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Ion Channels Induced in Planar Lipid Bilayers by the *Bacillus thuringiensis* Toxin Cry1Aa in the Presence of Gypsy Moth (*Lymantria dispar*) Brush Border Membrane

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Abstract. The apical brush border membrane, the main target site of *Bacillus thuringiensis* toxins, was isolated from gypsy moth (*Lymantria dispar*) larval midguts and fused to artificial planar lipid bilayer membranes. Under asymmetrical N-methyl-D-glucamine-HCl conditions (450 mM *cis*/150 mM *trans,* pH 9.0), which significantly reduce endogenous channel activity, trypsin-activated Cry1Aa, a *B. thuringiensis* insecticidal protein active against the gypsy moth in vivo, induced a large increase in bilayer membrane conductance at much lower concentrations (1.1–2.15 nM) than in receptor-free bilayer membranes. At least 5 main single-channel transitions with conductances ranging from 85 to 420 pS were resolved. These Cry1Aa channels share similar ionic selectivity with P_{Cl}/P_{NMDG} permeability ratios ranging from 4 to 8. They show no evidence of current rectification. Analysis of the macroscopic current flowing through the composite bilayer suggested voltage-dependence of several channels. In comparison, the conductance of the pores formed by 100–500 nM Cry1Aa in receptor-free bilayer membranes was significantly smaller (about 8-fold) and their P_{Cl}/P_{NMDG} permeability ratios were also reduced (2- to 4-fold). This study provides a detailed demonstration that the target insect midgut brush border membrane material promotes considerably pore formation by a *B. thuringiensis* Cry toxin and that this interaction results in altered channel properties.

Key words: *Bacillus thuringiensis* — Cry1Aa toxin — Lepidopteran larval midgut — Brush border membrane — Planar lipid bilayer — Gypsy moth

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Introduction

Bacillus thuringiensis is used worldwide as an effective biological control agent for many species of insects including agricultural and forest pests, and several vectors of human and animal diseases. The crystalline δ-endotoxins (also called Cry toxins) produced by this Gram-positive bacterium are known to act on the luminal brush border membrane of insect midgut epithelial cells by disrupting the permeability barrier after having been solubilized and protease-activated in the insect gut. The activated toxins first bind to specific, high-affinity receptors at the surface of the brush border membrane of midgut columnar cells and insert into the membrane to create aqueous pores (Schnepf et al., 1998). Pore formation disrupts the ionic gradients and osmotic balance across the luminal membrane and eventually causes the midgut epithelial cells to lyse (Knowles & Ellar, 1987).

Various activated Cry toxins, including Cry1Aa (Grochulski et al., 1995; Schwartz et al., 1997b), Cry1Ac (Slatin, Abrams & English, 1990; Smedley, Armstrong & Ellar; 1997; Schwartz et al., 1997a; Chandra et al., 1999), Cry1C (Schwartz et al., 1993; Racapé et al., 1997; Peyronnet et al., 2000a), Cry2Aa (English et al., 1994), Cry3Aa (Slatin et al., 1990) and Cry3B2 (Von Tersch et al., 1994), have the ability to form ion-permeable channels in receptor-free phospholipid vesicles and planar lipid bilayer membranes. However, the toxin concentrations needed to observe channel formation in artificial bilayer membranes are 100 to 1000 times higher than their in vivo insecticidal concentrations.

Epithelial midgut membrane receptors undoubtedly play an important role in the mode of action of *B. thuringiensis* toxins. Studies on the binding of many activated toxins to larval midgut brush border membrane vesicles have demonstrated that the presence of recep-

tor(s) for a specific toxin is essential for its toxicity (Hofmann et al., 1988a, b; Van Rie et al., 1989, 1990a). In addition, insect resistance to these toxins can, in many cases, be attributed to altered receptor binding properties or reduced binding-site concentration (Van Rie et al., 1990b; Ferré et al., 1991, 1995). In several lepidopteran insect species, evidence has been reported for the presence of different glycosylphosphatidylinositol-anchored aminopeptidases N and cadherin-like proteins that act as receptors for various Cry1A toxins (*see* Schnepf et al., 1998 for a review).

Toxins form channels far more efficiently in artificial lipid membranes into which midgut brush border membrane vesicles (English, Readdy & Bastian, 1991; Lorence et al., 1995; Martin & Wolfersberger, 1995) or a purified toxin receptor complex (Sangadala et al., 1994; Schwartz et al., 1997a) have been incorporated. Planar lipid bilayer studies have yielded somewhat conflicting conclusions, however, concerning the role of receptor proteins in the biophysical properties of toxin channels. For instance, while very large channels with conductances in the nS range were observed in studies using fused brush border membrane vesicles (Lorence et al., 1995; Martin & Wolfersberger, 1995), Cry1Ac formed channels with similar conductances in the presence or absence of its reconstituted 120-kDa aminopeptidase N receptor complex (Schwartz et al., 1997a).

Brush border membrane vesicles have the advantage of providing a full complement of toxin receptors and other proteins and lipids which could interact with the toxins and affect pore formation. They also possess a large number of endogenous channels, most of them being slightly selective for cations (Lorence et al., 1995; Peyronnet et al., 2000b). Interestingly, the activity of these endogenous channels can be significantly decreased by performing the experiments under N-methyl-D-glucamine-HCl conditions (Peyronnet et al., 2000b).

In the present study, we took advantage of this observation to investigate the interaction of Cry1Aa toxin with gypsy moth brush border membranes mechanically fused with planar lipid bilayers. Our results show that toxin receptors or other components of the apical membrane of insect midgut epithelial cells have a profound influence on both protein insertion efficacy and channel properties, including conductance and ionic selectivity.

Materials and Methods

Chemicals and Solutions

The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Salts, ethylenediaminetetraacetic acid (EDTA), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and *n*-decane were purchased from Sigma (St. Louis, MO).

Planar lipid bilayer experiments were conducted under asymmetrical conditions with buffered salt solutions composed of (in mM) 450/150 (*cis/trans*) N-methyl-D-glucamine-HCl (NMDG-Cl), 5 $CaCl_2$, 0.5 EDTA, 5 Tris-HCl (pH 9.0).

Cry1Aa Toxin Preparation

Cry1Aa toxin (from *B. thuringiensis* subsp. *kurstaki* HD-1) was trypsin-activated and purified by fast protein liquid chromatography as described elsewhere (Masson et al., 1989). The toxin was dialyzed extensively against water with continuous stirring until it precipitated. The precipitate was centrifuged and solubilized in 25 mM Tris-HCl pH 9.4 to prepare stock solutions (0.75–2 mg/ml). These were kept at −20°C and diluted daily to the appropriate final toxin concentration. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. Prior to use, the toxin solution was vortexed and sonicated during 30 seconds as a precaution to prevent possible toxin aggregation.

Insect and Brush Border Membrane Vesicle Preparation

Second-instar larvae of the European gypsy moth *Lymantria dispar* L. (Lepidoptera: Lymantriidae) were obtained from the insect-rearing facility of the Great Lakes Forestry Center (Natural Resources Canada, Sault Ste. Marie, Ontario, Canada) and reared at 18°C on a standard synthetic diet. Brush border membrane vesicles were prepared from isolated midguts of last-instar larvae (Peyronnet et al., 2000b) according to the $MgCl_2$ precipitation method of Wolfersberger et al. (1987). The vesicles were suspended in 10 mM HEPES/KOH (pH 7.5) to a final concentration of 15–20 mg membrane protein/ml, stored at −80°C and used within a month. Compared with the initial crude homogenate, the vesicle preparations were enriched 6- to 8-fold in alkaline phosphatase specific activity (Kelly & Hamilton, 1970).

Planar Lipid Bilayer Experiments

The procedure used to fuse vesicles to planar lipid bilayers has been described in detail elsewhere (Peyronnet et al., 2000b). Briefly, planar phospholipid bilayer membranes were formed with a 1:1 (wt:wt) mixture of POPE and POPC at a lipid concentration of 25 mg/ml in 99% *n*-decane. Membranes had a typical capacitance of about 250 pF and remained stable for several hours. Before vesicle fusion, bilayers were monitored under holding voltage conditions for more than 30 min to ensure that no contaminant-induced activity was present. Vesicles were then fused to the *cis* side of the bilayer by gently touching it with a 150 μm-diameter “finger plugger” dental probe (SDS Kerr, Orange, CA) previously dipped in the vesicle suspension (Denicourt et al., 1996). Proper fusion was evidenced by an increase in membrane conductance. Vesicle fusion was also favored by applying a transbilayer holding potential of −80 to −120 mV.

When the endogenous channel activity was sufficiently weak and stable for at least 30 minutes, trypsin-activated Cry1Aa (0.6 to 61.5 nM) was added directly to the *cis* chamber buffer, which was stirred using a small magnetic flea until toxin channel formation. If no change in this basal channel activity was observed within 30 minutes, more toxin was added to the *cis* chamber. For experiments done in the absence of fused vesicles, the toxin (2.15 to 500 nM) was added 30 minutes following bilayer formation. If channels failed to appear within 15 to 30 minutes,

the bilayer was ruptured and a new bilayer was immediately painted. All experiments were performed at room temperature (22 to 25°C).

Data Recording and Analysis

Holding voltages were applied to the *cis* chamber relative to the *trans* chamber, which was grounded. They were corrected for liquid junction potentials. Under asymmetrical NMDG-Cl conditions (450/150 mM *cis/trans*), the liquid junction potential, measured experimentally as described elsewhere (Laprade & Cardinal, 1983), was –13.8 mV (Peyronnet et al., 2000b). Single-channel currents were recorded with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 500 Hz and digitized off-line at 4 kHz as described elsewhere (Peyronnet et al., 2000b). Analysis was performed on a personal computer using a Digidata 1200 series interface, and Axotape and pClamp version 6.02 software (Axon Instruments).

Single-channel conductances were determined as follows. For each experiment, the current amplitude of the resolvable steps at each applied voltage was measured and averaged over 2–15 similar steps. Corresponding values obtained in different experiments were then averaged over the number of experiments in which they had been observed. The resulting data were plotted versus voltage to generate current-voltage relations from which the conductances (g) were derived as the slopes of the linear regressions on the data points. The zero-current reversal potential (V_R) of each channel was therefore the voltage for which the corresponding linear regression intersected the horizontal axis of the current-voltage plots. Channel selectivity to Cl^- over $NMDG^+$ was derived from the P_{Cl}/P_{NMDG} permeability ratios calculated using V_R values and the Goldman-Hodgkin-Katz equation (Hille, 1992). Voltage dependence of the channels, i.e., the dependence of their activity upon applied voltage, was derived from the overall open probability NPo calculated for each applied voltage as the ratio of the total time during which all N channels were open to the total recording time. NPo was used rather than single-channel open probability because the total number of channels in the bilayer was generally not known.

For recordings in which there were no direct transitions from the closed state, single-channel conductances corresponding to resolvable current steps, reversal potentials and permeability ratios were obtained as described above. In addition, the macroscopic current across the membrane was evaluated for each applied voltage and in each experiment as the mean value of the overall current over the length of the record. This current corresponded to the sum of the products, for each type of channel present in the membrane during an experiment, of the single-channel currents, the total number of channels of each type, and their probability to be open (Hille, 1992). Therefore, voltage dependence of the channels was simply related to the nonlinear nature of the macroscopic current plotted against voltage after correction for the macroscopic current resulting from brush border membrane endogenous channel activity.

Because of the multichannel nature of the recordings, kinetic analysis of the channels was not attempted. Conductances, reversal potentials, overall open probabilities and macroscopic currents are expressed as means ± SD.

Results

Effect of the Brush Border Membrane on Cry1Aa Channel Formation

The midgut brush border membrane of lepidopteran insects contains a variety of endogenous channels (Lorence et al., 1995; Peyronnet et al., 2000b) that may complicate the observation of toxin-induced channel activity. However, the endogenous activity was considerably reduced by performing the experiments in the presence of NMDG-Cl (Peyronnet et al., 2000b). Under these conditions, only small anionic channels were detected (Fig. 1*B*, trace T_0) and the macroscopic conductance of the bilayer was sufficiently low and stable to allow Cry1Aa-induced currents to be readily distinguished from those of these endogenous channels (Fig. 1*A* and 1*B*). As illustrated in Fig. 1*A,* the macroscopic current of the planar lipid bilayer membranes into which brush border membrane vesicles were incorporated increased sharply after a delay of several minutes following the addition of Cry1Aa to the aqueous phase on the *cis* side of the membrane. Afterwards, the membrane current continued to increase at a slower rate. Although no change in membrane current was detected during 30 minutes of exposure to 0.6 nM Cry1Aa, channel activity, in the presence of brush border membrane, was readily detected at toxin concentrations as low as about 1.1 nM (Fig. 2*A*). Above this concentration, the probability of detecting channel activity increased abruptly to a maximum of about 85–90%. The delay preceding the appearance of channel activity decreased rapidly as the toxin concentration was increased (Fig. 2*B*). In the absence of brush border membrane, channel activity also appeared after a delay of a few minutes (Fig. 2*B*), but was only detected in the presence of at least 100 nM Cry1Aa (Fig. 2*A*).

Cry1Aa Channel Properties in the Absence of Brush Border Membrane

Representative recordings of Cry1Aa channel activity observed in receptor-free bilayers are illustrated in Fig. 3*A*. Channel activity could vary from one experiment to another, but recordings were frequently characterized by long periods of silence interrupted by bursts of various single-channel events that could stay active for many seconds. In some cases, bursts were characterized by the superimposition of a few small channels as seen in the top trace of Fig. 3*A*. Some isolated and rapid single-channel events displaying different amplitudes could also be detected between bursts of channel activity. The channels displayed a variety of behaviors including rapid flickering and slow gating as seen in the bottom trace of Fig. 3*A*.

Analysis of data collected from 4 representative experiments led to the identification of at least 5 main conductance levels. The current-voltage relationships were linear with slope conductances varying from 11 to 52 pS (Fig. 3*B* and Table), indicating an ohmic behavior of the pores. The larger conductances were approximate multiples (2- to 5-fold) of the smaller 11 pS conductance, suggesting the oligomerization of small channel units. In

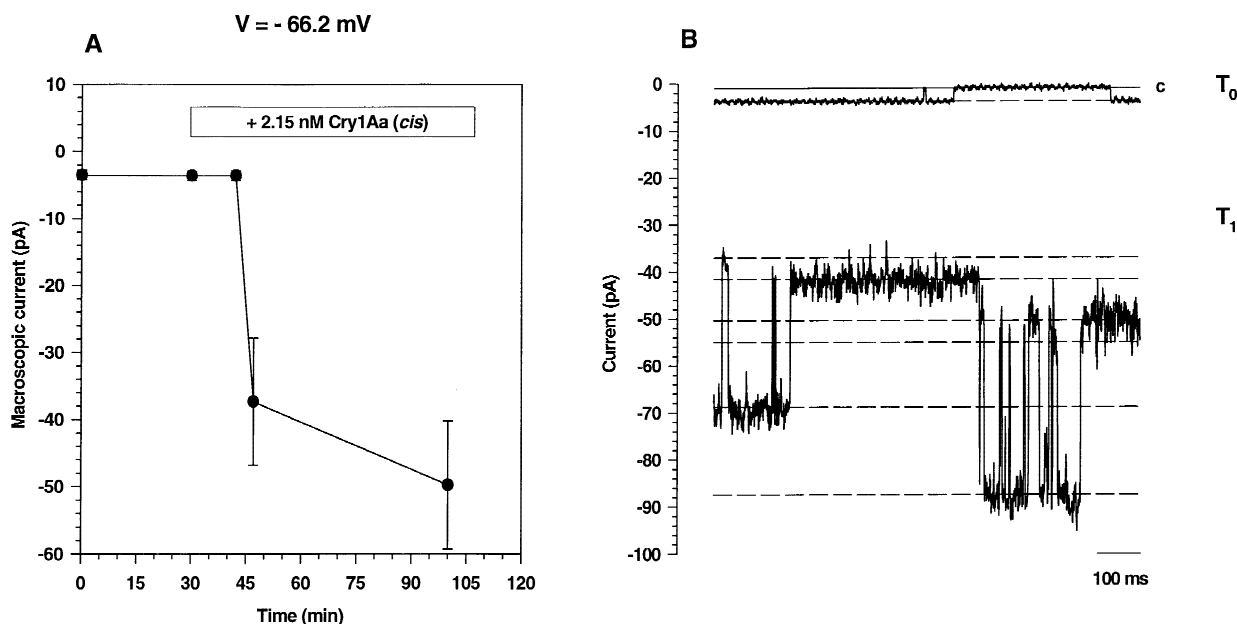

Fig. 1. Effect of Cry1Aa toxin on phospholipid bilayer membranes fused with gypsy moth brush border membrane vesicles. Bilayer membranes were formed in 450/150 mM NMDG-Cl (*cis/trans*), 5 mM $CaCl_2$, 0.5 mM EDTA and 5 mM Tris-HCl (pH 9.0), and fused mechanically with brush border membrane vesicles as described under Materials and Methods. (*A*) Macroscopic current flowing through the bilayer membrane held at –66.2 mV in a representative experiment. Error bars correspond to the SD of the macroscopic current measured over 60- to 90-sec typical recordings. (*B*) Current recordings in the absence (T_0) and presence (T_1) of Cry1 Aa toxin. T_0 is a representative segment of the channel current of the most commonly encountered endogenous channel in gypsy moth brush border membrane vesicles fused to a phospholipid bilayer, before Cry1Aa toxin addition and 30 minutes after fusion (*see* Peyronnet et al., 2000b for more detail). T_1 is a representative segment of a current recording obtained 70 minutes after addition of 140 ng of Cry1Aa (2.15 nM) to the *cis* side of the bilayer membrane. The different levels of current transitions are shown by the dashed lines. The letter *c* and the solid line indicate the closed state of all channels for both traces.

one experiment, a higher conductance of about 100 pS was detected at a very low frequency (*data not shown*). A closer observation of the current traces by filtering the recordings below 300 Hz suggested the presence of a low conductance (<10 pS) that was difficult to analyze in detail. The number of discrete conductance levels varied from one experiment to another.

Zero-current reversal potentials (V_R) ranged from +8 to +10 mV, demonstrating a higher permeability for Cl^- than for $NMDG^+$ since the Nernst potential for Cl,$^-$ under the conditions used, was +28.2 mV (Fig. 3*B* and Table). For each conductance level, the P_{Cl}/P_{NMDG} permeability ratio was calculated from V_R using the Goldman-Hodgkin-Katz equation (Hille, 1992). This ratio was approximately 2, indicating a small selectivity for Cl^- over $NMDG^+$ (Table). The selectivity of the smaller 11-pS channel was not determined, as its V_R value could not be precisely measured.

The overall open probability (*NPo*) of the Cry1Aa channels varied from 0.4 to about 1 with a tendency for a gradual decrease in *NPo* as the amplitude of the voltage was increased, suggesting that some of the channels may be voltage-dependent (Fig. 3). It remains possible, however, that the *NPo* reduction observed at both polarities could result from the insertion of channels in opposite directions relative to the plane of the membrane due to the fact that the membranes were ruptured and repainted during the experiments.

Cry1Aa Channel Properties in the Presence of Brush Border Membrane

Channel activity induced by Cry1Aa in planar lipid bilayers containing brush border membranes involved a macroscopic current with distinct large current levels indicating the insertion of numerous channels. Direct transitions from the different conductance levels to the closed state (where all channels are closed) were never observed. The channels were often very noisy and unstable due to their large number, making their characterization difficult. However, in 3 experiments (out of a total of 9), distinct single-channel events could be resolved. Current recordings representative of these experiments are shown in Fig. 1*B* (Trace T_1) and Fig. 4*A*. At least 5 main single-channel transitions were resolved with ohmic conductances ranging from 85 to 420 pS (Fig. 4*B* and Table). Each of them was observed in all three experiments analyzed. Close examination of the recordings showed small transitions of less than 80 pS

but, due to the current noise in these multi-channel recordings, they were impossible to analyze in detail. Interestingly, the larger conductances, like those observed in receptor-free bilayers, were multiples of the smallest resolved conductance level (85 pS). In the course of our experiments, we have not observed any increase in the amplitude of these conductances, suggesting that the oligomeric state of the channels remained constant with time.

Zero-current reversal potentials (V_R) ranged from +15 to +20 mV, demonstrating a higher permeability for Cl^- than for $NMDG^+$. P_{Cl}/P_{NMDG} permeability ratios ranged from 3.8 to 7.6, indicating that all channels have a small and relatively similar selectivity for Cl^- over $NMDG^+$ (Table).

Due to the complexity of the recordings, the voltage dependence and kinetic properties of these channels could not be analyzed directly. Therefore, voltage dependence was evaluated from the macroscopic current-voltage curves (Fig. 4*C*). The macroscopic conductance levelled off at voltages below −10 and above +30 mV. Since the single-channel conductances of the 5 main Cry1Aa channels were constant over the entire range of voltages tested (Fig. 4*B*), it appeared that the open probability of at least some channels was affected by the membrane voltage.

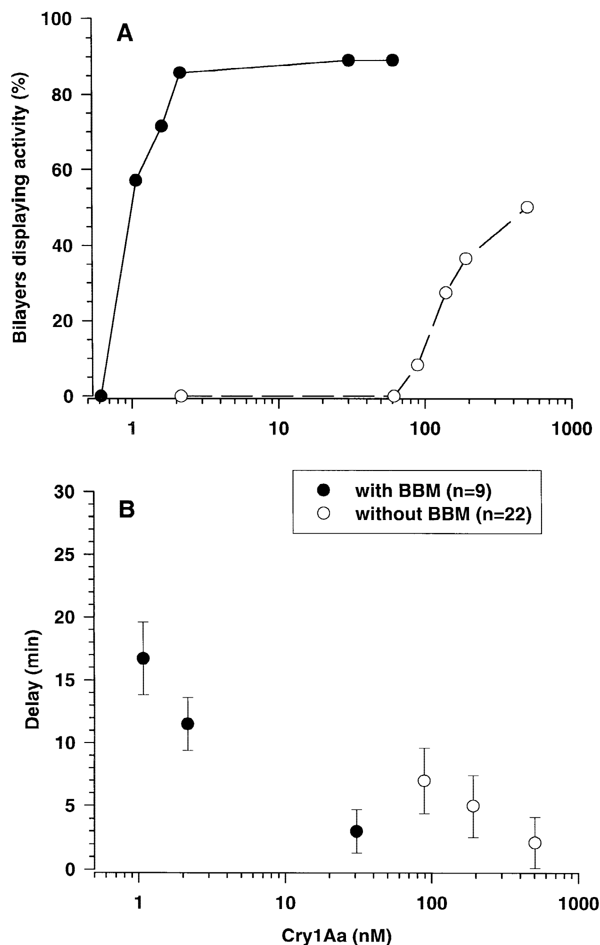

Fig. 2. Effect of Cry1Aa concentrations on the percentage of bilayers displaying toxin-induced channel activity (*A*) and on the delay preceding the onset of channel activity (*B*). Data were collected from bilayers to which brush border membranes were fused (filled circles, $n = 9$) or not (empty circles, $n = 22$). Other experimental conditions were identical to those described in the legend of Fig. 1. Cry1Aa (0.6 to 500 nM) was added directly to the *cis* side of the bilayers. The delay corresponds to the time separating the addition of Cry1Aa and the appearance of channel activity.

Discussion

The present study constitutes the first detailed comparison of the properties of the pores formed by a *B. thuringiensis* toxin in lipid bilayers in the presence and absence of fused midgut brush border membranes under otherwise identical experimental conditions. It clearly demonstrates that brush border membrane components not only greatly enhance the efficiency of Cry1Aa toxin channel formation, but also modify significantly the conductance and ionic selectivity of the channels.

Toxin Insertion Efficiency

Cry1Aa is among the most efficient *B. thuringiensis* toxins in killing gypsy moth larvae (van Frankenhuyzen et al., 1991, 1993; Lee et al., 1996a), in inhibiting transepithelial short-circuit current (Lee et al., 1996a) and depolarizing the apical membrane (Peyronnet et al., 1997) of their isolated midgut, and in permeabilizing vesicles prepared from this membrane (Peyronnet et al., 1996). While channel formation was extremely inefficient in receptor-free bilayers, as little as 1.1 nM Cry1Aa induced large macroscopic conductance increases, within 10–20 minutes, in bilayers to which brush border membrane vesicles were fused. This concentration was at least 100-fold less than that required to form pores in the absence of receptors and correlated well with in vivo lethal concentrations of this toxin (van Frankenhuyzen et al., 1991, 1993; Lee et al., 1996a). These results are consistent with those of earlier studies showing that nanomolar concentrations of toxin are sufficient to induce channel formation in planar lipid bilayers (Lorence et al., 1995; Martin & Wolfersberger, 1995; Schwartz et al., 1997a) or liposomes (English et al., 1991; Sangadala et al., 1994) to which midgut brush border membrane vesicles were fused or into which a purified receptor was reconstituted.

Toxin Channel Properties

B. thuringiensis toxin receptor function does not appear to be limited to binding the toxin and increasing its con-

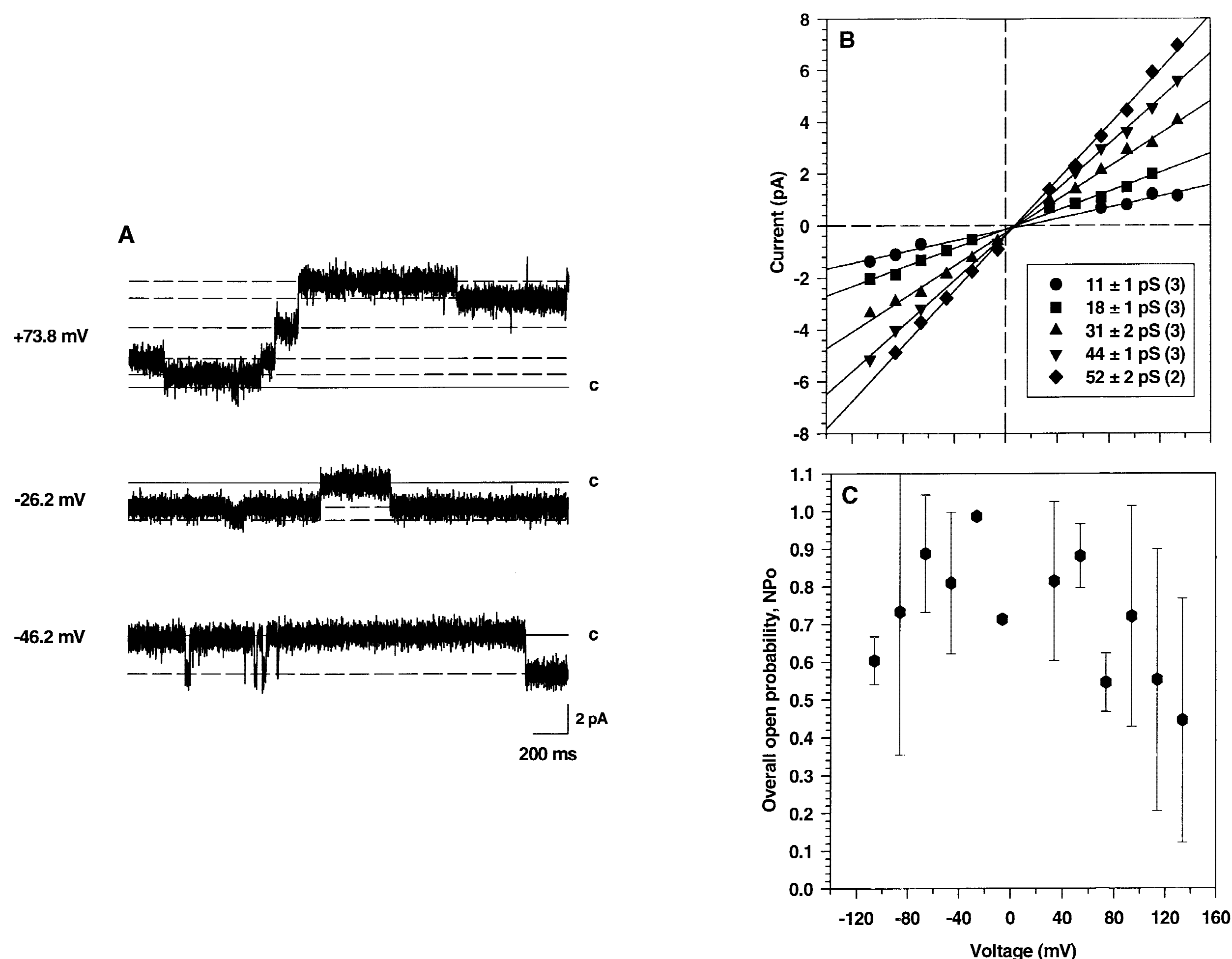

Fig. 3. Cry1Aa channels in receptor-free planar lipid bilayers. (*A*) Current traces at various holding voltages given at the left of the traces. The solid lines and the letter *c* at the right indicate the current level at which all channels were closed. The different levels of current transitions are indicated by dashed lines. (*B*) Current-voltage relationship of the main channels. For each experiment and for each voltage, current steps were measured and averaged over a number of similar steps. Each data point is the average of at most the number of experiments given in parentheses and error bars were omitted for clarity. For each experiment, the curves were fitted to the data points by linear regression. Data are derived from 4 experiments conducted at toxin concentrations in excess of 100 nM, with the number of experiments in which a given conductance level was detected in parentheses. (*C*) Overall open probability of the channels. *NPo,* the ratio of the total time spent in the open state by *N* distinct channels to the total recording time, was obtained for 30 to 120 sec current records.

centration in the vicinity of the membrane. The channels formed by Cry1Aa in brush border membrane-containing lipid bilayers had an 8-fold larger conductance and, for most channels, a 2- to 4-fold higher P_{Cl}/P_{NMDG} permeability ratio than those formed in receptor-free bilayers. The results of previous studies (Lorence et al., 1995; Martin & Wolfersberger, 1995) also suggest that the channels formed by *B. thuringiensis* toxins in lipid bilayers containing fused brush border membrane vesicles have a higher conductance than those formed in receptor-free bilayers. For instance, the conductance of Cry1C-induced channels ranged from 50 pS to 1.9 nS in bilayers containing brush border membranes (Lorence et al., 1995) and from 20 to 250 pS in the absence of receptor (Schwartz et al., 1993; Racapé et al., 1997; Peyronnet et al., 2000a).

These conductance values, as well as those previously reported for Cry1Aa channels formed in receptor-free bilayers (Grochulski et al., 1995; Schwartz et al., 1997a, b), are several fold higher than those measured in the present study. Although previous single-channel conductance measurements were carried out in the presence of KCl, a more highly conducting salt than NMDG-Cl, the specific conductance of equimolar solutions of these salts only differ by a factor of about 2 (*results not shown*). The conductance of Cry1Aa channels is therefore not proportional to the specific conductance of the electrolyte solutions bathing the lipid bilayer, indicating

Table. Conductance and ionic selectivity of the most common Cry1Aa-induced current transitions observed under asymmetrical NMDG-C1 conditions in the absence or presence of gypsy moth brush border membranes (BBM)

Without BBM (n = 4) Cry1Aa [100–500 nM]			With BBM (n = 3) Cry1Aa [1.1–2.15 nM]			g_{+BBM}/g_{-BBM}
g_{-BBM} (pS)	V_R (mV)	P_{C1}/P_{NMDG}	g_{+BBM} (pS)	V_R (mV)	P_{C1}/P_{NMDG}	
11 ± 1	n.d.*	n.d.*	85 ± 2	20 ± 1	7.6	7.7
18 ± 1	8 ± 3	1.9	158 ± 5	16 ± 2	4.3	8.8
31 ± 2	10 ± 3	2.3	240 ± 20	15 ± 3	3.8	7.8
44 ± 1	10 ± 4	1.9	330 ± 5	18 ± 2	5.6	7.5
52 ± 2	8 ± 3	1.9	420 ± 5	20 ± 1	7.6	8.1

Conductance (g) and reversal potential (V_R) values are given as means ± SD. Conductance values correspond to the slopes of the curent-voltage curves. Permeability ratio (P_{C1}/P_{NMDG}) values were calculated from the reversal potentials using the Goldman-Hodgkin-Katz equation (Hille, 1992). n indicates the number of experiments.
* Not determined.

that these channels are not ideal aqueous pores. Hence the smaller single-channel conductances measured in the presence of $NMDG^+$ probably result from a stronger interaction of this larger ion with the inner wall of the channel.

The conductance values reported by Martin and Wolferberger (1995) for the smallest current steps induced by Cry1Ac (2 and 13 nS, depending on pH) are considerably higher than those observed for *B. thuringiensis* toxin channels in any other lipid bilayer study. The use by these authors of an osmotic swelling procedure to promote fusion of brush border membrane vesicles to the planar lipid bilayer suggests that these very large conductance values could have resulted from the spontaneous fusion of vesicles, into which a large number of toxin channels were already formed, rather than from the insertion of toxin channels directly into the lipid bilayer. In contrast, the use of a mechanical fusion procedure (Denicourt et al., 1996; Peyronnet et al., 2000b) reduces considerably the probability of such fusion events by allowing measurements of toxin activity to be performed in the presence of a minimal number of vesicles remaining in the aqueous phase.

In a previous study (Schwartz et al., 1997a), a *Manduca sexta* receptor complex containing the 120-kDa glycosylphosphatidylinositol-linked aminopeptidase N, previously identified as a receptor for Cry1Ac (Sangadala et al., 1994), and which also recognizes Cry1Aa (Schwartz et al., 1997a), was reconstituted into planar lipid bilayers. The conductance of the main channels formed by Cry1Aa was about 2-fold smaller (200 pS) in the presence of this receptor complex than in receptor-free bilayers (450 pS). While channels formed by Cry1Ac in the absence of the receptor displayed linear current-voltage relationships, those formed in the presence of the receptor complex rectified strongly the passage of cations (Schwartz et al., 1997a). It should be pointed out, however, that in gypsy moth, while Cry1Ac binds to a 100-kDa aminopeptidase, Cry1Aa and Cry1Ab recognize a 210-kDa protein receptor (Valaitis et al., 1995; Lee et al., 1996b). On the other hand, the present study shows a large increase in conductance most likely attributable to the presence of membrane receptor(s), but no rectification of single-channel conductance. Taken together, these results indicate that the biophysical properties of the channels formed by *B. thuringiensis* toxins depend not only on the presence of receptor proteins and possibly other brush border membrane components, but also on the specific receptors with which the toxins interact.

Voltage-dependence of Cry1Aa Channels

The present study also shows that the channels formed by Cry1Aa are slightly voltage-dependent. In receptor-free bilayers, although channel activity was often triggered by voltages above ± 80 mV, a decrease in the overall open probability of the channels was also observed at voltage above ± 60 mV (Fig. 3*C*). In brush border membrane-containing bilayers, the observed rectification of macroscopic current (Fig. 4*C*) also strongly suggests a decrease in the open state probability of the Cry1Aa channels. In receptor-free lipid bilayers, a slight voltage dependence has been previously reported for Cry1Aa (Schwartz et al., 1997b) and Cry2Aa (English et al., 1994). Our results provide the first documented evidence of a voltage dependence for channels formed by a *B. thuringiensis* toxin in the presence of the midgut brush border membrane.

Toxin Oligomerization

In this study, Cry1Aa induced at least 5 main single-channel conductances, either in the presence or absence of gypsy moth brush border membranes. In both cases,

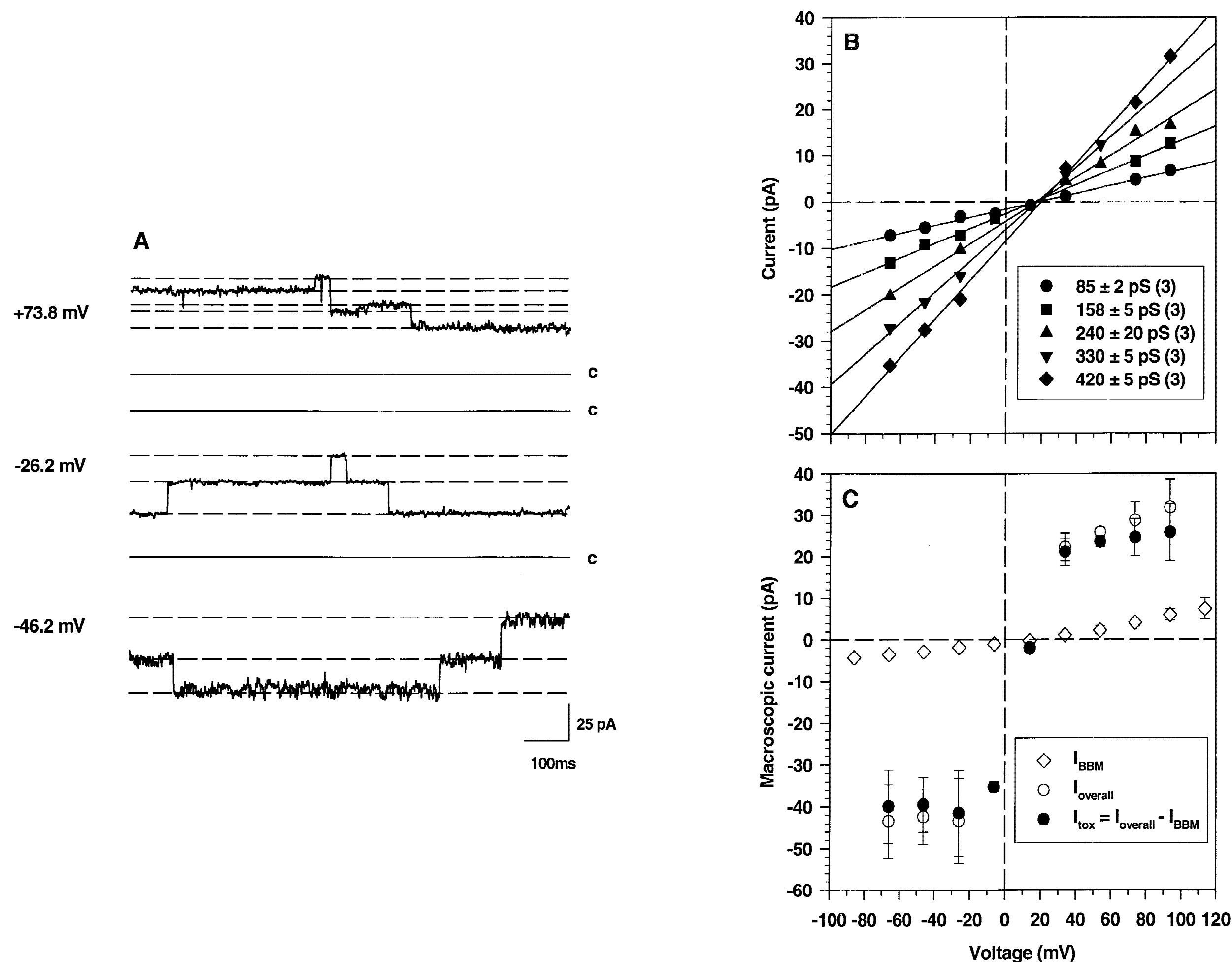

Fig. 4. Cry1Aa channels in bilayers containing *L. dispar* brush border membrane. (*A*) Representative segments of typical current traces recorded at various holding voltages given at the left of each trace. The solid line and the letter *c* at the right indicate the current level at which all channels were closed. The different levels of current transitions are indicated by dashed lines. (*B*) Current-voltage relationship of the main channels. Each data point was calculated as described in the legend of Fig. 3 and curves were fitted to the data points by linear regression. Results are derived from 3 experiments conducted at toxin concentrations of 1.1 to 2.15 nM. (*C*) Macroscopic currents through the bilayers before (I_{BBM}, empty diamonds) and after Cry1Aa addition ($I_{overall}$, empty circles) at various holding voltages. The macroscopic current induced by the Cry1Aa toxin (I_{tox}, filled circles) was obtained by subtracting I_{BBM} from $I_{overall}$. Results are derived from 2 experiments. For I_{BBM}, the SD values were smaller than the width of the symbols.

the channels displayed conductance levels corresponding to a multiple of the smallest observed current transition. Although it cannot totally be excluded that the lower conductances represented subconductance states, these findings suggest that the channels, like those formed by several other bacterial toxins (Lesieur et al., 1997), result from a multimeric assembly of a variable number of toxin molecules (Aronson, Geng & Wu, 1999; Schwartz & Laprade, 2000; Soberón et al., 2000). The presence of brush border membrane components is not necessary for toxin channel formation, but the fact that the conductance of the channels in brush border membrane-containing lipid bilayers was about 8-fold larger may indicate that native membrane material favored the formation of stable channels made of a larger number of toxin monomers. The nature of these multiple conductance levels remains unclear. They could result from the formation of various multimeric pore structures differing in channel diameter or from the synchronous gating of several identical channel units forming clusters within the lipid bilayer, as was reported for various types of ion channels (Krasilnikov & Sabirov, 1992; Larsen et al., 1996; Kaulin et al., 1998; Goudet et al., 1999). In agreement with the latter possibility, the ionic selectivity of the various Cry1Aa channels observed in artificial membranes was about the same, and though higher, it was also similar for the channels formed by the toxin in the presence of brush border membranes. Further work is presently being carried out to distinguish between these possibilities.

In summary, this study shows that native midgut brush border material affects the channels formed by Cry1Aa toxins and provides a detailed description of their biophysical properties. More work is needed to ascertain which midgut membrane components are responsible for these changes and what is the structural origin of these functional alterations.

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