1% Tween-20) for 1 h, followed by three washes with TBS-T. For ligand blotting, filters were bathed with ^{125}I-labeled toxin (2.3×10^6 cpm, 0.14 nM). After 3 h incubation at room temperature with gentle shaking, filters were washed three times (10 min per wash) with TBS-T, blotted dry, and exposed to X-ray film at −80°C.

2.2. Binding experiments

The conditions used in this study are similar to those described elsewhere [35]. Briefly, binding of Cry toxins to the purified *M. sexta* protein complex was monitored by a surface plasmon resonance (SPR) detector system (BIAcore, Pharmacia Biosensor, Piscataway, NJ). Approximately 3000 resonance units of the protein complex (dissolved as a 0.1 mg/ml stock solution in ammonium acetate 20 mM, pH 5.0), was amine-coupled to the activated carboxymethylated dextran surface of a CM5 sensor chip. The running buffer contained 150 mM NaCl, 3.4 mM EDTA, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4 to which 0.05% BIAcore surfactant P20 was added. The regeneration buffer contained 150 mM NaCl, 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11.0. A reagent flow rate of 5 μl/min was used during all experiments.

2.3. Proteoliposome preparation

A 4:1 (wt/wt) mixture of phosphatidylcholine (PC) and phosphatidylserine (PS) lipids (Avanti Polar Lipids, Alabaster, AL, USA) in chloroform was dried under nitrogen and hydrated at a concentration of 9.1 g/l in a buffer solution containing 150 mM NaCl, 1 mM EGTA, 10 mM HEPES, pH 7.4 and 0.15 g/l of receptor complex. The final protein:lipid ratio was therefore 1:60. Following a 5 min vortexing period, the mixture was freeze-thawed five times in a dry ice/ethanol bath. Large unilamellar vesicles were then produced by extrusion through 0.1 μm polycarbonate filters (Nucleopore, Costar, Cambridge, MA, USA) mounted in a dual, 250-μl syringe (Unimetrics, Shorewood, IL, USA) mini-extruder apparatus (Avanti Polar Lipids, Alabaster, AL, USA) [36].

2.4. Planar lipid bilayers

The general technique of *Bt* toxin reconstitution in planar lipid bilayer has been described in detail elsewhere [25]. Briefly, phospholipid membranes were formed from a 7:2:1 (wt/wt) lipid mixture of phosphatidylethanolamine (PE), PC and cholesterol (Avanti Polar Lipids, Alabaster, AL, USA). Typical membrane capacitance was 150 to 250 pF. After 10–15 min following membrane formation, proteoliposomes were added to the *cis* chamber to a final concentration of 90–130 μg/ml. Vesicle fusion to PLBs was promoted by the establishment of a transbilayer osmotic gradient by addition of 400 mM urea in the *cis* chamber, by the presence of calcium ions and by stirring [37]. Channel activity following addition of 0.33 to 85 nM of activated toxins to both bilayer chambers was monitored by step changes in the current recorded during holding test voltages across the PLB. All experiments were performed at room temperature (20–22°C) in buffer solutions containing either 150 or 450 mM KCl, 10 mM $CaCl_2$ and 5 mM TRIS, pH 9.0. Single channel currents were recorded with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Analysis was performed on a personal computer using pClamp and Axotape software (Axon Instruments, Foster City, CA, USA).

3. Results and discussion

3.1. Separation of M. sexta APN receptor complex

M. sexta BBMV proteins were fractionated by gel filtration on Sephacryl S-300 and fractions analyzed by SDS-PAGE and ligand blotting. A broad peak eluting near the void volume contained the 120-kDa CryIAc-binding protein (Fig. 1). Other BBMV proteins co-eluting with the 120-kDa toxin-binding protein include the 110-, 106-, 85-, 65-, 54- and 47-kDa proteins.

3.2. Control PLB experiments

Separate experiments were designed to develop appropriate conditions for proteoliposome fusion. PC:PS:ergosterol liposomes (64:16:20, wt/wt) were prepared as described in Materials and Methods for PC:PS liposomes. They were loaded with 10 μg/mg nystatin, a sterol-dependent oligomeric channel-forming antibiotic. Nystatin liposome fusion to the PLB was electrically monitored in the absence of *Bt* toxin and toxin receptor material by the appearance of current jumps or more often, current spikes, indicative of nystatin channels being formed in the PLB [38] (data not shown). Reliable fusion was best observed with 400 mM urea and 10 mM $CaCl_2$ in the *cis* chamber. In control experiments conducted under these conditions but without liposomes, PLB currents displayed by 85 nM CryIC or CryIAa were identical to those recorded under standard conditions [21,25] (data not shown). Once the conditions for liposome fusion had been set, nystatin and ergosterol were subsequently omitted from liposomes to

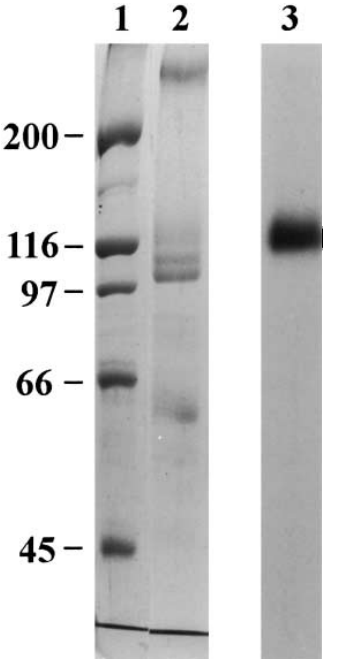

Fig. 1. SDS-PAGE and ligand blot analysis of toxin-binding protein in S-300 column fractions. Lanes 1 and 2 were stained with Coomassie blue. Lane 1 contains the molecular size markers and Lane 2 pooled 120-kDa toxin-binding protein fractions. Lane 3 is a ^{125}I-CryIAc ligand blot of the 120-kDa toxin-binding protein fractions.

prevent channel contamination and data interpretation difficulties. It was verified that in the absence of *Bt* toxin no channel activity was detectable for at least 1 h following fusion of receptor-enriched liposomes to the bilayer, demonstrating that the receptor complex does not possess operative endogenous ion channels.

3.3. Channel activity induced by CryIAc toxin

In receptor-free PLBs, CryIAc induced channel activity within minutes following addition of the protein to the *cis* bath and at doses similar to those reported elsewhere [26] (i.e. 85–170 nM) (Fig. 2). Under symmetrical KCl conditions and at pH 9.0, the current–voltage relation of the channel was linear with a conductance of 461 pS. The channel displayed several subconducting states (Fig. 2A). Under non-symmetrical conditions, i.e. with 450 mM KCl on the *cis* side and 150 mM KCl on the *trans* side of the bilayer, the channel conductance increased to 688 pS and the zero-current reversal potential shifted towards negative voltage by 22 mV, demonstrating selectivity of the channel to cations (Fig. 2B). The properties of CryIAc channels in PLBs were generally comparable to those of CryIAa [21]. However, the conductance of the channels was significantly less than that reported by Slatin et al. [24]. This may be related to differences in lipid composition of the membranes: in [24], PLBs were made of asolectin, asolectin and cholesterol, or diphytanoyl-PC, while in the present study we used a mixture of PE, PC and cholesterol. Since lipids from insect midgut bind *Bt* toxins (S.F. Garczynski, personal communication), it cannot be excluded that channel conductance may partly depend on the lipid environment of the pore.

In PLBs where the *M. sexta* receptor complex was reconstituted by proteoliposome fusion, channel activity was observed within minutes following addition of the toxin at doses as low as 0.33 nM (Fig. 3A). This concentration was about 250-fold less than that required to form pores in the absence of receptors. In the experiment illustrated in Fig. 3A, close observation of the fine structure of the channel current (5th trace from top, expanded time scale) revealed the presence of different channel conductances, or more likely, of subconducting states. The channels were not voltage-dependent and displayed kinetic properties similar to those found in receptor-free membranes. Fig. 3B is a current–voltage plot of the mean amplitude of the current flowing through the main open channel (Fig. 2A) for different applied voltages. The current–voltage relationship was not linear, indicating that in the presence of the receptor complex, CryIAc channels displayed current rectification. In this experiment, the conductance ranged be-

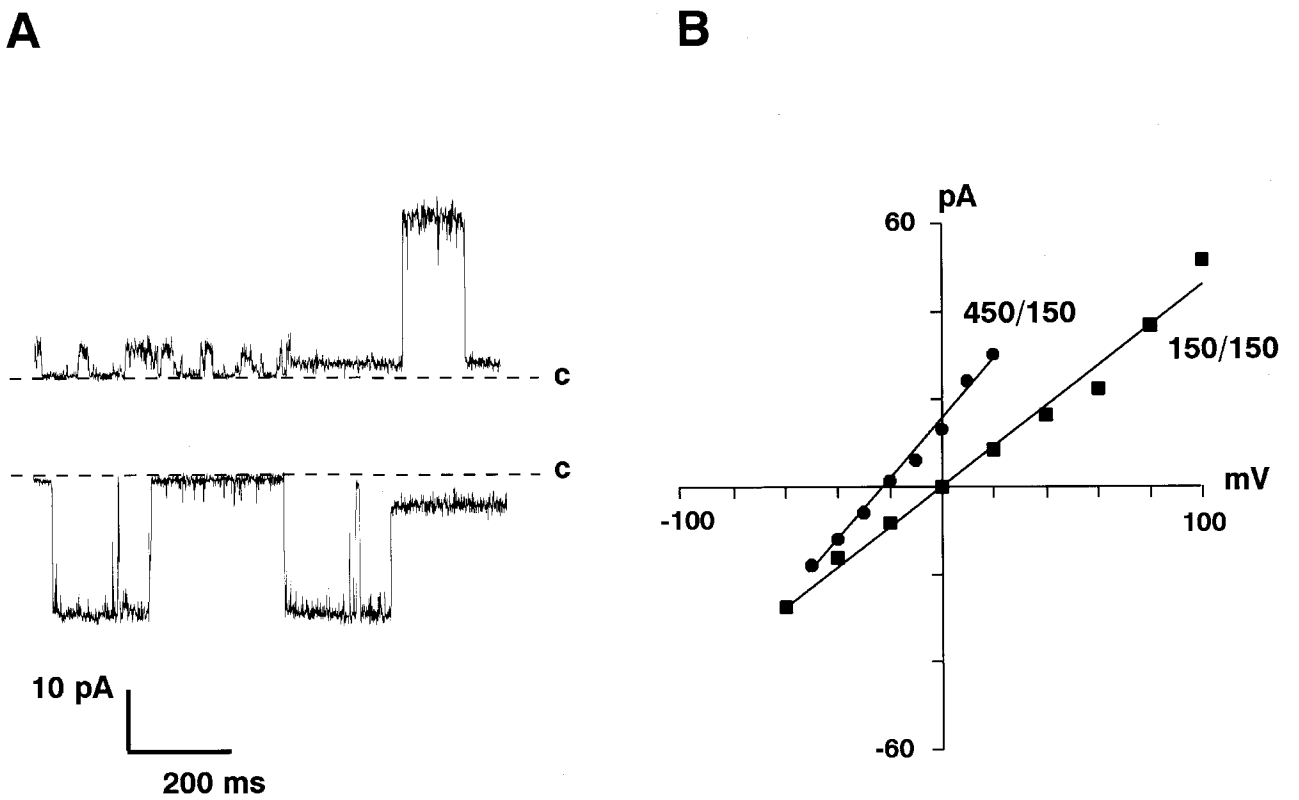

Fig. 2. CryIAc channels in receptor-free PLBs. (A) Current traces at +60 mV (upper trace) and −60 mV (lower trace) applied voltages under symmetrical 150 mM KCl conditions at pH 9.0. Dashed line and letter c indicate the current level corresponding to the closed state of the channel. Applied voltages are defined with respect to the *trans* chamber which was held at virtual ground potential. Positive currents, i.e. currents flowing through the planar bilayer from the *cis* to the *trans* chamber, are shown as upward deflections. The direction of current flow corresponds to positive charge movement. Note the presence of intermediate conductance levels corresponding to substrates. Data are representative of three similar experiments conducted at toxin doses in excess of 80 nM. (B) Current–voltage relations obtained from current data of experiment illustrated in panel A under symmetrical KCl conditions (filled squares), then under non-symmetrical conditions (450 mM KCl *cis*, 150 mM KCl *trans*, filled circles). In this particular experiment, the channel conductance (principal conducting state) was 461 pS under symmetrical conditions and 688 pS under non-symmetrical conditions. Note that under these conditions, the curve was shifted to the left by 22 mV. Data points were fitted by linear regression. Under 150/150 mM KCl conditions, the conductance of CryIAc reconstituted in receptor-free PLBs was 457 ± 13 pS ($n = 3$, mean ± standard error of the mean, S.E.M.).

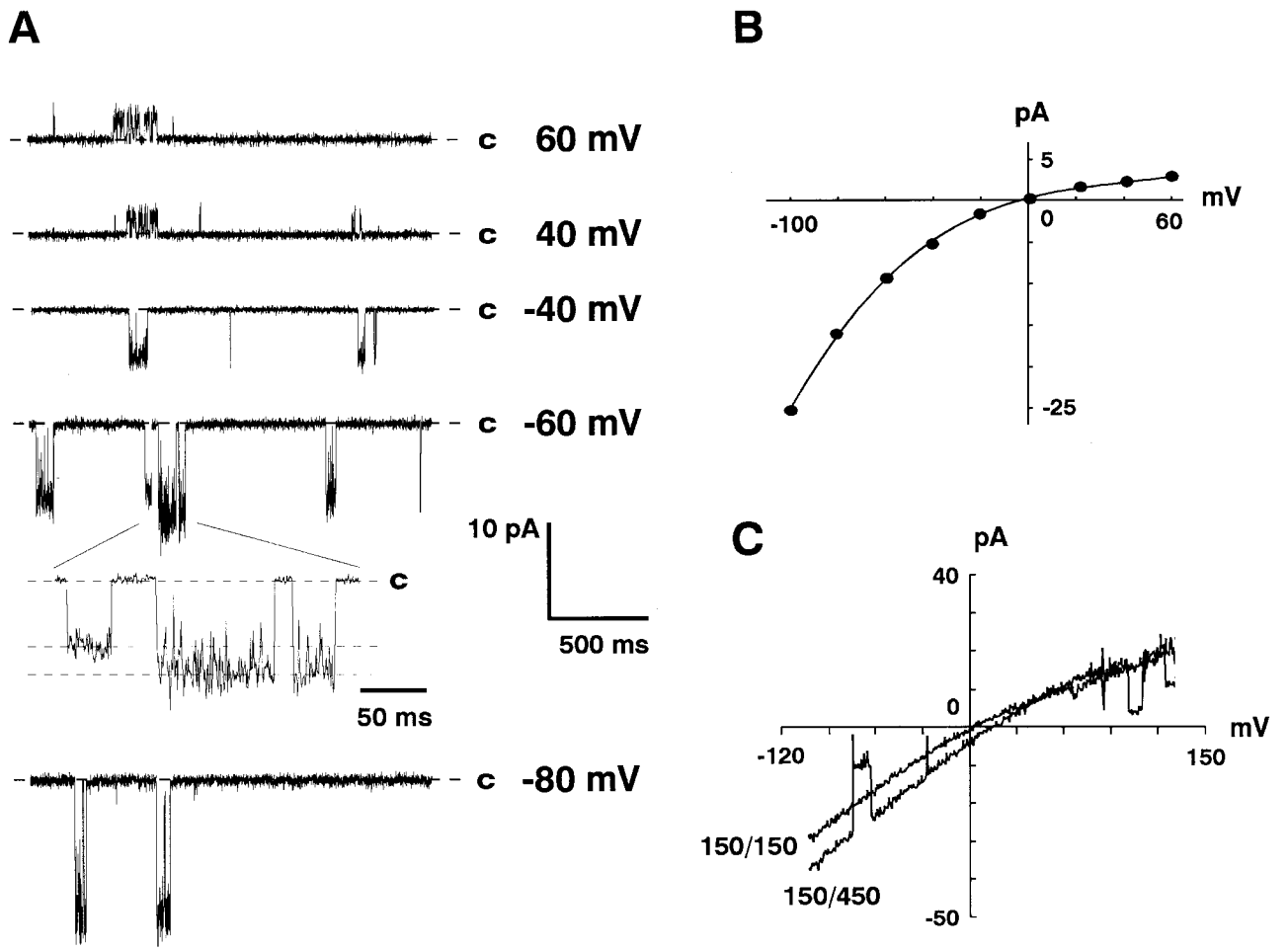

Fig. 3. CryIAc channels in PLBs containing the *M. sexta* receptor complex. (A) Current traces recorded at various applied voltages under symmetrical 150 mM KCl conditions at pH 9.0. Dashed line and letter c indicate the current level corresponding to the closed state of the channel. The 5th trace (from the top) is an expanded time scale representation of the fine structure of the channel current recorded at −60 mV. Data are representative of eight similar experiments conducted at toxin doses comprised between 0.33 and 0.5 nM. (B) Current–voltage relations obtained from current data of experiment illustrated in panel A. The curve was fitted through data points using a natural cubic spline algorithm. Maximum conductance: 438 pS, $V<0$; minimum conductance: 27 pS, $V>0$. (C) Instantaneous current–voltage relations of CryIAc channels recorded under symmetrical (150/150 trace) and non-symmetrical (150/450 trace) conditions. A 20 mV/s ramp was applied to the bilayer. The 150/450 trace was shifted to the right by approximately 18 mV, as expected from a cation-selective channel with 150 mM KCl on the *cis* side and 450 mM KCl on the *trans* side. Note the occasional closures of the channel (upward current jump at negative voltages and downward jumps at positive voltages). Under 150/150 mM KCl and with the receptor reconstituted in PLBs, the maximum conductance of CryIAc was 432 ± 32 pS for one voltage polarity, and the minimum conductance was 27 ± 5 pS for the opposite voltage polarity ($n=8$, mean ± S.E.M.).

tween 27 pS at positive voltages and 438 pS at negative voltages (Fig. 3B). Ion selectivity was investigated by recording channel current in response to voltage ramps under various KCl concentration gradients across the bilayer. In the experiment illustrated in Fig. 3C, a 20-mV/s, positive-going voltage ramp was applied to the membrane which was initially under symmetrical 150 mM KCl conditions (150/150 trace). The second trace (150/450 trace) corresponded to the current recorded after the KCl concentration was raised to 450 mM in the *trans* chamber. This experiment showed that the instantaneous current–voltage relation was shifted to positive voltages, demonstrating that in the presence of toxin receptors the channels were selective to cations. Interestingly, this voltage ramp experiment also showed that channels remained mostly open for the duration of the test ramp, suggesting that rectification did not originate from voltage polarity-dependent substrate occupancy. Finally, considering that all experiments were conducted in the presence of 10 mM $CaCl_2$, it can be concluded that the channels are not blocked by this divalent ion.

In two experiments, the 115-kDa APN, which serves as a CryIAc receptor in *M. sexta* and lacks a GPI-anchor [11,31,35], was co-sonicated with liposomes and subsequently fused to PLBs. Channel activity was observed upon addition of low doses (3–8 nM) of CryIAc, but without current rectification or reduction in channel conductance, compared to CryIAc activity in membranes enriched with the receptor complex (data not shown). It cannot be excluded that lipids remained associated with the purified 115-kDa protein (S.F. Garczynski, personal communication), which would have promoted its incorporation (or adherence) to liposomes. However, the fact that channels were similar to those recorded in receptor-free bilayers suggests that while the 115-kDa receptor catalyzed channel formation, the APN did not appear to in-

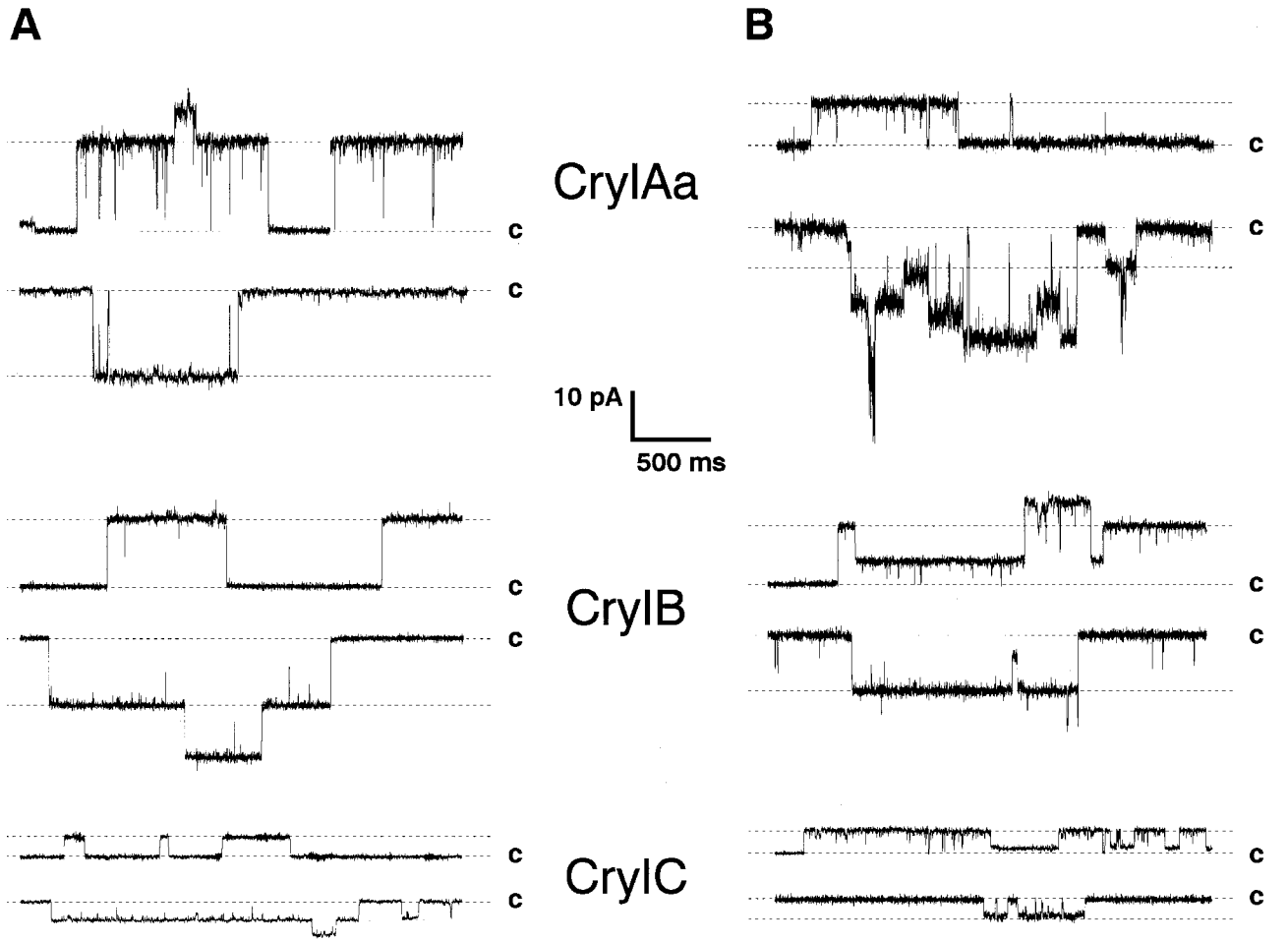

Fig. 4. CryIAa, CryIB and CryIC channels in receptor-free PLBs (A) and in PLBs into which the *M. sexta* receptor complex was fused (B). Currents were recorded with +40 (upper traces in pairs) and −40 mV (lower traces) applied voltages. The conductances of the principal channels were as follows: CryIAa, 450 pS and 200 pS, CryIB, 350 pS and 300 pS, and CryIC, 90 pS and 100 pS, in receptor-free and receptor-enriched PLBs, respectively. Data are representative of three experiments for CryIAa, two experiments for CryIB and two experiments for CryIC.

teract functionally with the channels formed by CryIAc under these conditions.

Our results are consistent with those from previous studies in other laboratories showing that CryIAc strongly catalyzed ion transport in the presence of receptor-rich membranes. In PLBs to which *M. sexta* [30] or *Spodoptera frugiperda* [29] BBMVs had been fused, very low doses of CryIAc or CryIC induced channel activity. Similarly, in liposomes in which the *M. sexta* receptor complex was reconstituted, permeability to rubidium ions was significantly increased by nanomolar doses of CryIAc toxin [11]. However, under these conditions, it was reported that the toxins formed very large pores in the nanosiemens range [29,30]. In our experiments, the interaction between the toxin and the receptor complex was different, as demonstrated by the fact that the channel conductances were not larger than those produced in receptor-free PLBs and that the channel current displayed rectification, a feature that has never been reported before in either receptor-free bilayers or BBMV-enriched membranes.

3.4. Channel activity induced by other Cry toxins

Among *Bt* toxins, CryIAc is the most active toxin against *M. sexta* larvae, followed by CryIAa, CryIAb and CryID, while CryIC and CryIE are about ten-fold less potent and CryIB being non-toxic [6,12,39,40]. The possibility that in vivo toxicity and channel properties may be related was further investigated by conducting experiments with CryIAa, CryIB and CryIC. Previous work has shown that CryIAa and CryIC form channels in receptor-free PLBs [21,25]. As illustrated in Fig. 4A, similar channel activity was induced by CryIB in phospholipid membranes. The conductance of the channel was around 350 pS under symmetrical 150 mM KCl conditions, the current–voltage relation was linear, the channel was cationic and subconducting states were occasionally observed (data not shown). Fig. 4B shows current records from experiments conducted with CryIAa, CryIB and CryIC in PLBs in which the *M. sexta* receptor complex was incorporated. Like CryIAc, CryIAa formed channels at extremely low doses (0.33 nM). However, contrary to CryIAc, the current–voltage relation of the channel was linear (not shown). Furthermore, CryIAa channel conductance was reduced when the complex was present (205 pS, compared to 450 pS in receptor-free bilayers). Although CryIC channels displayed similar properties with or without the receptor complex, it took 50-fold less toxin (1.7 nM) to trigger activity in receptor-enriched membranes than in pure phospholipid bilayers. Finally, CryIB channels were only recorded at toxin concentrations in excess of 80 nM with the presence of reconstituted receptors

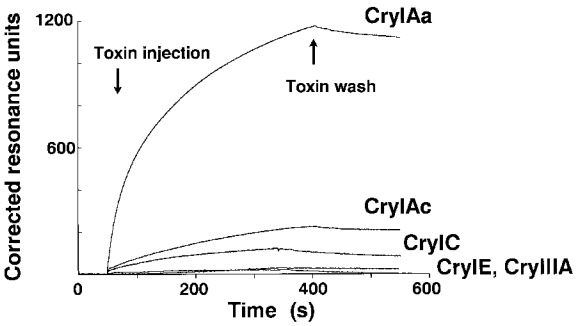

Fig. 5. Composite binding curve of Cry toxins. Various toxin concentrations were injected over a surface of immobilized receptor complex. The accumulation of toxin mass on the sensor chip surface was recorded as resonance units versus time [35].

not affecting the channel properties. From these experiments, it can be concluded that the presence of the receptor complex in PLBs catalyzed channel formation by CryIAa and CryIC, two *M. sexta* bioactive toxins but did not assist incorporation of CryIB, which is not toxic against the tobacco hornworm. Furthermore, the reduction observed in channel conductance when CryIAa was reconstituted in the presence of the receptor complex suggests that there was a functional interaction between the toxin and the receptor complex, possibly related to a higher occupancy rate for a subconducting state. Therefore, this interaction appears to be of a different nature from that observed with CryIAc, because no rectification was observed in current–voltage relations.

3.5. Kinetic binding of Cry toxins to M. sexta receptor complex

Different Cry toxins were injected over a surface of immobilized receptor complex to assess their binding capability by SPR. Fig. 5 shows that 500 nM CryIAa, 500 nM CryIAc and 400 nM CryIC, three proteins that are toxic to *M. sexta*, bound to the receptor complex, while 1.5 μM CryIE, which is poorly active against the tobacco budworm [40], and 1.5 μM CryIIIA, a coleopteran *Bt* toxin, displayed little or no SPR response. However, it was not possible to accurately determine the respective affinity of the toxins to the receptors, since they were tested on a mixture of binding proteins and possibly lipids. Qualitatively however, these experiments demonstrated that only bioactive toxins bound to the receptor complex.

In SPR-binding experiments conducted with the 115-kDa, GPI-cleaved purified receptor, it was shown that this protein recognized CryIAa, CryIAb and CryIAc, but not CryIC [35]. While CryIAa and CryIAb bound to a single site, CryIAc interacted with two distinct sites [35]. In view of these results and our channel data, it is tempting to speculate that facilitated partitioning of CryIAa resulted from the binding of the toxin to its site on the 120-kDa, GPI-linked receptor, and that the receptor–toxin interaction was responsible for reduction of channel conductance, compared to that measured in receptor-free bilayers. The presence of two binding sites for CryIAc would assist toxin intercalation into the membrane and cause current rectification.

Using a purified *M. sexta* receptor complex reconstituted in artificial phospholipid membranes, we demonstrated clearly that in addition to binding, some of the bioactive *Bt* toxins interacted functionally with their receptors to form channels with altered biophysical properties. These changes may be related to the toxin adopting a different conformation in the presence of the receptor. We have shown elsewhere that conformational changes are a prerequisite for toxin integration into lipid bilayers [41]. They may also reflect structural interactions involving parts of the receptor molecule contributing to the channel architecture and functional properties.

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