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Lepidopteran-Specific Crystal Toxins from *Bacillus thuringiensis* Form Cation- and Anion-Selective Channels in Planar Lipid Bilayers

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Summary. Previous studies in our laboratory have shown that CryIC, a lepidopteran-specific toxin from *Bacillus thuringiensis*, triggers calcium and chloride channel activity in SF-9 cells (*Spodoptera frugiperda*, fall armyworm). Chloride currents were also observed in SF-9 membrane patches upon addition of CryIC toxin to the cytoplasmic side of the membrane. In the present study the ability of activated CryIC toxin to form channels was investigated in a receptor-free, artificial phospholipid membrane system. We demonstrate that this toxin can partition in planar lipid bilayers and form ion-selective channels with a large range of conductances. These channels display complex activity patterns, often possess subconducting states and are selective to either anions or cations. These properties appeared to be pH dependent. At pH 9.5, cation-selective channels of 100 to 200 pS were most frequently observed. Among the channels recorded at pH 6.0, a 25–35 pS anion-selective channel was often seen at pH 6.0, with permeation and kinetic properties similar to those of the channels previously observed in cultured lepidopteran cells under comparable pH environment and for the same CryIC toxin doses. We conclude that insertion of CryIC toxin in SF-9 cell native membranes and in artificial planar phospholipid bilayers may result from an identical lipid-protein interaction mechanism.

Key Words *Bacillus thuringiensis* · toxin · lipid bilayer · pH · ion channel

Introduction

The crystal produced by the gram-positive bacterium *Bacillus thuringiensis* (*B. thuringiensis*) during sporulation is a large protein that has been shown to be toxic to a number of insect larvae spanning three insect orders—lepidoptera, diptera and coleoptera [1, 4, 16]. In recent years, several toxin genes of this organism have been characterized and cloned. They have been assembled into 13 different classes or subclasses based on insecticidal spectrum and genetic data [16]. Major interest in this entomopathic toxin stems from its importance in agriculture and forestry, because of its action on specific target insects and their relatively limited resistance to it, and the well-developed fermentation and formulation technology allowing economical production of the toxin and widespread application with a minimum of environmental impact.

The exact mode of action of the insecticidal proteins produced by *B. thuringiensis* is not known [7, 9]. It is generally accepted that they disrupt the ion balance of the midgut cells of target insects after interaction with specific cell surface receptors. The cell membrane is made permeable to small ions through the formation of small, nonspecific pores allowing a net uptake of ions into cells, followed by water, which results in cell swelling and eventual lysis [19]. Different mechanisms of toxin action involving potassium channels [6, 29, 35] and interaction with sodium and potassium transporters [10, 13, 34] have been proposed.

Ionic channel formation by Cry toxins has been directly demonstrated by single channel current recordings using artificial lipid membranes [31]. In that study it was reported that CryIA(c) and CryIIIA toxins formed potassium-selective channels and that channel activity could only be observed at alkaline pH (above 9.5). Other toxins from *B. thuringiensis*, although structurally unrelated to Cry toxins, have also been shown to form ionic channels in planar lipid bilayers (PLBs). One study was performed on the 27 kD CytA toxin from *B. thuringiensis* var. *israelensis* [17]. The protein formed cation-selective channels of about 40 pS, which were more selective for potassium than sodium. Recently, another report demonstrated low-conductance, cation-selective

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channel formation by a 22.5-kD cytolytic toxin from *B. thuringiensis* var. *kyushuensis* [18]. However, until lately, there was no direct evidence on the involvement of ion channels in live cells of target insects.

In the first electrophysiological and microspectrofluorometric study on single cultured lepidopteran insect cells (*Spodoptera frugiperda,* SF-9), we disclosed several steps which may be involved in the early action of the entomopathic protein CryIC [30]. They include an initial rise of intracellular calcium (monitored using the fluorescent intracellular free calcium probe Fura-2), followed by activation of chloride-selective ion channels in the plasma membrane. The most striking—and yet unexplained—observation we made is that the *B. thuringiensis* toxin seems to have a dual mode of action: on the one hand, it induces chloride channel activity by what appears to be a second messenger mechanism, as observed in the cell-attached configuration of the patch-clamp technique, with the toxin in the bath; and on the other hand, it triggers identical chloride channel activity when applied directly to the cytoplasmic side of excised cell membrane patches, as seen in the inside-out, patch-clamp configuration. The ability of CryIC to form channels in the plasma membrane of cultured lepidopteran cells prompted us to investigate its interaction with a receptor-free, artificial lipid membrane system.

We report here the results of the study of CryIC channel formation in artificial phospholipid membranes. The toxin was inserted in a planar lipid bilayer and single channel ion movements across the bilayer were monitored under various environmental conditions using the voltage-clamp recording technique [23].

Materials and Methods

Chemicals and Solutions

TRISMA base (Tris), HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), MES (2-[N-morpholino]ethanesulfonic acid), PIPES (1,4-piperazinediethanesulfonic acid) and CAPS (3-[cyclohexylamino]-1-propane-sulfonic acid) were purchased from Sigma, St Louis, MO. Phospholipids were obtained from Avanti Polar Lipids, Birmingham, AL. Renografin was obtained from Squibb, Montreal, Quebec. All other materials were of reagent grade. In the standard experimental conditions, both chambers contained 4 ml of either a 150 mM KCl, 5 mM MES, pH 6.0 solution or 150 mM KCl, 5 mM Tris, pH 9.5 solution. For ion selectivity determinations, transmembrane salt gradients were established using concentrations of KCl from 300 to 600 mM in the *cis* chamber while keeping 150 mM KCl in the *trans* chamber. In some experiments, either potassium gluconate or choline chloride were used instead of KCl. Changes in pH were realized by adding precalibrated aliquots of either HCl or Tris solutions to the bilayer chambers to reach the desired final pH.

Toxin Purification

Inclusion bodies were produced from the cloned CryIC gene (*B. thuringiensis* var. *aizawai*) in *Escherichia coli* (HB101) and were purified as described elsewhere [22]. Activated recombinant CryIC toxin was purified by solubilization of inclusion bodies in 10 mM NaOH followed by the addition of trypsin to a 1% (w/v) final concentration. The mixture was left for 5–12 hr at ambient temperature followed by centrifugation at $2 \times 10^5 \times g$ for 2 hr at 20°C to remove any membranous material. The digested toxin preparation was passed four times through a centricon-30 (Amicon, W.R. Grace, Danvers, MA) in order to remove small peptides and to change the buffer to 50 mM CAPS pH 11. The 65-kilodalton (kD) toxin was further purified by FPLC. In some experiments, we used trypsin-activated CryIC toxin isolated from *B. thuringiensis* var. *entomocidus* crystals. The purity of the recombinant or crystal-derived CryIC activated toxin was examined by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [20]. Fractions containing the purified toxins were further treated by centrifugation through centricon-30 in order to change the solvent to distilled water. The water solubilized toxins were stored at 4°C. All protein concentrations were determined by a protein-dye method [2] using bovine serum albumin as a standard.

Planar Lipid Bilayers

Planar lipid bilayers [24] were formed from a 7 : 2 : 1 lipid mixture of phosphatidylethanolamine, phosphatidylcholine and cholesterol. The final lipid concentration was 25 mg/ml dissolved in decane. The 250-μm orifice drilled in a Delrin cup was pretreated with the same lipid mixture dissolved in chloroform. The bilayer was painted across the orifice using a Teflon stick dipped in the decane lipid solution. Membrane thinning was assayed by applying a triangular test pulse. Typical capacitance values were between 250 and 400 pF. Toxin incorporation was performed either by adding aliquots of the protein (10–60 μg) to the 4-ml *cis* chamber after bilayer formation or, prior to bilayer formation, by microinjecting 0.5 μl of an aqueous solution of the toxin (0.5 mg/ml) in the hole of the Delrin cup following chloroform evaporation of the lipid pretreated cup. The latter procedure increased the success rate of toxin incorporation and observation of channel activity. Both incorporation procedures resulted in identical channel behavior. Incorporation of the toxin was either spontaneous or induced by application of an 80-mV holding potential across the bilayer. Channel activity was monitored by step changes in the current recorded during holding test voltages across the planar lipid bilayer. All experiments were performed at room temperature (20–22°C).

Recording and Data Analysis

Single channel currents were recorded with a Dagan 8900 patch-clamp amplifier (Dagan Corp, Minneapolis, MN). Currents, filtered at 1 kHz, were displayed on an oscilloscope (Kikusui 5040, Tokyo, Japan) and simultaneously stored on videotape, using a digital data recorder (DAS/VCR900, Unitrade, Minneapolis, MN), and on a digital chart recorder (Dash II MT, Astromed,

West Warwick, NJ). They were played back and filtered at 400 Hz by an analog, 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized using a Labmaster TL-1 interface and Axotape software (both from Axon Instruments, Foster City, CA). Analysis was performed on a personal computer using pCLAMP software (Axon Instruments, Foster City, CA) or programs kindly supplied by Dr. M. Nelson, University of Vermont, Burlington, VT.

Subconductance states were recognized using the following identification criteria [11]: (i) direct transitions from subconductance levels to main conductance levels were observed; (ii) subconductance states were never observed in the absence of the main conductance state, and (iii) the main conducting state did not result from the superposition of two or more independent channel openings.

Applied voltages are defined with respect to the *trans* chamber which was held at virtual ground. Positive currents (i.e., currents flowing through the planar lipid bilayer from the *cis* chamber to the *trans* chamber) are shown as upward deflections in the figures. The direction of current flow corresponds to positive charge movement (anions move in the opposite direction).

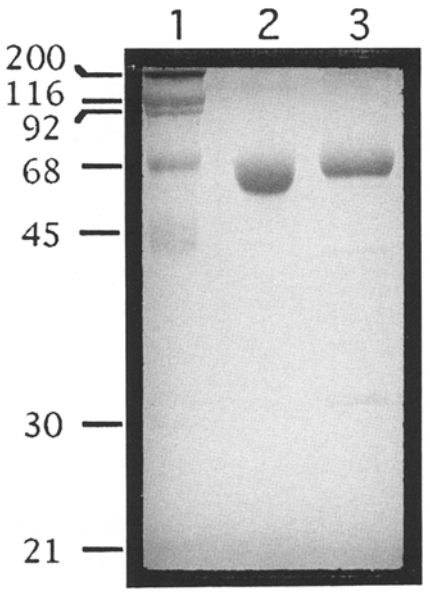

Fig. 1. SDS-PAGE of FPLC-purified, trypsin-activated CryIC toxins. Lane *1*: Molecular weight markers. The molecular weights are indicated by the numbers (in kD) on the left. The protein bands for the 45, 30 and 21 kD markers were underloaded and are barely visible. Lane *2*: 2 μg of recombinant CryIC toxin from *B. thuringiensis* (HD-133) cloned into *E. coli*. Lane *3*: 1 μg of activated toxin from purified *B. thuringiensis* var. *entomocidus* crystals. The proteins were visualized by Coomassie brilliant blue staining.

Results

CryIC Toxin Purity

To ascertain that the observed channel activity can be attributed to the toxin itself and not to impurities in the toxin preparations, the toxin stocks were examined by SDS-PAGE. As shown in Fig. 1, the recombinant CryIC protein stock consisted of only a single 65-kD polypeptide band (Lane *2*). However, a few faint bands can be seen with the crystal-purified toxin (Lane *3*). These bands are presumed to be toxin breakdown products. Their intensity usually increases with time. This result is not surprising since crystals from *B. thuringiensis* typically harbor crystal-associated proteases [26]. It is important to note that any CryIC toxin prepared from crystals was used immediately for bilayer experiments and not stored for periods of time in excess of one week. More importantly, no differences were seen in channel formation whether the recombinant protein or the crystal protein were used.

CryIC Toxin Forms Ionic Channels in Planar Lipid Bilayers

In symmetrical 150 mM potassium chloride solutions, various types of channels were observed shortly (within minutes) after addition of the toxin to the *cis* chamber of the PLB. Channel activity was present in either alkaline (pH 9.5, Fig. 2*A*) or acidic environments (pH 6.0, Fig. 2*B*), but the channel conductances, kinetic properties and ionic selectivities appeared to be pH dependent. In general, at alkaline pH, the number of distinct, main conductance opening levels increased with time, without, however, exceeding six or seven, suggesting that progressive, but rather limited incorporation took place in the PLB. The channels displayed a variety of kinetic behaviors, including a rapid flickering (Fig. 2*A*, upper traces) or slow gating modes, with the appearance of large amplitude, short-lived events together with long-lasting subconducting states (Fig. 2*A*, lower traces). Conductances at this pH and in symmetrical 150 mM KCl solutions ranged between 12 and 200 pS. At acidic pH, the elementary currents were different from those recorded at higher pH. There were either low-conductance channels (25 to 35 pS) with rapid transitions between states (Fig. 2*B*, upper trace) or high-conductance channels (100 to 140 pS) which remained closed for a very long period of time (open time probability less than 10% at -100 mV; Fig. 2*B*, lower traces). Under these acidic pH conditions, the occurrence of single electrical events was less frequent, and in several cases, only one or two opening levels were observed. This low level of channel activity at pH 6.0 may have resulted either from the reduced solubility of CryIC

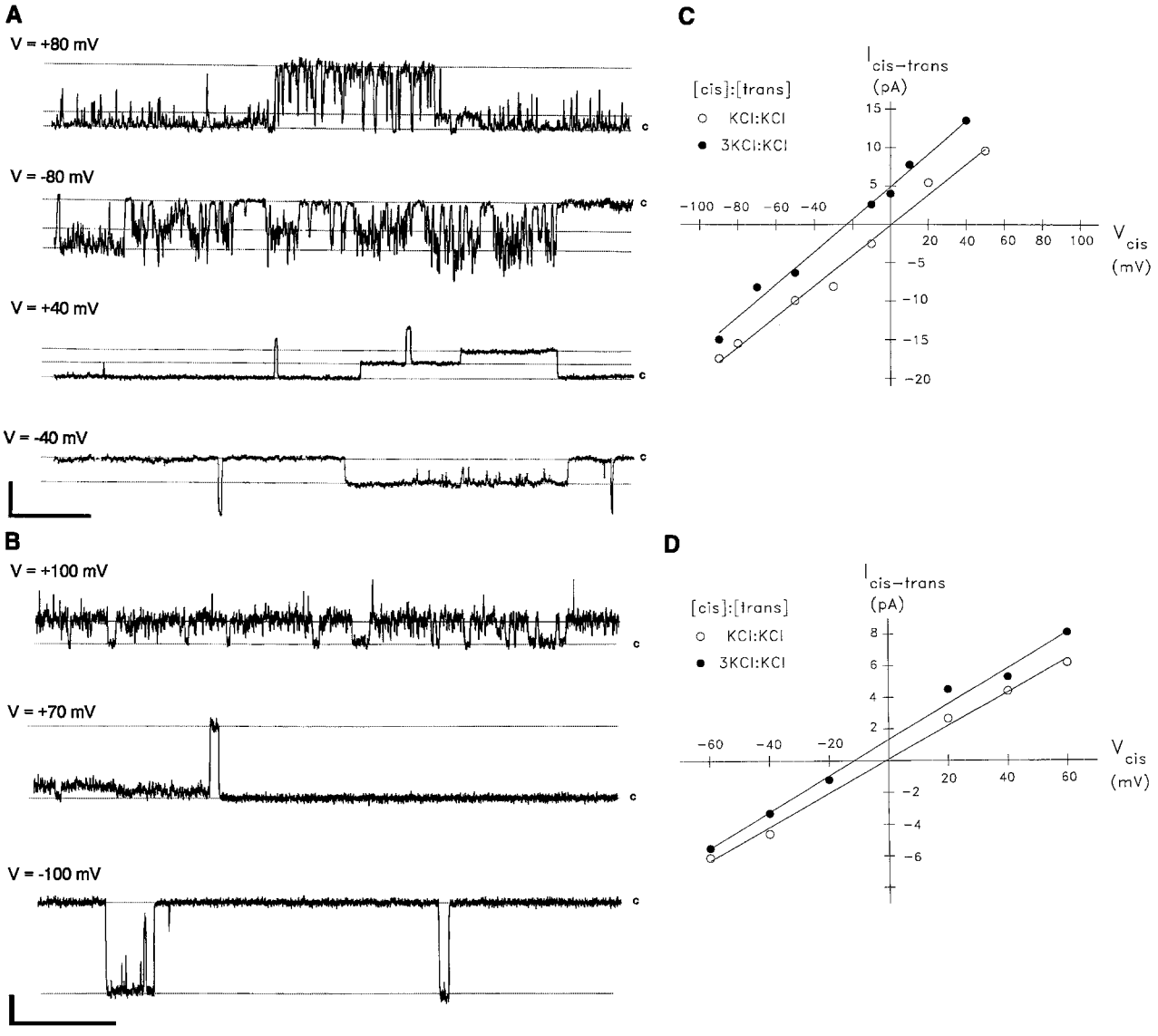

Fig. 2. Single channel currents observed after toxin addition to the *cis* side of the bilayer in KCl solutions and in either alkaline or acidic environment. (*A*), pH 9.5. Representative records at four different voltages (indicated next to the traces). Note in the two lower traces the existence of substates. The main conductance of this channel was evaluated to be around 120 pS, with two subconductances levels at 40 and 80 pS. The letter *c* indicates the closed state of the channels. Vertical bar = 4 pA. Horizontal bar = 500 msec. (*B*) pH 6.0. Representative records at three different voltages (indicated next to the traces). The letter *c* indicates the closed state of the channels. Vertical bar = 4 pA. Horizontal bar = 500 msec. (*C*) Current-voltage relations at pH 9.5 from single channel currents recorded in a PLB bathed in symmetrical (150 mM : 150 mM) and asymmetrical (450 mM : 150 mM) KCl solutions (two experiments). The straight line was fitted by linear regression to the data points. The reversal potential was equal to 23 mV in the cation-selectivity direction. (*D*) Current-voltage relations at pH 6.0 from single channel currents recorded in a PLB bathed in symmetrical (150 mM : 150 mM) and asymmetrical (450 mM : 150 mM) KCl solutions (two experiments). The straight line was fitted by linear regression to the data points. The reversal potential was equal to 12 mV in the cation-selectivity direction.

at that pH, or from modified partitioning properties of the toxin protein in this acidic environment, or from both. However, while in several experiments performed in symmetrical potassium chloride solutions at pH 6.0 the bilayer remained silent after toxin addition to the *cis* chamber, brief openings of single ion channels could be detected after the pH was raised to 9.5, suggesting a possible causal relation between pH and channel insertion and/or formation.

Ion selectivity was tested by performing experiments using KCl gradients at both pHs. At pH 9.5, under symmetrical 150 mM KCl conditions, a 192 pS channel was observed. When the concentration of KCl was raised to 450 mM in the *cis* compartment, the conductance of the channel increased to 220 pS.

The channel reversal potential shifted by 23 mV in the negative direction (Fig. 2*C*), which is consistent with the −27.7 mV equilibrium potential calculated by Nernst equation applied to a monovalent cation under these conditions. This establishes clearly that these channels are selective to cations. The selectivity of the larger channels was tested at pH 6.0 using the same experimental approach. The conductance of a 106 pS channel observed in symmetrical 150 mM KCl increased to 114 pS in a 450 mM: 150 mM KCl gradient and its reversal potential shifted by 12 mV in the negative direction (Fig. 2*D*). This result indicates that this channel is also cation-selective, but to a lesser extent than the 192 pS channel recorded at pH 9.5. It appears, therefore, that the pH environment of the toxin incorporated in the PLB affects its selectivity.

Anion Channel Formation after Toxin Incorporation at Acidic pH

Patch-clamp experiments demonstrated anion-selective channel formation by CryIC toxin in the plasma membrane of lepidopteran SF-9 cells [30]. Whether CryIC forms similar channels in an artificial membrane system was further investigated by the following experiments in which permeant potassium ions were excluded from the solutions bathing the bilayer. In experiments in symmetrical potassium-free, choline-chloride solutions at pH 6.0, channel activity was detected following toxin addition to the *cis* bath. More than one type of channel was observed. They included very small channels with very little activity and conductances ranging between 8 and 13 pS, larger channels which were the most frequently seen, with conductances around 25 pS and, occasionally, even larger channels whose conductances ranged between 60 and 120 pS.

Only the intermediate conductance channels (around 25 pS) were studied further. These channels remained open for long periods of time and displayed numerous short-lived events with no voltage dependence (Fig. 3*A*). Fig. 3*B* shows the current-voltage relation for these channels in symmetrical 150 mM choline chloride (24 pS, $n = 4$). Selectivity was investigated in experiments using a 300 mM *cis:* 150 mM *trans* choline chloride gradient. This resulted in the displacement of the reversal potential by 16 mV, consistent with the 17.5-mV Nernst potential of a monovalent anion electrode (Fig. 3*C*), suggesting that the channel is selective for anions.

A number of ion channels are affected by zinc. In particular, some chloride channels are blocked by this divalent cation [3]. In view of the anion-selective nature of some of the CryIC channels at pH 6.0, we investigated the possible effect of zinc on these channels. When added to the *cis* side of the PLB, 1 mM Zn^{++} reduced significantly the probability of channel opening at negative voltages, from 0.4 to less than 0.05 at −80 mV. It was less effective when added to the *trans* side of the artificial bilayer (*data not shown*).

Cation Channel Formation by Toxin Incorporation at Alkaline pH

Having established that at alkaline pH CryIC forms only cation-selective channels, additional experiments were performed in the absence of permeant anions. Following toxin insertion in the PLB bathed in symmetrical chloride-free, potassium gluconate solutions at pH 9.5, electrical activity was consistently observed (Fig. 4). The toxin formed various types of channel with different kinetic behaviors. Figure 4*A* shows the unitary currents flowing through at least two single channels of large conductance (144 pS) which displayed marked voltage dependency. A more complex recording of single channel currents involving different channels or channels possessing different conductance levels is seen in Fig. 4*B*. The presence of very short-lived states is also apparent in these records. The existence of several subconducting states is shown in Fig. 4*C*, in which five equidistant levels are easily resolved. In this particular experiment, we evaluated the conductance of the "fully open" channel to be 90 pS and the subconductances 18, 36, 54 and 72 pS. The current-voltage relations of the most commonly encountered channels (169, 101 and 52 pS) are shown in Fig. 4*D*. These experiments, which were performed in the absence of any permeant ions except potassium, confirm the cationic nature of the channels observed at alkaline pH.

Since channel activity was observed at acidic pH in symmetrical KCl (Fig. 2*B*), it was relevant to examine if such cation-selective channels could be observed at pH 6.0 in the absence of chloride. After toxin incorporation at pH 6.0 in symmetrical potassium gluconate, the channel displayed long periods of silence interrupted by a few short openings (Fig. 5, left panel). This channel behavior was similar to that shown in Fig. 2B (lower traces). Addition of 100 mM of choline chloride to both sides of the artificial membrane at pH 6.0 had two effects: the amplitude of the unitary current flowing through the channel augmented and the probability of opening of the channel increased significantly in a voltage-dependent manner (Fig. 5, right panel). It is not known whether chloride ions reactivated the cation-selective channels observed at acidic pH or permeated

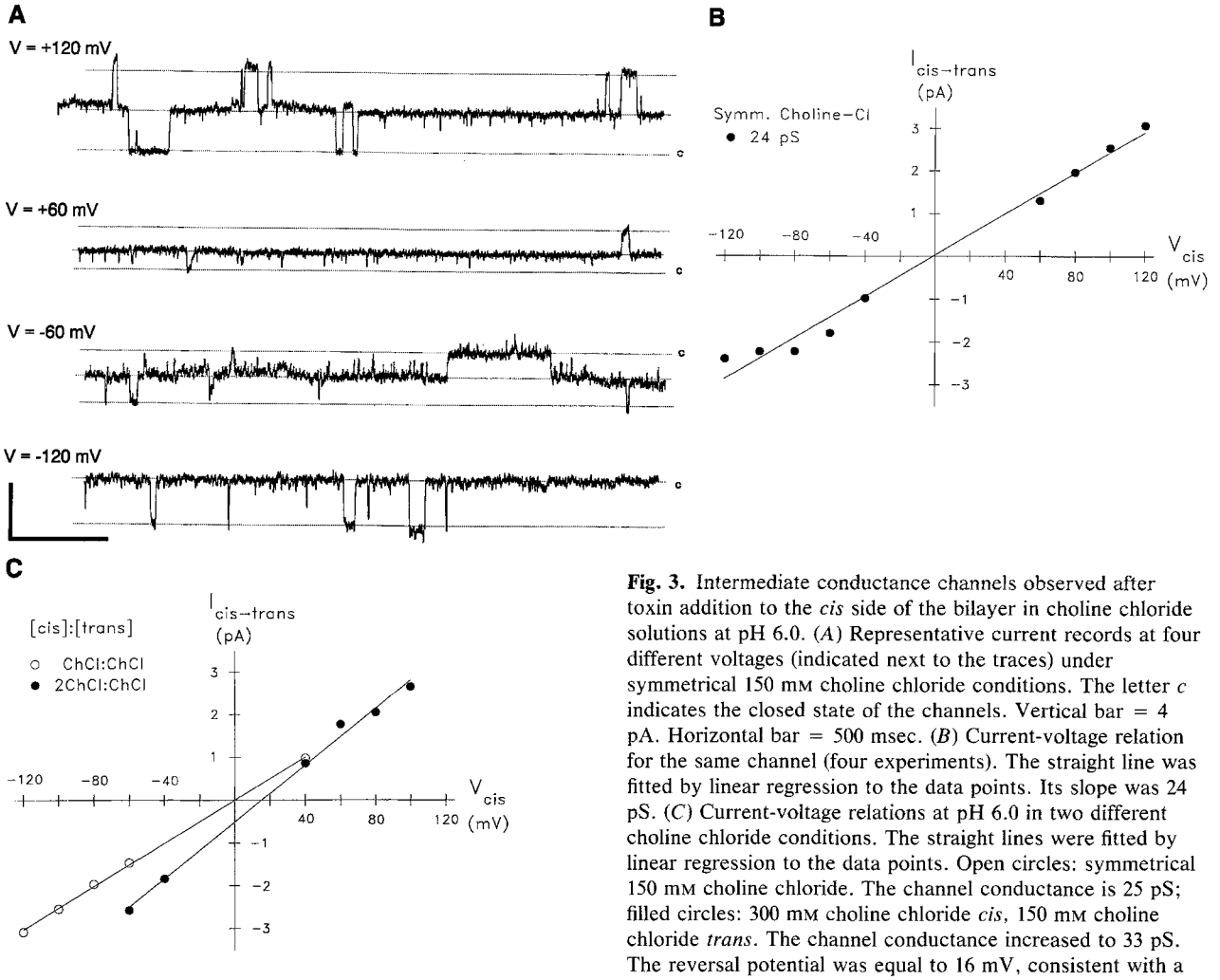

Fig. 3. Intermediate conductance channels observed after toxin addition to the *cis* side of the bilayer in choline chloride solutions at pH 6.0. (*A*) Representative current records at four different voltages (indicated next to the traces) under symmetrical 150 mM choline chloride conditions. The letter *c* indicates the closed state of the channels. Vertical bar = 4 pA. Horizontal bar = 500 msec. (*B*) Current-voltage relation for the same channel (four experiments). The straight line was fitted by linear regression to the data points. Its slope was 24 pS. (*C*) Current-voltage relations at pH 6.0 in two different choline chloride conditions. The straight lines were fitted by linear regression to the data points. Open circles: symmetrical 150 mM choline chloride. The channel conductance is 25 pS; filled circles: 300 mM choline chloride *cis*, 150 mM choline chloride *trans*. The channel conductance increased to 33 pS. The reversal potential was equal to 16 mV, consistent with a monovalent anion electrode.

through anion-selective channels already inserted in the artificial bilayer at acidic pH and in the absence of chloride.

Discussion

This study demonstrates that the trypsin-activated CryIC gene product, a lepidopteran-specific *B. thuringiensis* toxin, forms ion channels in planar lipid bilayers. These channels are selective to either anions or cations and display complex activity patterns which appear to be pH dependent.

The major finding of this study is that, for the first time, a *B. thuringiensis* toxin is shown to form ion channels in artificial membranes at pH values as low as 6.0. The purity of the activated toxin used in this study rules out channel formation by another protein. Although solubility of Cry toxins is significantly reduced at lower pH, the amount of CryIC toxin in solution was sufficient for the protein to partition into the lipid membrane. CryIC forms a variety of channels in PLBs, with conductances (or subconductance levels) ranging from 7 to 250 pS. Among them, a 25–35 pS anion-selective channel, which displayed permeation and kinetic properties similar to those of the channels observed in a previous study on SF-9 cells, both in the cell-attached and the inside-out configurations of the patch-clamp technique [30] was often seen at acidic pH. It is remarkable that under a comparable pH environment and for the same CryIC toxin doses a similar class of channels was recorded in both PLBs and cultured lepidopteran cells. This suggests a common mechanism of toxin insertion in the plasma membrane of SF-9 cells and in artificial membranes. It also confirms that channel formation in both systems is possible, even in the absence of receptors.

SF-9 cells are not of midgut origin. However, CryIC is far more toxic to these cells than CryIA(b), and CryIA(a) and CryIA(c) are not toxic at all

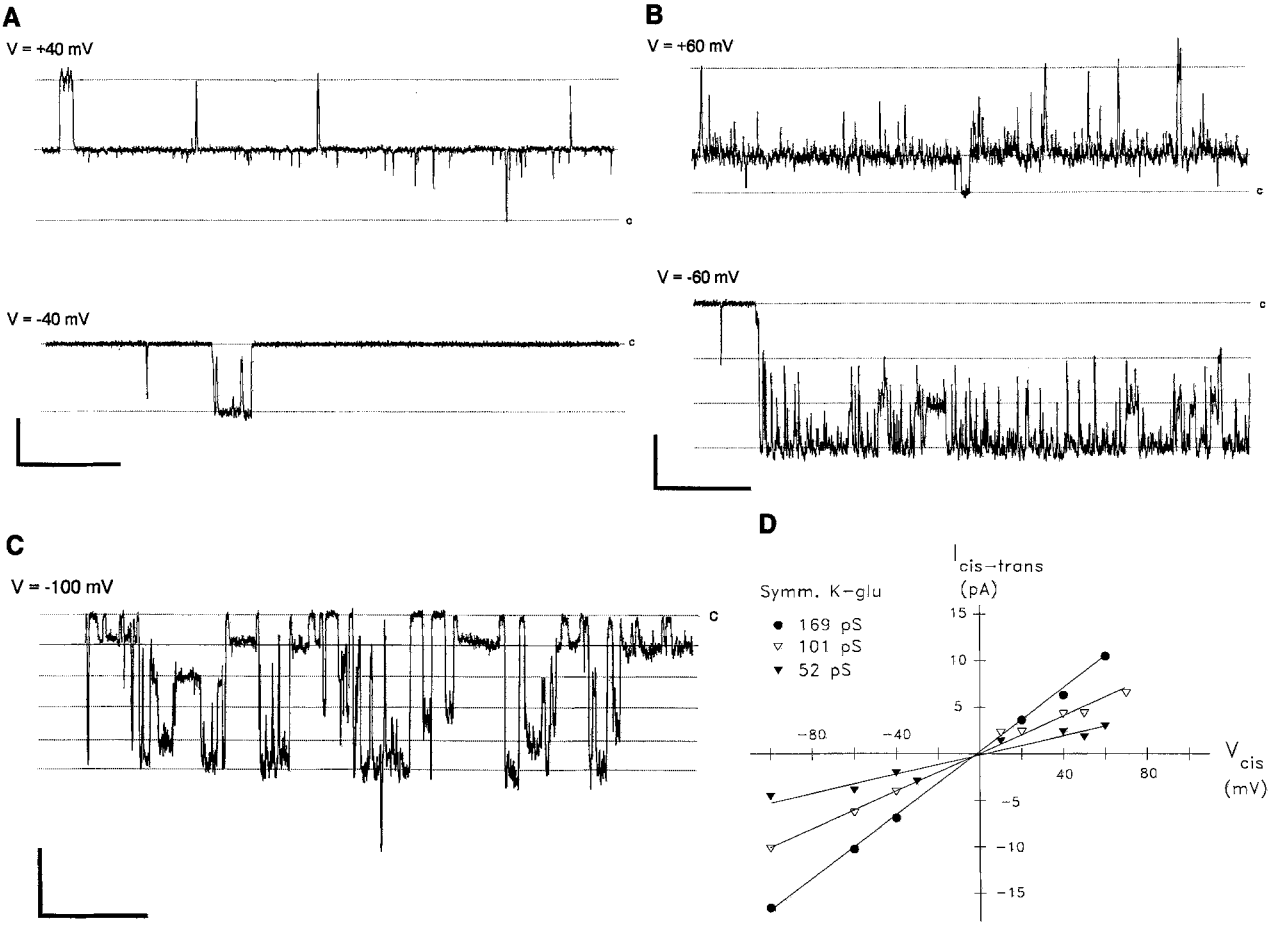

Fig. 4. Single channel currents observed after toxin addition to the *cis* side of the bilayer setup in symmetrical 150 mM potassium gluconate solutions at pH 9.5. (*A*) Representative records at two different voltages (indicated next to the traces) of a 144-pS conductance channel with marked voltage dependency. The letter *c* indicates the closed state of the channels. Vertical bar = 4 pA. Horizontal bar = 500 msec. (*B*) Representative records at two different voltages (indicated next to the traces) of currents from different channels or channels possessing different levels of conductance. The letter *c* indicates the closed state of the channels. Vertical bar = 4 pA. Horizontal bar = 500 msec. (*C*) Representative record of the current flowing through a 90 pS channel including five 18-pS subconducting states. The voltage is indicated next to the trace. The letter *c* indicates the closed state of the channels. Vertical bar = 4 pA. Horizontal bar = 500 msec. (*D*) Current-voltage relations obtained from currents recorded from the most common channel observed in symmetrical 150 mM potassium gluconate solutions at pH 9.5. The straight lines were fitted by linear regression to the data points. Filled circles: 169 pS, two experiments; open triangles: 101 pS, two experiments; filled triangles: 52 pS, two experiments.

(Table), indicating specificity of various Cry toxins to this cell line. This would normally imply that SF-9 cells possess surface receptors specific to CryIC. However, we have shown that similar doses of CryIC induced comparable channel activity in inside-out patches of SF-9 native membrane and in PLBs, in both cases without receptors. It cannot be excluded that in midgut, the initial steps of the mode of action of the *B. thuringiensis* toxin may be different from those observed in nonmidgut cells of susceptible insects. In the former case, surface receptors would be responsible for toxin specificity while in the latter case, favorable pH and/or phospholipid environments [32] would account for it. These factors could play a significant role in the properties of the ion pores induced by CryIC and presumably other *B. thuringiensis* toxins.

The results of this study indicate that conductance, selectivity, kinetic properties and the existence of subconducting states of CryIC channels formed in PLBs are sensitive to pH. Titration of charged groups located at the mouth or in the wall of the channel provides a simple explanation for this dependence, which has been reported for several bacterial pore-former toxins [5, 14, 25, 27, 28]. However, in a few preliminary experiments, it was observed that the anionic or cationic selectivity of CryIC channels appeared to be determined by the initial pH and did not change when the pH was

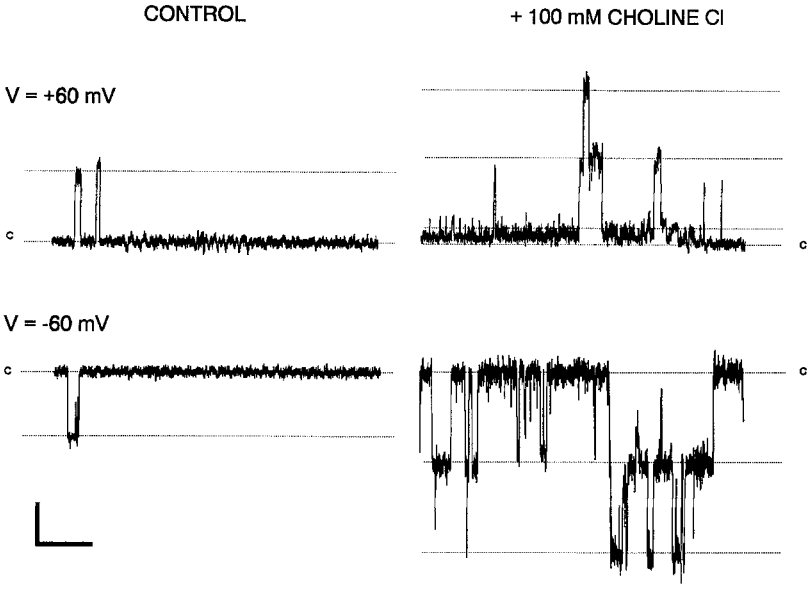

Fig. 5. Single channel currents observed in symmetrical 150 mM potassium gluconate solutions at ph 6.0 (left panel) and effect of addition of 100 mM choline chloride to each side of the artificial membrane (right panel). Representative records at two different voltages (indicated next to the traces). The letter *c* indicates the closed state of the channels. Vertical bar = 4 pA. Horizontal bar = 800 msec.

Table. Specificity of various CryI toxins to cultured cells of lepidopteran insects

Activated toxin[a]	Lawn assay threshold values[b] (ng)	
	Choristoneura fumiferana (CF-1)	*Spodoptera frugiperda* (SF-9)
CryIA(a)	Nontoxic	Nontoxic
CryIA(b)	500	250
CryIA(c)	0.1	Nontoxic
CryIC	6.0	0.2

[a] A twofold dilution series of each toxin was made in 50 mM CAPS buffer (pH 10.5). One microliter from each dilution was spotted onto the surface of immobilized insect cells and the plates were incubated and stained as described in [12].
[b] The toxicity threshold values are defined as the lowest amount of activated toxin in the dilution series creating a visible Trypan Blue lytic spot.

shifted to other values. This suggested that, in addition to charge titration, conformational changes may be involved. The pH-dependent mechanism of insertion of colicin A into membranes has been shown to include a transition from the native to a molten globular state [33]. It has been proposed that colicin E1 channel selectivity is also determined by a pH-dependent conformational change and that these channels may stay in an irreversible selective state depending on their phospholipid environment [5]. Similar mechanisms may control CryIC channel formation and selectivity in PLBs. This would explain, in addition to the often observed preference for anions over cations at acidic pH, the fact that, under appropriate conditions at the time of insertion in the lipid bilayer, channels remain in a "locked" conformational state and therefore retain their initial selectivity at a different pH.

Our results are at variance with the only report on *B. thuringiensis* Cry toxin channel formation in PLBs. This study was performed on two different toxins from the Cry family, CryIA(c), a 55-kD lepidopteran-specific toxin and CryIIIA, a 67-kD coleopteran-specific toxin [31]. It described potassium channel formation by both toxins, with very large conductances (ranging from 200 to 4,000 pS). Channel activity could only be observed at alkaline pH (pH 9.5 or higher). The present work demonstrates that CryIC is capable of partitioning in lipid membranes for a much wider range of pH values than the other *B. thuringiensis* toxins studied so far. Moreover, in separate experiments performed with trypsin-activated CryIA(c) toxin, we have observed a 29 pS channel at pH 6.0 in symmetrical salt solutions (*not illustrated*). This indicates that in our hands CryIA(c) can also partition in lipid bilayers

at acidic pH and form ion-selective channels. For this particular toxin, the differences between our results and those reported by other investigators remain unexplained.

Computer analysis of Cry toxins sequences revealed that the pore-forming capability of the first 600 residues of these cytolytic proteins may be predicted by the presence of six amphiphilic helical segments [15]. We have confirmed, using a similar approach [8], that indeed CryIC possesses six putative α-helices that could span a lipid bilayer. In particular, the helices 2, 4 and 5 (residues 96 to 113, 159 to 176 and 193 to 211, respectively) present large hydrophobic faces and are therefore good candidates, if associated in a multimeric manner, for transmembrane pore formation. The ionic channels reported in this study could result from the oligomerization of several proteins inserted in the bilayer [11], as suggested by the existence of subconducting states of the cation-selective channels observed at pH 9.5.

It cannot be excluded, however, that the CryIC toxin pore could be constructed by an appropriate transmembrane arrangement of the putative helices of a single molecule. Such a mechanism of pore formation has been proposed for the coleopteran-specific toxin CryIIIA, whose X-ray crystal structure has recently become available [21]. The CryIIIA structure shows a bundle of seven α-helices, one of which is completely surrounded by the other six. It has been proposed that, upon binding of the toxin to its receptor, this bundle may rearrange itself to create an ion channel through displacement of the central α-helix. A similar hypothesis can be proposed for the CryIC toxin in view of its significant homology with CryIII in terms of amino acid sequence. The two sequences show 37.7% identity and 58.6% similarity when a computer alignment is made for amino acids 1 and 600 of CryIC and amino acids 44 and 652 of CryIIIA [8]. Furthermore, helical wheel drawings of the regions corresponding to the seven α-helices of CryIIIA show similar, although not identical, distribution of hydrophobic faces [8] (*data not shown*).

Alternatively, it may be that the mode of channel formation could differ widely from toxin to toxin within the Cry family. It is also possible that, depending on the conditions prevailing at the time of insertion (pH environment, membrane composition), a channel would be formed by either a single protein or the oligomeric association of several of them. This would result in channels displaying different permeation, selectivity and gating behaviors. Differentiation between the monomeric mode of pore formation or the multimeric model will require further work.

In summary, the present study demonstrates that *B. thuringiensis* CryIC toxin forms ion channels in planar lipid bilayers. In an alkaline environment, 100–200 pS cation-selective channels were regularly observed. Moreover, at pH 6.0, the toxin forms anionic channels similar to those recorded in native membranes of SF-9 lepidopteran cells at pH 6.3. It is tempting to speculate that in SF-9 cells the membrane composition and its environment play a significant role in CryIC specificity and that surface receptors may not be necessarily involved. Whether the present observations are restricted to the SF-9 cellular model and to the CryIC toxin remains to be clarified.

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