δ-Endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar lipid bilayers

Argelia Lorence, Alberto Darszon, Claudia Díaz, Arturo Liévano, Rodolfo Quintero, Alejandra Bravo*

Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca 62271, Mor., México

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Abstract **Membrane potential measurements using a fluorescent dye indicated that two specific toxins active against *Spodoptera frugiperda* larvae (CryIC and CryID) cause immediate permeability changes in midgut epithelial brush border membrane vesicles (BBMV). The initial response and the sustained permeability change are cationic, not very K^+ selective, and occur at in vivo lethal doses (nM). The toxin response has a different ion selectivity and is more sensitive to Ba^{2+} than the intrinsic cation permeability of BBMV. Experiments incorporating BBMV into planar lipid bilayers (PLB) demonstrated that these vesicles contain cation channels (31, 47 and 76 pS). A 2–40 fold conductance increase was induced by nM concentrations of toxin in PLB containing BBMV. Cationic single channel transitions of 50, 106, 360 and 752 pS were resolved. Thus, *Bacillus thuringiensis* δ-endotoxins induce an increase in cation membrane permeability involving ion channels in BBMV-containing functional receptors.**

Key words: δ-Endotoxin; Mode of action; Ionic channel; *Bacillus thuringiensis*

1. Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium that during sporulation produces crystalline inclusions formed by proteins called δ-endotoxins or insecticidal crystal proteins (ICP), which are toxic to the larvae of various insects, many of which are disease vectors or major crop pests. Numerous ICP genes have been cloned, sequenced, and classified into five groups and several subgroups on the basis of sequence homology and toxicity spectra [1].

ICP are synthesized in the form of inactive protoxins that are solubilized and proteolytically activated in the insect gut. The activated toxin binds to specific receptors located in the apical microvilli of epithelial cells in the insect's midgut. After binding, the toxin inserts irreversibility into the cell plasma membrane [2] and forms a pore or lesion that allows net uptake of ions and water, leading to midgut cell swelling and eventual lysis. It has been proposed that K^+-selective pores are involved in this process [3–7].

The purified ICP have the ability to induce ion leakage in synthetic phospholipid vesicles [8,9]. In the absence of specific receptors CryIA(c), CryIIIA, CryIIIB2 and CryIC toxins form cation-selective channels at alkaline pH; CryIC also forms anion-selective channels at pH 6.0, in planar bilayers [10–13]. The toxin concentrations needed to exert these effects are at least 1000-times higher than the in vivo biocide concentration. It has been proposed that the receptor functions as a binding protein to increase the local concentration of the toxin in the vicinity of the membrane [11]. Recently the receptor for CryIA(c) toxin in *Manduca sexta* has been identified as a metalloprotease aminopeptidase N [14].

Recent in vitro assays using brush border membrane vesicles (BBMV) have shown permeability changes induced by Cry toxins at physiological concentrations (nM) [15,16]. However, the toxin effects were recorded after 1 h of incubation with the vesicles, missing the initial response produced by the toxin once it interacts with its receptor.

Here we show that nM concentrations of ICP cause an immediate change in membrane potential, measured with a potential-sensitive fluorescent dye, in BBMV isolated from the midgut of *Spodoptera frugiperda* larvae. Two specific toxins active against this insect (CryIC and CryID) and two non-toxic ICP (CryIIIA and CryIA(c)) were used. We studied the ionic basis of the toxin-induced membrane potential changes and explored their pharmacology. Having found specific toxin responses in these BBMV, we incorporated them into planar bilayers where they displayed intrinsic single channel cation conductances mainly of 31, 47 and 76 pS. A 2–40 fold conductance increase was observed when nM concentrations of toxin were added to bilayers containing BBMV. The toxin-induced conductance increase was cationic, and single channel transitions of 50, 106, 360 and 752 pS could be resolved. These results indicate that the toxin receptor is fundamental for efficient toxin-induced cation pore formation.

2. Materials and methods

2.1. Preparation of the brush border membrane vesicles

BBMV from 5th instar *S. frugiperda* larvae were prepared as reported [17]. The vesicles were dialyzed overnight against 400 vols. of 150 mM KCl, 2 mM EGTA, 0.5 mM EDTA, 10 mM HEPES-HCl, pH 7.5 (Sigma, St. Louis, MO), and sonicated for six 30-s periods at 25°C (Branson 1200 sonic bath; Danbury, CT) in the same solution. The BBMV used in the planar lipid bilayer experiments were loaded overnight with 1.26 M sucrose at 0°C and stored at −70°C until used.

BBM enrichment was estimated according to the alkaline phosphatase (AP)-to-cytochrome *c* oxidase activity ratio (9.5–12.5 fold increase/mg protein relative to the initial homogenate), which were measured as reported [18,19]. Vesicle orientation was determined from the AP activity in the presence and absence of 0.02% Triton X-100 (Sigma).

Receptor concentration in the vesicles and the homogenate was measured after CryIC binding using monoclonal antibody 20A6E9 against the toxin (supplied by Dr. M. Peferoen, Plant Genetic Systems, Gent, Belgium) and a second antibody coupled to peroxidase (data not shown).

*Corresponding author. Fax: (52) (73) 172 388.
E-mail: bravo@pbr322.ceingebi.unam.mx

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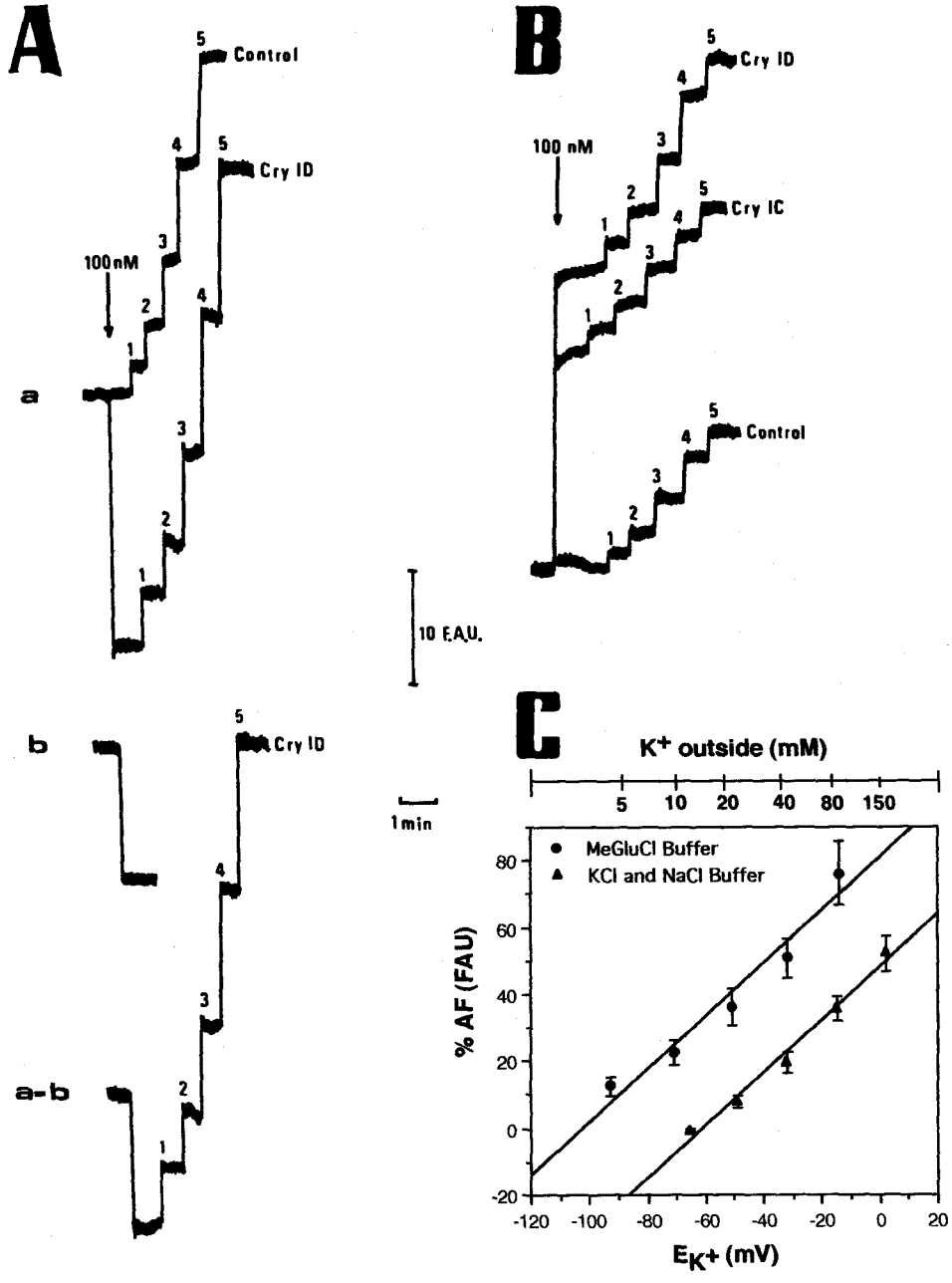

Fig. 1. Effect of the CryIC and CryID toxins on the membrane potential in *Spodoptera frugiperda* midgut brush border membrane vesicles. Membrane potentials of BBMV (20 μg) loaded with (mM) 150 KCl, 2 EGTA, 0.5 EDTA, 10 HEPES-HCl, pH 7.5, were recorded as described in section 2. Pre-equilibration with 1.5 μM Dis-C_3-(5) (9 min) and the effect of 0.5 μM oligomycin are not shown. An upward deflection indicates a membrane potential depolarization. The arrow on top of the traces corresponds to the time of toxin or buffer A addition. FAU, fluorescence arbitrary units. (A) CryID response of BBMV suspended in 140 mM methylglucamine chloride, 10 mM HEPES-HCl, pH 7.5. (Aa) Final K^+ concentrations were (mM): 1 = 4; 2 = 13; 3 = 30; 4 = 64 and 5 = 133. In the control, buffer A was added instead of toxin. (Ab) Interaction of the CryID toxin with the dye in absence of BBMV. (B) CryIC and CryID effects upon substitution of methylglucamine chloride incubation buffer by 10 mM KCl, 130 mM NaCl, 10 mM HEPES-HCl, pH 7.5, buffer. Final K^+ concentrations were (mM): 1 = 23; 2 = 44; 3 = 85; 4 = 165; 5 = 367. The control trace was obtained with buffer A addition, as well as with CryIIIA, CryIA(c) and boiled CryIC and CryID toxins additions. (C) Resting membrane potential was determined as described in section 2.3 from a ΔF (%) vs. E_{K^+} (mV) curve. In the presence of external 10 mM KCl, 130 mM NaCl, the measured resting membrane potential was -59 ± 3 mV ($n = 13$), and -99 ± 5 mV ($n = 38$) in external 140 mM MeGluCl.

2.2. Preparation of insecticidal crystal proteins

Escherichia coli recombinant strains containing the *cry*IC and *cry*ID genes, derived from *Bt* var. *entomocidus* and *aizawai*, respectively, were kindly supplied by Dr. M. Peferoen. The inclusion bodies were solubilized, activated and purified as in [20]. Crystals from *Bt* var. *tenebrionis* strain BTS1, containing CryIIIA protein, and from *Bt* var. *kurstaki* strain HD-73, containing CryIA(c) protein, were produced and purified as described in [21]. Toxins were dialyzed against 200 vols. of 200 mM NaCl, 20 mM Tris-HCl, pH 8.6 (buffer A), and maintained at 4°C at most for a month. Activated toxin purity was examined by SDS-PAGE [22] and by Western blots [23]. The CryIC and CryID toxins were denatured by boiling them for 30 min in 90 mM NaOH and then

neutralized with HCl. The denatured toxins lost their insecticidal activity for *S. frugiperda*. Insect toxicity was assayed with 1st instar *S. frugiperda* larvae according to [24]. Protein was measured as described in [25] using BSA as a standard (New England BioLabs, Beverly, MA).

2.3. Fluorescence measurements

Membrane potential was monitored with the fluorescent positively charged dye, 3,3′-dipropylthiodicarbocyanine (Dis-C_3-(5); Molecular Probes, Eugene, OR.; 1.5 μM final, 1 mM stock in DMSO). Fluorescence was recorded at the 620/67 nm excitation/emission wavelength pair using a Hansatech system (Norfolk, England), as in [26]. Hyperpolarization causes dye internalization into the BBMV and a fluorescence decrease; depolarization causes the opposite effect. Dye calibration and determination of resting membrane potential were performed in the presence of valinomycin (2 μM) by successive additions of KCl to BBMV (20 μg) suspended in various media (700 μl). Neither carbonyl cyanide *m*-chlorophenyl hidrazone (FCCP, 1 μM); Calbiochem), a mitochondrial uncoupler, nor oligomycin (0.5 μM; Sigma), an ATPase inhibitor, altered the membrane potential measurements, indicating that there is no mitochondrial contribution to the recorder signals (data not shown). All determinations were made at 30°C with constant stirring. Time 0 (t_0) was considered to be when the BBMV were added, and the KCl additions were made after 9 min. Membrane potential determinations were done 10 times with 4 different vesicle batches under the different experimental conditions used. Cation substitutions in the incubation buffer were made by replacing the *N*-methyl-D-glucamine chloride (MeGluCl) by the indicated salt(s) (mol per mol), maintaining the osmolarity (~150–160 mOsm). Cl^- was substituted with methanesulfonate$^-$.

K^+ channel blockers Ba^{2+} and Cs^+ were from J.T. Baker, Phillipsburg, NJ, tetraethylammonium chloride (TEA) and 4-aminopyridine (4-AP) were from Alomone Labs, Jerusalem, Israel.

2.4. Planar lipid bilayer experiments

Planar lipid bilayers (PLB) were made by the Müeller and Rudin method [27], as in [28], with diphytanoyl-phosphatidyl choline (diPPC) (Avanti Polar Lipids, Birmingham, AL; 20 mg/ml in *n*-decane). Typical bilayer capacitance values were between 150 and 250 pF. Buffer solution (300 μl of 150 mM KCl, 5 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) was added to each of the cell compartments. Once the bilayer was formed, a transmembrane salt gradient was established raising [KCl] to 550 mM in *cis*, the compartment where sucrose loaded BBMV (0.2–3.7 μg protein) were added for fusion; *trans* was held at virtual ground. Vesicle incorporation was assayed after application of a 50-mV holding potential across the PLB. Toxin (0.6–10.6 nM) was added to the *cis* compartment. All experiments were performed at room temperature.

Single channel currents were recorded with a Dagan 3900A patch-clamp amplifier (Dagan Corp., Minneapolis, MN) as in [28]. Currents were filtered at 200 or 500 Hz and digitized on-line at 1 or 2 kHz, respectively, and analyzed on a personal computer using a Digidata 1200 interface and Axotape and pClamp software (Axon Instruments, Foster City, CA).

3. Results and discussion

3.1. The resting membrane potential of BBMV

The resting membrane potential of BBMV loaded with 150 mM KCl, and assayed with the membrane potential-sensitive fluorescent dye Dis-C_3-(5), is not far from the K^+ equilibrium potential (E_k) calculated by the Nernst equation. In the presence of external 10 mM KCl, 130 mM NaCl, the measured resting membrane potential was -59 ± 3 mV ($n = 13$), and -99 ± 5 mV ($n = 38$) in external 140 mM MeGluCl (Fig. 1C). The calculated E_k were −71 and −108 mV, respectively. This suggests that in the BBMV of *S. frugiperda*, K^+ permeability contributes significantly to the resting membrane potential. K^+ addition experiments without valinomycin indicated that the vesicles have intrinsic channels permeable to K^+ (Fig. 1A and B, control traces).

3.2. Effect of δ-endotoxins

The CryIC toxin was about 2 fold more active than CryID toxin against *S. frugiperda* larvae (LD_{50}'s of 31.2 ng/cm^2 with a 18–48 Cl_{95}, and 77.6 ng/cm^2 with a 17–147 Cl_{95}, respectively), whereas the CryIA(c) and CryIIIA toxins showed no activity.

Addition of 100 nM of either of the active toxins (approximately 2.5 pmol toxin/μg BBMV protein) to BBMV suspended in 140 mM MeGluCl produced a fast hyperpolarization. After toxin exposure, the vesicles also increased their response to KCl additions, when compared to the control to which the same amount of buffer was added (Fig. 1Aa). Experiments in the absence of BBMV with an equivalent concentration of fluorescent dye, showed that both toxins induce a fast fluorescence decrease, smaller than the hyperpolarization seen with the vesicles (Fig. 1Ab). This artifact, which may result from electrostatic interaction of the toxins with the dye, was determined under each ionic condition and subtracted in all subsequent experiments (Fig. 1Aa,b). The hyperpolarization (9 ± 3 mV, $n = 23$) and the higher sensitivity of the vesicles to external K^+ are consistent with a toxin-induced increase in K^+ permeability, possibly mediated by ion channel opening.

3.3. Ion selectivity and specificity

It has been reported that CryIA(a) toxin increases cation permeability non-selectively, even for divalents, in BBMV from *Bombyx mori* [15]. To explore the selectivity of CryIC and CryID responses in *S. frugiperda* BBMV, MeGluCl was replaced by NaCl in the external media. Under this condition, which is more physiological, both CryIC and CryID toxins induced a depolarization, even though the toxin addition artifact decreases fluorescence (Fig. 1B). This response is due to the electrogenic uptake of Na^+, and suggests that the toxin-induced permeability pathway is not very selective among monovalent cations.

As mentioned above, the cation permeability of BBMV increases after toxin addition (100 nM CryID, see Fig. 1A). In this condition ion selectivity can be estimated from the ratio of the slope of the fluorescence rises which result from additions of increasing concentration of a certain cation vs. that of K^+ (m_{X^+}/m_{K^+}). Since BBMV have intrinsic cation permeabilities, the response to serial additions of each cation was determined in the absence of toxin, as described earlier, and the slope subtracted from those obtained in its presence. Table 1 shows the selectivity series for the intrinsic permeability of BBMV

Table 1
Relative ion permeability

Cation	BBMV intrinsic permeability (m_{X^+}/m_{K^+})	CryID-induced permeability (m_{X^+}/m_{K^+})*
K^+	1.00	1.00
Na^+	0.83	0.70
Li^+	0.98	0.56
Cs^+	1.15	0.32
Rb^+	0.83	0.65
NH_4^+	1.25	0.79

Calculations were as described in section 3.3. m_{X^+}, slope of the fluorescence changes induced by different XCl additions to BBMV; X^+, different monovalent cations. m_{K^+}, slope for KCl additions. *Estimated relative permeability of the toxin CryID-induced pores after subtracting the intrinsic channel relative permeability. All standard deviations were less than 5% ($n = 5$).

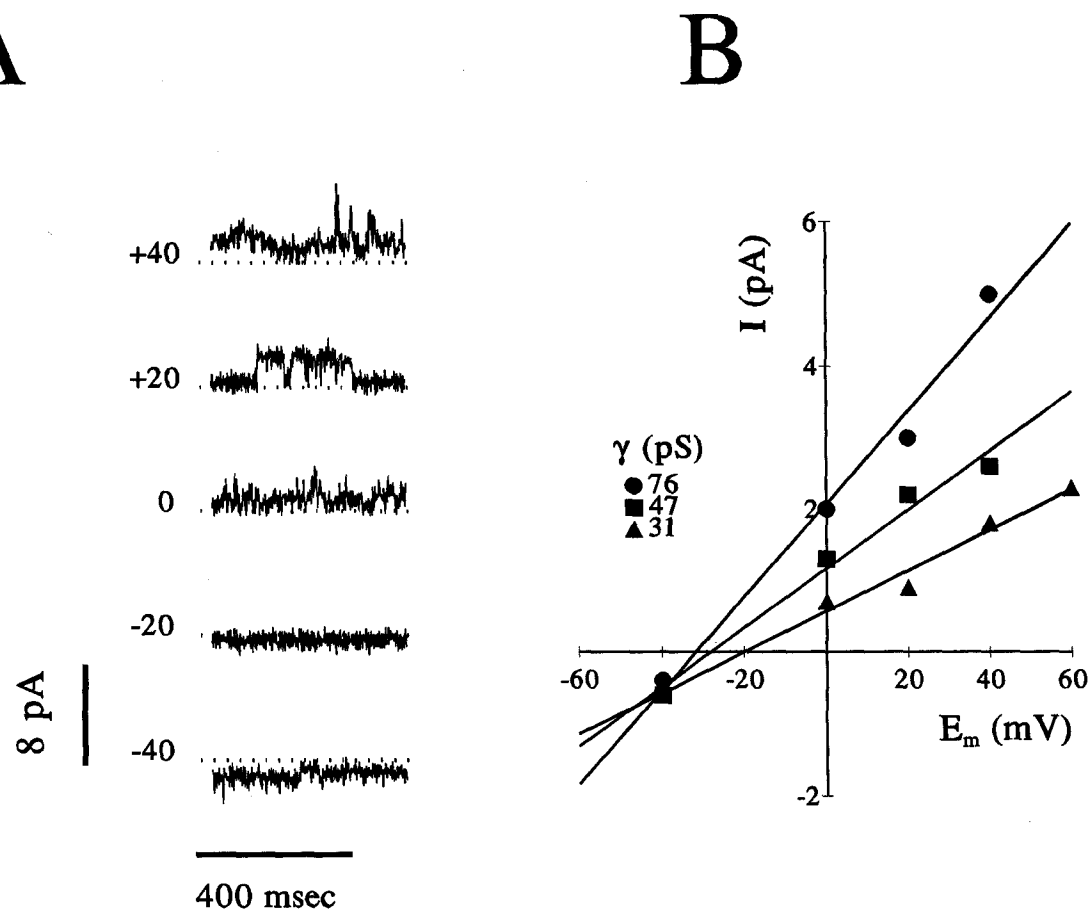

Fig. 2. Representative single channel records (A) and *I–V* curves from the most common current transitions (B) of BBMV from *S. frugiperda* fused to a diPPC lipid bilayer. Records were obtained in 550 mM KCl, 5 mM Tris-HCl, 0.5 mM EDTA, pH 9.0, *cis*; 150 mM KCl, 5 mM Tris-HCl, 0.5 mM EDTA, pH 9.0, *trans*. Holding potentials in mV are indicated on the left of each record. Zero current level is indicated by dashed lines. E_R was around −20 mV.

($NH_4^+ > CS^+ > K^+ > Li^+ > Rb^+ = Na^+$) and for that induced by the toxin ($K^+ > NH_4^+ > Na^+ > Rb^+ > Li^+ > Cs^+$). As the initial response triggered by the toxin, the sustained permeability change is cationic, although not very K^+ selective.

Osmotic swelling experiments in BBMV from *Manduca sexta* have indicated that CryIA(c) toxin increases anion permeability [16]. Experiments where MeGluCl was substituted by MeGlu-methanesulfonate in the incubation buffer showed that the toxin does not induce anion permeability changes, but a hyperpolarization (8 ± 2 mV, $n = 3$) due to K^+ efflux (as in Fig. 1A). However, intrinsic anionic channels may exist in these vesicles.

The coleopteran-specific toxin (CryIIIA) and the non-toxic lepidopteran-specific toxin (CryIA(c)) were used to determine the specificity of the effect. Neither toxin affected BBMV intrinsic permeability, even at higher concentrations (500 nM and 200 nM, respectively). In addition, the CryIC and CryID toxins lost their insecticidal activity and their capacity to change the permeability of the vesicles when boiled.

Although the CryIC toxin is 2 fold more toxic to *S. frugiperda* than CryID toxin, it is less potent in inducing permeability changes to the BBMV. However, the toxicity assays were performed with 1st instar larvae, in contrast to the permeability assays which were performed with BBMV purified from the last instar. In BBMV isolated from the 2nd and 3rd instar we have found that CryIC and CryID have similar effects (data not shown). It is possible that the CryIC and CryID receptor density in BBMV varies during larval development.

3.4. Pharmacological characterization

Previously it was reported that the CryIAs-induced permeability increase is very sensitive to external Ba^{2+} and Ca^{2+} [3]. Table 2 compares the effect of some K^+ channel blockers and Ca^{2+} on the intrinsic channels of BBMV and on their response to CryID toxin. The intrinsic channels are more sensitive to

Table 2
Effect of K^+ channel blockers and Ca^{2+} on the in vitro activity of the CryID toxin

K^+ channel blocker	Intrinsic channels		CryID channels	
	A	B	A	B
Ba^{2+}	400 ± 45 μM	(50%)	200 ± 20 μM	(50%)
Ca^{2+}	400 ± 40 μM	(50%)	600 ± 40 μM	(50%)
Cs^+	10 ± 2.5 mM	(50%)	100 ± 10 mM	(0%)
TEA	30 ± 5.0 mM	(50%)	200 ± 20 mM	(26%)
4-AP	4 ± 0.5 mM	(50%)	30 ± 5 mM	(0%)

A, channel blocker concentration (± S.D., $n = 5$). B, % of K^+ permeability inhibition. IC_{50} were determined from dose–response curves.

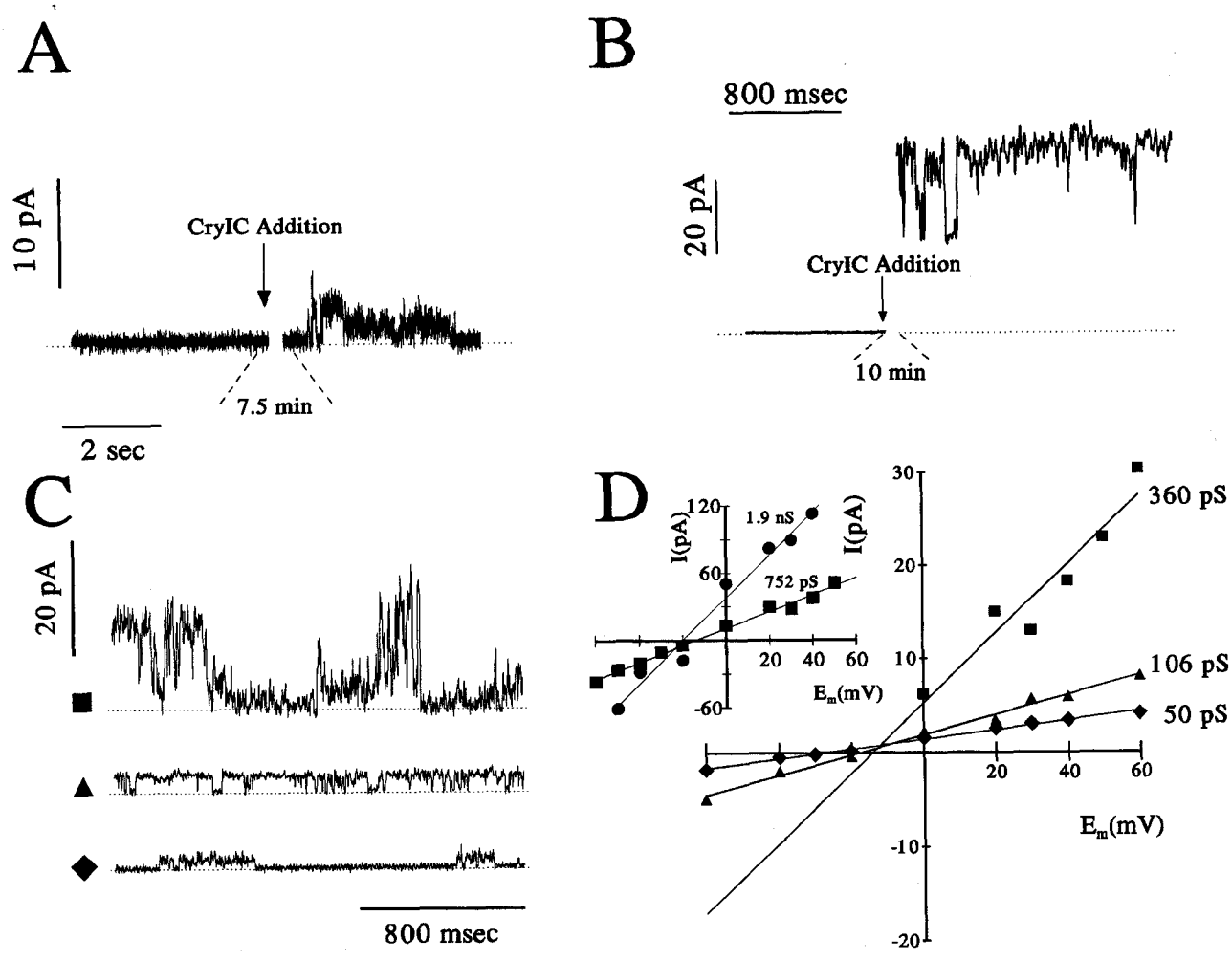

Fig. 3. Cation channels formed by CryIC toxin in bilayers with fused BBMV. Ionic conditions were as in Fig. 2. (A) Single channel transitions observed 7.5 min after 10.6 nM CryIC addition. (B) Macroscopic response observed at 10 min with the same toxin concentration. (C) Representative single channel transitions at 0 mV from bilayers containing BBMV exposed to 1.3–10.6 nM CryIC toxin. The dotted lines indicate the zero current level. E_R were close to −20 mV, indicating that these channels are cationic. (D) *I–V* plot of the single channel transitions presented in C. The insert in the left side also shows the *I–V* curve of a macroscopic response (γ = 1.9 nS).

Ca^{2+}, Cs^+, TEA^+ and 4-AP, but less sensitive to Ba^{2+}. The distinct ion selectivity and sensitivity towards various blockers of the intrinsic and the CryID-induced permeability changes suggest that they are different.

3.5. Toxin responses of planar lipid bilayers containing BBMV

The previous results suggest that cation channels mediate the toxin effects. Thus, toxin-induced single channel activity was examined in planar bilayers. Fig. 2A illustrates the most frequent single channel transitions detected in PLB to which BBMV were fused (see section 2). These experiments were performed having a 3.7 fold higher KCl concentration in *cis* than in *trans*. Single channel conductances of 31, 47 and 76 pS were calculated from *I–V* curves of these transitions which have reversal potentials of ~−20 mV, indicating that these channels are cation-selective with a P_{K^+}/P_{Cl^-} ~ 5.7 (Fig. 2B).

The endogenous BBMV channels washout, lasting only 1–2 min. This makes their characterization difficult; however, in 2/3 experiments made in 100 mM CsCl *cis*/100 mM KCl *trans*, no single channel activity was detected after BBMV addition, although channels appeared after nM toxin addition. These preliminary results suggest that the endogenous channels are blocked by Cs^+ while those induced by the toxin are not, in agreement with the membrane potential experiments.

It has been shown that the purified toxins alone can induce single channel activity in PLB [10–13]. We repeated these experiments and never saw channel activity (n = 25) unless >5 μM toxin was used. The single channel transitions observed with μM concentrations of toxin were cationic and similar to those described earlier [10–13].

The response to CryIC of PLB containing BBMV was tested. Two types of toxin responses are illustrated in Fig. 3: one involving few channels (Fig. 3A,C), and a macroscopic conductance increase (Fig. 3B) where some large single channel transitions can be resolved. *I–V* curves of the toxin-induced single channel events (Fig. 3D) or from macroscopic responses (Fig. 3D insert, 1.9 nS curve) measured under a 3.7 fold KCl gradient indicated that the toxin-activated channels are cationic. Toxin responses were observed in 15/17 experiments where the conductance increase ranged from 2 to 40 fold. The

time to induce a response in bilayer experiments depended on the toxin concentration. Low concentrations (0.6–3 nM) required up to 45 min ($n = 13$), while at higher concentrations (5–10.6 nM) the response was seen after 0–10 min ($n = 3$). Fig. 3C shows examples of the toxin-induced single channel transitions 752, 360, 106 and 50 pS in bilayers containing BBMV. The symbol on the left side of each current record corresponds to the I–V curve for that channel (Fig. 3D).

This work presents additional evidence that BBMV contain a functional *Bt* toxin receptor. Both in vesicles and in PLB, ICP after interacting with their receptors induce an increase in cation membrane permeability involving ion channels. The responses were observed at toxin concentrations (nM) which correlate with the in vivo lethal doses and with the range needed to inhibit amino acid uptake into BBMV of other insects [7]. The results indicate that midgut cell lysis results from receptor mediated toxin-induced cation pore formation.

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