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# Analysis of the Properties of *Bacillus thuringiensis* Insecticidal Toxins Using a Potential-sensitive Fluorescent Probe

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**Abstract.** A potential-sensitive fluorescent probe, 3,3′-dipropylthiadicarbocyanine iodide, was used to analyze, at pH 7.5 and 10.5, the effects of *Bacillus thuringiensis* toxins on the membrane potential generated by the efflux of $K^+$ ions from brush border membrane vesicles purified from the midgut of the tobacco hornworm, *Manduca sexta*. Fluorescence levels were strongly influenced by the pH and ionic strength of the media. Therefore, characterization of the effects of the toxins was conducted at constant pH and ionic strength. Under these conditions, the toxins had little effect on the fluorescence levels measured in the presence or absence of ionic gradients, indicating that the ionic selectivity of their pores is similar to that of the intact membrane. Valinomycin greatly increased the potential generated by the diffusion of $K^+$ ions although membrane permeability to the other ions used to maintain the ionic strength constant also influenced fluorescence levels. In the presence of valinomycin, active toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1C and Cry1E) efficiently depolarized the membrane at pH 7.5 and 10.5.

**Key words:** Insecticidal toxins — Membrane potential — Brush border membrane vesicles — diS-$C_3(5)$ — *Bacillus thuringiensis* — *Manduca sexta*

## Introduction

*Bacillus thuringiensis* is a widely used alternative to chemical insecticides for the control of insect pests. During sporulation, this Gram-positive bacterium produces crystalline parasporal inclusion bodies containing insecticidal proteins (δ-endotoxins). Following their ingestion by insect larvae, the proteins are solubilized and converted to active toxins by midgut proteases. Activated toxins act by forming pores after binding to specific receptors at the surface of the insect midgut luminal membrane [22, 37, 38].

The pH of the lepidopteran midgut lumen varies from about 8 to above 12 depending on species and region of the midgut [9, 10]. The alkaline and reducing conditions found in the lepidopteran midgut play an important role in the solubilization of the crystals and proteolytic activation of the toxins [1, 19]. High pH is also thought to play an important role in the activity of the activated toxins [37, 38, 56], although most previous studies have been carried out at near neutral pH values. Recently, however, the effect of alkaline pH was found to vary considerably for different toxins [45].

The pores formed by *B. thuringiensis* toxins have been shown, using different experimental approaches, to allow the passage of a variety of neutral and charged solutes [6–8, 15, 21, 23, 45, 48, 49, 52]. They are nevertheless recognized as being cation-selective [21, 26, 39–41, 43, 47], although anion-selective pores have also been observed at pH 6.0 [39]. Fluorescence measurements using a membrane potential-sensitive probe, 3,3′-dipropylthiadicarbocyanine iodide (diS-$C_3(5)$), have been used to characterize pore formation by *B. thuringiensis* toxins in insect brush border membrane vesicles [26, 47]. Conclusions drawn from such experiments, regarding the biophysical properties of the pores, were based on the assumption that the observed changes in fluorescence were due exclusively to changes in membrane potential. Other factors, such as pH and ionic strength, could nevertheless influence fluorescence measurements by

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altering the state of aggregation of the dye and the extent of dye binding to membranes and proteins [16, 53, 58].

In the present study, fluorescence measured with diS-$C_3$(5) and vesicles isolated from the midgut of *Manduca sexta* was shown to be strongly influenced by these factors and non-linearly related to membrane potential. The intrinsic cationic selectivity of the membrane was poorly affected by the toxins, but could be increased substantially by addition of the $K^+$ ionophore, valinomycin. Active toxins efficiently reduced the membrane potential generated by valinomycin-induced efflux of $K^+$ ions from the vesicles, at both pH 7.5 and 10.5.

## Materials and Methods

### Preparation of Membrane Vesicles

Whole midguts were isolated from 5th-instar *M. sexta* larvae (Carolina Biological Supply Company, Burlington, NC), freed of attached Malpighian tubules and luminal contents, rinsed thoroughly with ice-cold 300 mM sucrose, 17 mM Tris/HCl (pH 7.5) and 5 mM EGTA, and stored at −80°C until use. Brush border membrane vesicles were prepared as described previously [57]. The final membrane preparation was resuspended in 10 mM HEPES/KOH (pH 7.5) and stored at −80°C until use. In preparation for the experiments, vesicles were centrifuged at 12,200 × *g* for 20 min, resuspended at 2.0 mg protein/ml in the solution with which they were to be loaded, as specified in the figure legends, and allowed to equilibrate overnight at 4°C. Vesicles were kept at 4°C until injected into the spectrometer cuvette, except in the case where a 60 min incubation at room temperature was performed.

### Toxins

Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C and Cry1E toxins were produced as insoluble inclusions in *Escherichia coli*, solubilized, trypsin-activated, and purified by fast protein liquid chromatography as described earlier [29].

### Fluorescence Measurements

Fluorescence measurements were carried out at 23°C in a Spex Fluorolog CM-3 spectrofluorometer (Jobin Yvon Horiba, Edison, NJ) at a frequency of 10 Hz, an excitation wavelength of 620 nm and an emission wavelength of 670 nm. Polystyrene cuvettes containing 1.5 μM of diS-$C_3$(5) (Molecular Probes, Eugene, OR), from a 1 mM stock solution in dimethyl sulfoxide, and the appropriate buffer solution, as specified in the figure legends, were stirred for 10 min, in the dark, before each experiment, to allow fluorescence to reach a steady level [17, 42]. A few seconds after the beginning of the recording, vesicles were injected into the cuvette at a final concentration of 10 μg protein/ml. In some experiments, 7.5 μM valinomycin (Sigma, St. Louis, MO) was added to the cuvette, from a 5 mM stock solution in ethanol. Fluorescence values were normalized relative to the level measured before adding the vesicles. For each experimental condition tested, the fluorescence level attained in the absence of a membrane potential was measured by injecting vesicles into the same solution as that with which they were loaded. Unless specified otherwise, data are means ± SEM of 3 experiments, each performed with a different vesicle preparation. Experimental values for each individual experiment consisted of the average of four replicates obtained using the same vesicle preparation. Statistical comparisons were made with the two-tailed unpaired *t* test.

## Results

### Fluorescence Measurements Are Influenced by pH and Ionic Strength

Vesicles loaded with 150 mM KCl were first injected into cuvettes containing either an isotonic solution of sucrose or the same solution as that with which they were loaded (Fig. 1*A*). This provided a measurement of the fluorescence levels attained in the presence and absence of a membrane potential. At both pH 7.5 and 10.5, a stronger decrease in fluorescence intensity was observed in the presence of a membrane potential than in its absence, as expected when using a potential-sensitive dicarbocyanine probe. Preferential efflux of potassium ions, relative to chloride ions, generates an inside-negative potential that causes the positively charged dye to accumulate within the vesicles and to associate with their membrane. Concentration of the dye results in fluorescence quenching due to the aggregation of dye molecules [36, 42, 53]. The generation of an inside-negative membrane potential (Fig. 1*A*) confirms that the membrane has a higher permeability to $K^+$ than to $Cl^-$ ions [26, 35].

A much more substantial drop in fluorescence was observed at pH 10.5 than at pH 7.5, following the injection of vesicles (Fig. 1*A*). The fluorescence of diS-$C_3$(5) is known to be independent of pH in solution, but strongly pH-dependent in the presence of cells or cell membranes [11, 16, 53]. A direct effect of pH on the fluorescence of the dye itself would not be apparent in the figures presented herein because of the normalization of the data. The difference between fluorescence levels observed at pH 7.5 and pH 10.5 can therefore be attributed to an alteration of the proportion of dye molecules that bind to the vesicles, due to the titration of charges at the surface of the membrane.

To examine the additional possibility of an effect of ionic strength, a series of experiments similar to that illustrated in Fig. 1*A* was performed with vesicles loaded with different concentrations of KCl and injected in isotonic solutions of sucrose (Fig. 1*B*). Under these conditions and according to the Goldman–Hodgkin–Katz equation, membrane potential depends on the ratio of intravesicular and extravesicular KCl concentrations. Because under each set of experimental conditions an equal volume of vesicles was injected into the cuvettes, the final ratio of intravesicular and extravesicular KCl concentrations was constant and either equal to 1, in the absence of a membrane potential (*open bars*), or to approximately

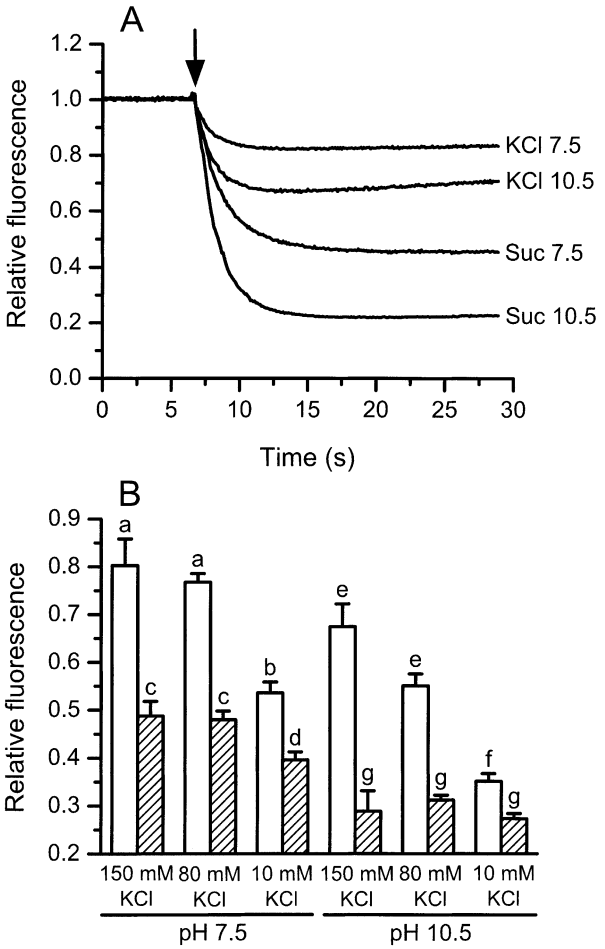


**Fig. 1.** Effect of ionic strength. (*A*) The vesicles were loaded with 150 mM KCl and 10 mM of either HEPES/tetramethylammonium hydroxide (TMAOH) (pH 7.5) or CAPS/TMAOH (pH 10.5). At the time indicated by the arrow, vesicles were injected into cuvettes containing either the same solution as that with which they were loaded (KCl) or 300 mM sucrose (*Suc*) and 10 mM of either HEPES/TMAOH (pH 7.5) or CAPS/TMAOH (pH 10.5). Each trace corresponds to the average of 4 experiments performed with the same vesicle suspension. (*B*) The vesicles were loaded with KCl at the indicated concentrations and 10 mM of either HEPES/TMAOH (pH 7.5) or CAPS/TMAOH (pH 10.5), and injected into cuvettes containing either the same solution as that with which the vesicles were loaded (*open bars*) or an isotonic solution of sucrose (*hatched bars*). Data are means ± SEM of the minimum relative fluorescence recorded after injection of the vesicles, measured in 3 experiments, each performed in quadruplicate with a different vesicle preparation. Means labeled with the same letter are not significantly different ($p > 0.05$).

150, under conditions used to generate a membrane potential (*hatched bars*), independently of the ionic strength. Fluorescence levels decreased markedly as the ionic strength of the solution was decreased, at both pH values (Fig. 1*B*). This effect was more pronounced in the absence of membrane potential (*open bars*), where ionic strength is varied in both the intravesicular and extravesicular solutions, than in its presence (*hatched bars*), where ionic strength is varied inside the vesicles, but very little in the extravesicular milieu.

## Membrane Permeability to Ions Other than $K^+$

Given this strong effect of ionic strength, similar experiments were carried out under conditions where ionic strength was maintained constant and equal on both sides of the membrane with tetramethylammonium chloride (TMACl) (Fig. 2*A*). In the absence of a membrane potential (*open bars*), the decrease in fluorescence levels with decreasing KCl concentration was greatly attenuated in comparison with that observed in the previous experiment (Fig. 1). In the presence of a membrane potential generated by a $K^+$ concentration ratio of approximately 150, however, fluorescence levels increased gradually as the KCl concentration was decreased (*hatched bars*, Fig. 2*A*). This result is in contradiction to the Nernst equation, but consistent with the Goldman–Hodgkin–Katz equation, indicating that the membrane has a non-negligible permeability to either $Cl^-$ or $TMA^+$ ions or both.

Under conditions of constant ionic strength, the difference between the fluorescence levels measured in the presence and absence of a membrane potential was also considerably reduced, suggesting that the potential generated was relatively small. The membrane potential generated by the efflux of $K^+$ ions was nevertheless greatly increased in the presence of the $K^+$ ionophore valinomycin, as evidenced by the much larger differences observed between the fluorescence values measured under conditions designed to generate a potential (*hatched bars*) or not (*open bars*) (Fig. 2*B*). As was observed in the absence of valinomycin, these differences were also gradually attenuated as the $K^+$ concentration was reduced, indicating that even in the presence of valinomycin, the membrane permeability to $TMA^+$ or $Cl^-$ or both is non-negligible. Transmembrane potentials generated in the presence of valinomycin and estimated from fluorescence measurements were not increased by replacing TMACl by the chloride salts of either sodium, guanidinium, choline or N-methyl-D-glucamine (NMDGCl), or by tetramethylammonium gluconate (*data not shown*).

## Relationship between Fluorescence and $K^+$ Transmembrane Gradient

Evidence for a complex relationship between fluorescence and membrane potential is apparent from calibration curves obtained at constant ionic strength in the presence of valinomycin (Fig. 3). Relative fluorescence intensity varied as a sigmoid-like function of the logarithm of the intravesicular to extravesicular $K^+$ concentration ratio ($K_i/K_o$), both at pH 7.5

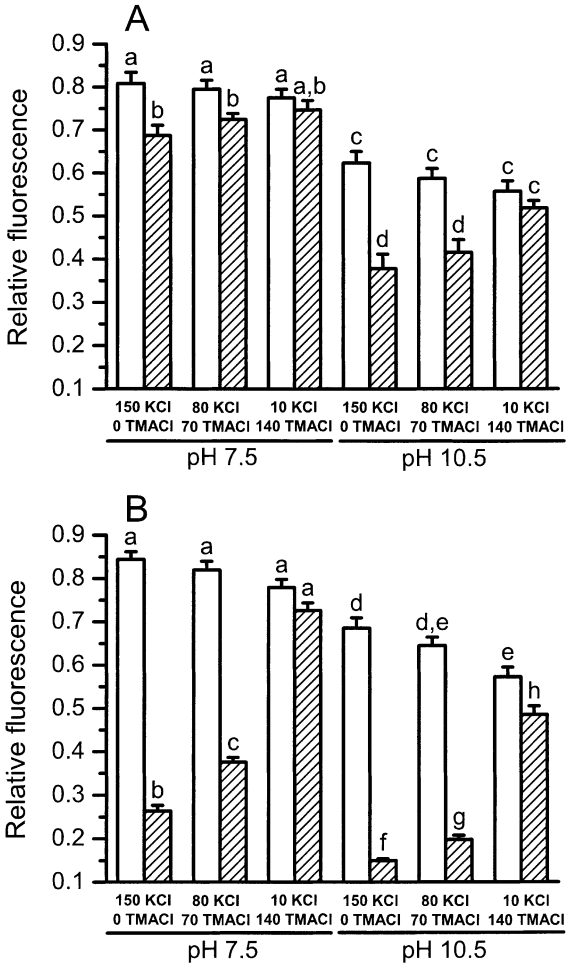


**Fig. 2.** Effect of valinomycin. Vesicles were loaded with the indicated concentrations of KCl and TMACl (in mM) and 10 mM of either HEPES/TMAOH (pH 7.5) or CAPS/TMAOH (pH 10.5). They were injected into solutions identical to those with which they were loaded (*open bars*) or in 150 mM TMACl and 10 mM of either HEPES/TMAOH (pH 7.5) or CAPS/TMAOH (pH 10.5) (generating an intravesicular/extravesicular $K^+$ ratio of approximately 150, *hatched bars*). Experiments were carried out either without valinomycin (*A*) or with 7.5 μM of the ionophore (*B*). Means labeled with the same letter are not significantly different ($p > 0.05$).

and 10.5. This relation clearly contrasts with the Nernst equation, since a linear relationship is only observed at $K_i/K_o$ ratios of up to approximately 5. For larger ratios, up to about 30, the slope of the curve gradually becomes steeper. Finally, for still larger ratios, the slope levels off gradually and monotonously due to the conductance of the membrane to ions other than $K^+$, as predicted by the Goldman–Hodgkin–Katz equation. Although non-linear calibration curves are not uncommonly found with a variety of cyanine dyes [4, 11, 17, 33, 46], linear relationships have been reported for experiments performed with diS-$C_3$(5) and brush border membrane vesicles isolated from the midguts of lepidopteran insects [26, 47]. These latter calibration curves, however, extended over a narrower range of intravesicular to extravesicular $K^+$ ratios than those shown in Fig. 3, thus excluding the higher $K^+$ ratios at which the curves depart most from linearity.

Considering the complexity of the calibration curves, it appears hazardous to calculate membrane potentials from such fluorescence measurements, especially in the absence of reliable data on the permeabilities of the different ionic species present. However, reliable values can be obtained for fluorescence levels corresponding to the linear region of the calibration curve. For instance, at pH 7.5 in the absence of valinomycin (Fig. 2*A*), membrane potentials of −43, −25 and −11 mV are obtained for the conditions where the vesicles were loaded with 150 mM KCl, 80 mM KCl/70 mM TMACl and 10 mM KC1/140 mM TMACl, respectively. These potentials are in good agreement with the low $K^+$ selectivity reported for the intrinsic channels of the brush border membrane of *Spodoptera frugiperda* ($P_K/P_{Cl} \sim 5.7$) [26] and *Lymantria dispar* ($P_K/P_{Cl} = 1.5$–$8.0$) [35]. Indeed, using the mean $P_K/P_{Cl}$ permeability ratio reported for the ionic channels found in the brush border of *L. dispar*, $P_K/P_{Cl} \cong 4.3$ [35], and assuming, for the sake of simplicity, a negligible permeability for $TMA^+$ ions, the Goldman–Hodgkin–Katz equation predicts potentials of −42, −30 and −6 mV, respectively, for the experimental conditions described above.

## Effect of *B. thuringiensis* Toxins

Five *B. thuringiensis* toxins, Cry1Aa, Cry1Ab, Cry1Ac, Cry1C and Cry1E, which are active against *M. sexta*, and Cry1B, which is inactive against the same insect [18, 50, 51], were first assayed for their effects on membrane potential by injecting vesicles, loaded with KCl and preincubated for an hour with one of the toxins, into cuvettes containing an isotonic solution of TMACl, in the absence of valinomycin (Figs. 4*A* and *B*). At both pH 7.5 and 10.5, all toxins tested had little effect on the small membrane potential generated by the outwardly-directed 150 mM $K^+$ gradient. Similar results were obtained when TMACl was replaced by NMDGCl (Fig. 4*C*), although experiments with this latter salt were only carried out at pH 7.5 because the N-methyl-D-glucamine ion, which has a pKa of 9.62 [20], is predominantly uncharged at pH 10.5. Despite similar conditions, Fig. 4*A* and 4*B* (no toxin) show somewhat smaller membrane potentials than Fig. 2*A* (150 mM KCl). This difference appears to result from the fact that, in the experiments presented in Fig. 4, the vesicles were preincubated for 60 min at room temperature before the fluorescence measurements. This incubation appears to slightly, but consistently,

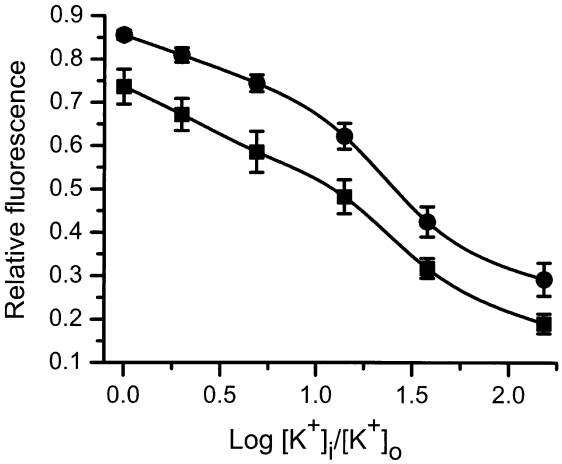


**Fig. 3.** Calibration of fluorescence as a function of the transmembrane potassium concentration ratio. Vesicles were loaded with 150 mM KCl and 10 mM of either HEPES/TMAOH (pH 7.5) or CAPS/TMAOH (pH 10.5). They were injected into cuvettes containing the appropriate concentration of KCl, enough TMACl to maintain osmolarity and ionic strength constant, 7.5 μM valinomycin and 10 mM of either HEPES/TMAOH (pH 7.5) (●) or CAPS/TMAOH (pH 10.5) (■).

modify the ionic selectivity of the membrane and thus slightly reduce the potential generated. In agreement with this conclusion, rundown of intrinsic brush border membrane channels has been previously reported [26, 35].

On the other hand, in the presence of valinomycin, Cry1Aa, Cry1Ab, Cry1Ac, Cry1C and Cry1E caused a strong and dose-dependent increase in fluorescence corresponding to a decrease in membrane potential at both pH values (Fig. 5). In contrast, the non-toxic Cry1B [18] was unable to depolarize the membrane.

Similar results were obtained at pH 7.5 in the presence of NMDGCl instead of TMACl (*data not shown*). Noteworthy is the fact that among active toxins, Cry1C appears relatively insensitive to pH while the other toxins show a lower activity at pH 10.5 than at pH 7.5. The saturation of the curves around 50 pmol toxin/mg membrane protein shows that there is a limited number of toxin molecules that can insert into the membrane and form pores, due to the presence of a finite number of binding sites. It is also interesting to note that the toxins were not able to depolarize the membrane completely, showing that the membrane permeability induced by the toxins to the different ions does not overcome that of valinomycin for $K^+$ ions.

## Discussion

The results of the present study demonstrate that membrane potential measurements using a fluorescent probe, in insect midgut brush border membrane vesicles, are strongly influenced by pH and ionic strength, as previously reported for other systems [16, 53, 58]. Keeping these factors constant allowed the design of an efficient and reliable method for assaying the pore-forming activity of *B. thuringiensis* toxins. Using this assay, membrane permeability was shown to be slightly greater for $K^+$ than for $Cl^-$ ions, as previously reported [26, 35]. The present study also demonstrates that active toxins have little effect on the small membrane potentials generated by $K^+$ efflux from intact vesicles, but strongly reduces the larger potentials generated in the presence of valinomycin.

The fact that membrane potential is increased substantially in the presence of valinomycin may suggest that this factor could modulate the properties of the pores formed by these toxins. Although this possibility cannot be excluded, membrane potential is clearly not required for toxin activity since these proteins can increase considerably the permeability of brush border membrane vesicles in its absence, as observed in light scattering experiments performed with non-electrolytes [6, 8, 45, 49]. Therefore, in the absence of valinomycin, the lack of a strong effect of the toxins on membrane potential, despite the large increase in permeability, indicates that the ionic selectivity of the pores formed by *B. thuringiensis* toxins does not differ significantly from that of the midgut brush border membrane. In agreement with this observation, similar reversal potentials were reported for the endogenous channels of insect midgut brush border membranes fused to planar lipid bilayers [26, 35] and for those formed by toxins in these membranes [26]. Our results are also consistent with the fact that the pores formed by *B. thuringiensis* toxins, despite having a measurable cation selectivity [21, 26, 39–41, 43, 49], allow the passage of a variety of cations and anions across the membrane [6–8, 15, 21, 23, 45, 48, 49, 52]. Active toxins increase not only the efflux of $K^+$ ions from the vesicles, but also the influx of $TMA^+$ or $NMDG^+$ and the efflux of $Cl^-$, thus preventing the generation of a strong membrane potential. In the midgut epithelium, membrane potential and a strong potassium electrochemical gradient are generated by a vacuolar-type proton ATPase coupled with a $K^+/H^+$ antiporter [54, 55]. Both are efficiently abolished in the presence of the poorly selective pores formed by the toxins [24, 34]. In the midgut, the toxins clearly act by increasing membrane permeability to various ions rather than by modifying the membrane's ionic selectivity (Fig. 4). Accordingly, active toxins completely abolish membrane potential in isolated midguts [34] but only partially in experiments performed with brush border membrane vesicles (Fig. 5). In these latter experiments, the final potential depends on the relative contribution of intrinsic membrane channels, toxin, and ionophore to the permeability and ionic selectivity of the membrane.

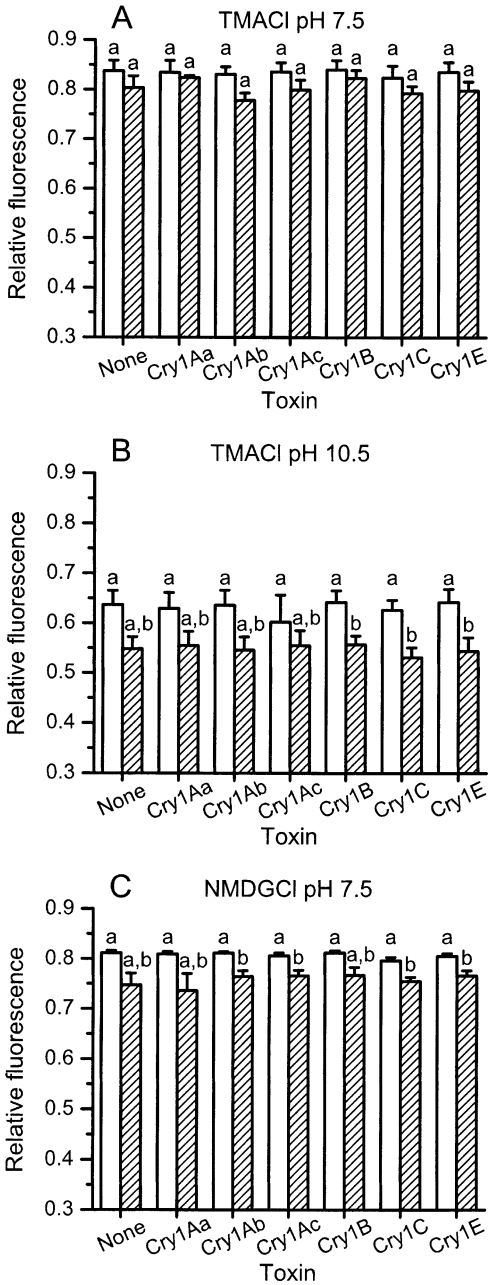


**Fig. 4.** Effects of *B. thuringiensis* toxins on the membrane potential of *M. sexta* brush border membrane vesicles. The vesicles were loaded with 150 mM KCl and 10 mM of either HEPES/TMAOH (pH 7.5) (*A*, *C*) or CAPS/TMAOH (pH 10.5) (*B*). They were preincubated for 60 min with 150 pmol toxin/mg membrane protein and injected into cuvettes containing a solution identical to that with which they were loaded (*open bars*) or 150 mM of either TMACl (*A*, *B*) or NMDGCl (*C*) and 10 mM of either HEPES/TMAOH (pH 7.5) or CAPS/TMAOH (pH 10.5) (*hatched bars*). Means labeled with the same letter are not significantly different ($p > 0.05$).

The above finding of a negligible effect of toxins on membrane potential, in the absence of an artificial membrane potential generated by an ionophore, contradicts those of previous reports in which it was concluded that *B. thuringiensis* toxins cause either a hyperpolarization [2, 3, 12, 13, 25, 26, 30–32, 44, 47, 59] or a depolarization [25, 26] in lepidopteran insect midgut brush border membrane vesicles. Furthermore, while in our experiments fluorescence was measured after the vesicles had preincubated for one hour with the toxins, to allow ample time for the pores to form, in most previous fluorescence studies [2, 3, 12, 13, 25, 26, 30–32, 44, 59], the observed changes in fluorescence occurred instantaneously upon addition of the toxin to the vesicles. In our hands, injection of toxin into cuvettes, after fluorescence levels were allowed to stabilize following the addition of the vesicles, never caused more than extremely small changes in fluorescence, comparable to the noise level observed in traces such as those shown in Fig. 1*A*. Rapid hyperpolarization was only observed when valinomycin was added instead of toxin. The absence of an instantaneous effect of *B. thuringiensis* toxins is in agreement with the results of numerous other studies, using different experimental systems, in which toxin-induced increases in membrane permeability only became apparent after approximately 10 seconds to several minutes [5, 6, 8, 14, 24, 34, 45, 48, 49].

In most previous fluorescence studies [2, 3, 12, 13, 25, 26, 31, 32, 44, 59], conclusions regarding the pore-forming ability of *B. thuringiensis* toxins as well as the properties of their pores were based on comparisons of the slopes of curves obtained by plotting changes in fluorescence levels, which occur in response to sequential additions of KCl to the vesicle suspension, against the $K^+$ equilibrium potential, calculated with the Nernst equation. Such calculations, however, are based on the assumption that the membrane has a negligible permeability to all ions except $K^+$. This assumption is not only contradicted by the results of the present study, but is also, in principle, incompatible with the data from light-scattering experiments in which osmotic swelling, in the presence of a salt, can only be detected if the membrane is permeable to both the anion and the cation [6, 21, 45]. In addition, sequential addition of KCl not only modifies the ionic strength of the solution in which the vesicles are suspended, but also its osmolarity. As clearly demonstrated in the present study, fluorescence levels are strongly affected by changes in ionic strength. Also changes in osmolarity undoubtedly bring about modifications in the volume of the vesicles and, consequently, in the intravesicular KCl concentrations on which calculations of $K^+$ equilibrium potentials are based. Because active toxins increase considerably the permeability of the membrane for KCl, such osmotic effects must be more pronounced in control vesicles than in those in which toxins are inserted. Finally, a clear indication that fluorescence measured under these conditions does not depend exclusively on membrane potential is

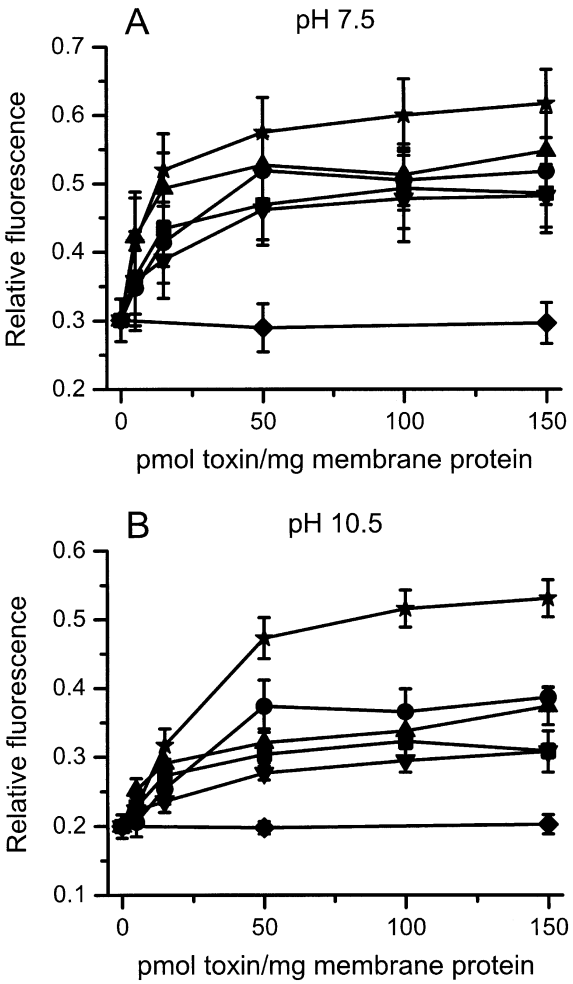


**Fig. 5.** Effects of *B. thuringiensis* toxins on the membrane potential generated by a $K^+$ gradient in the presence of valinomycin. Membrane vesicles loaded with 150 mM KCl and 10 mM of either HEPES/TMAOH (pH 7.5) (*A*) or CAPS/TMAOH (pH 10.5) (*B*) were preincubated for 60 minutes with the indicated concentrations of Cry1Aa (●), Cry1Ab (■), Cry1Ac (▲), Cry1B (◆), Cry1C (★) or Cry1E (▼). They were injected into cuvettes containing 150 mM TMACl, 7.5 μM valinomycin and 10 mM of either HEPES/TMAOH (pH 7.5) (*A*) or CAPS/TMAOH (pH 10.5) (*B*). Relative fluorescence levels measured in the absence of a membrane potential were 0.88 ± 0.01 at pH 7.5 (*A*) and 0.73 ± 0.01 at pH 10.5 (*B*).

apparent from the figures of several studies [2, 3, 26, 31, 32, 44] in which substantially different fluorescence levels are reported for measurements carried out under conditions where membrane potential must be equal to zero since identical $K^+$ concentrations are present on both sides of the membrane.

Although, in the present study, toxins had little effect on the membrane potential generated in intact vesicles, they strongly reduced the potential generated in the presence of valinomycin, indicating that the magnitude of the membrane permeability induced by the toxin is comparable to that of the ionophore although the ionic selectivity of the toxin pores is much smaller. The depolarizing effects of the toxins observed in the presence of valinomycin (Fig. 5) are qualitatively in agreement with published bioassay data [18, 50, 51], but do not reflect the approximately 4- to 12-fold higher $LC_{50}$ values reported for Cry1C and Cry1E than for Cry1Aa, Cry1Ab and Cry1Ac. Although several other cases have been described in which toxins readily form pores in brush border membrane vesicles [8, 27, 45, 49], isolated larval midguts [34] or dissociated midgut epithelial cells [28], despite a relatively modest toxicity toward larvae of the corresponding insect species, such differences remain to be explained. On the other hand, the effect of Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, and Cry1E, as measured by the present fluorescence assay (Fig. 5), correlates well with the toxins' pore-forming ability evaluated previously, using a light-scattering assay [45]. However, the lower pore-forming ability observed for Cry1C at pH 10.5, using the latter assay [45], was not observed with the current fluorescence assay. In fact, the fluorescence levels measured in the presence of Cry1C at pH 10.5 were significantly higher ($p < 0.05$) than those measured with the other five toxins. The major differences between light-scattering and fluorescence measurements are the magnitude and direction of the membrane potentials generated, and the fact that preincubation of the vesicles with the toxins is done at low ionic strength for light-scattering experiments and at high ionic strength for fluorescence experiments. Pore formation by Cry1C, and possibly other toxins, as well as the effect of pH on their activity, could therefore be influenced by the ionic strength of the buffer solutions and the membrane potential. These results stress the need for a detailed analysis of the effects of various physico-chemical factors found in the midgut lumen, including not only ionic strength and membrane potential, but also the presence of midgut proteases, for a better understanding of the mode of action of *B. thuringiensis* toxins.

In conclusion, the results of the present study illustrate the influence of pH and ionic strength on the measurements made with a membrane potential fluorescent probe on insect midgut brush border membrane vesicles and show that the relation between the fluorescence and membrane potential is relatively complex. However, taking these factors into account allowed the design of an improved fluorometric method for assaying toxin activity and studying the properties of their pores.

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