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BARIUM AND CALCIUM BLOCK *BACILLUS THURINGIENSIS* SUBSPECIES *KURSTAKI* δ-ENDOTOXIN INHIBITION OF POTASSIUM CURRENT ACROSS ISOLATED MIDGUT OF LARVAL *MANDUCA SEXTA*

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Summary

Ba^{2+} and Ca^{2+} prevent and reverse the Btk δ-endotoxin inhibition of the short-circuit current in isolated lepidopteran midgut. These findings support the K^+ pump–leak steady-state model for midgut K^+ homeostasis and the K^+ channel mechanism of Bt toxin action. They provide a new tool with which to study the interactions between Bt toxin and midgut cell membranes.

Introduction

The midgut epithelium of lepidopterous insects (Fig. 1) is refreshingly different from the gastrointestinal epithelia of vertebrates. This epithelium possesses a novel, electrogenic K^+ pump (Harvey & Nedergaard, 1964). The pump, with a unique K^+-ATPase, is restricted to the goblet cell (GC) apical membrane (GCAM) (Dow, Gupta, Hall & Harvey, 1983; Wieczorek, Wolfersberger, Cioffi & Harvey, 1986). The pump-generated potential difference (PD) gradient across the apical membrane exceeds 180 mV (lumen positive) which is sufficient to maintain passively a 1000-fold H^+ gradient (lumen alkaline), thought to be generated by the secondary transport of carbonate (Dow, 1984). Amino acid/K^+ symports, located in columnar cell (CC) apical membrane (CCAM), use the PD for nutrient uptake (Giordana, Parenti, Hanozet & Sacchi, 1985). The K^+ pump–leak steady state maintains a sevenfold elemental potassium concentration gradient (lumen high). The K^+ activity gradient is less than the elemental potassium gradient because the activity coefficient for K^+ in the alkaline lumen is low (Dow, 1986). The resulting alkalinity and high $[K^+]$ of the lumen are adaptations to the high tannin and potassium contents of dietary foliage (Dow, 1986). Agents which disrupt these vital PD, H^+ and K^+ gradients are candidates for environmentally safe insecticides (Harvey, Cioffi & Wolfersberger, 1986).

Many *Bacillus thuringiensis* subspecies produce bipyramidal parasporal crystals, composed of P1 protoxin protein molecules, which are lethal to susceptible lepidopterous larvae. Ingested crystals dissolve in the alkaline midgut contents and

Key words: K^+ channel, lepidopteran midgut, short-circuit current, K^+ transport.

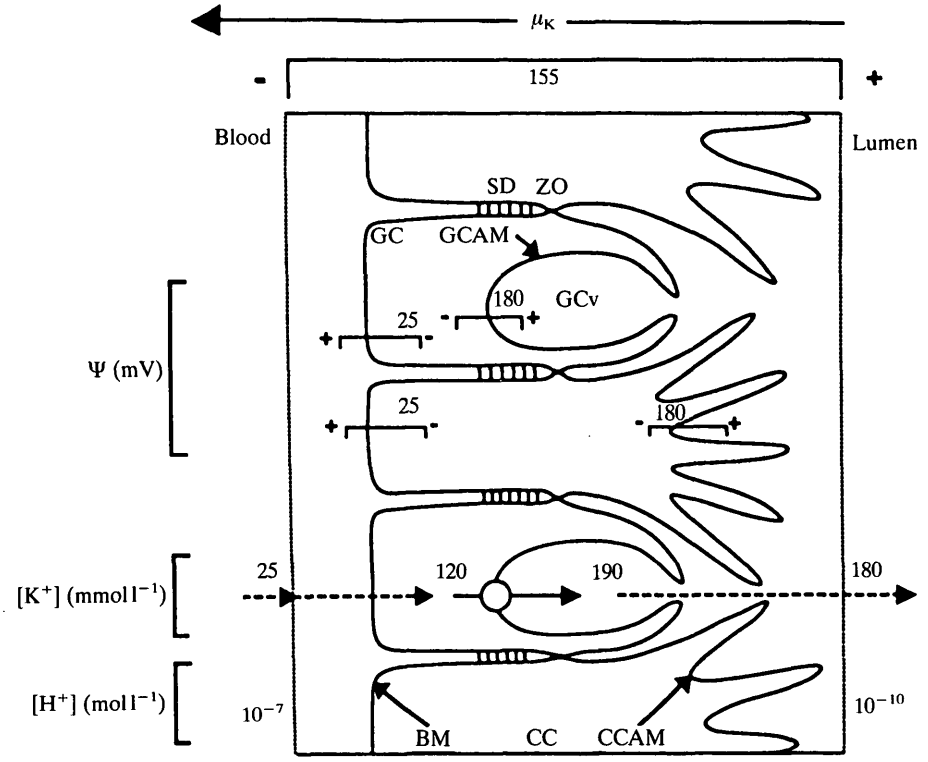

Fig. 1. Model for K^+ homeostasis in lepidopteran midgut (modified from Harvey, Cioffi & Wolfersberger, 1986). GC, goblet cell; GCAM, goblet cell apical membrane; CC, columnar cell; CCAM, columnar cell apical membrane; GCv, goblet cell cavity; BM, basement membrane; Ψ, transepithelial potential difference; μ_K, electrochemical gradient; SD, septate desmosome; ZO, zonula occludens.

the 130–140 kDa protoxin is digested by midgut proteases to a 55–70 kDa protease-resistant toxin (Aronson, Beckman & Dunn, 1986). Unlike the large protoxin protein, the smaller toxin diffuses readily across the peritrophic membrane (Wolfersberger, Spaeth & Dow, 1986*b*) and interacts with specific receptors on the CCAM of midgut cells (Luethy *et al.* 1986). Sacchi *et al.* (1986) have shown that lepidopteran-specific *Bacillus thuringiensis* δ-endotoxins (Bt toxin) from subspecies *thuringiensis* and *kurstaki* (Btk toxin) specifically increase the K^+ conductance of CCAM in the midgut of susceptible insects. This finding fulfils the prophecy made by Angus (1968) that Bt toxin might act 'on the membrane of the midgut epithelial cells affecting their selective permeability'. Wolfersberger, Hofmann & Luethy (1986*a*) suggest that the primary pathological action of Bt toxin is to form K^+ channels in midgut CCAM. The K^+ channels would shunt the pump-generated PD which, in turn, would disturb ion gradients, pH regulation and nutrient uptake, with eventual cytolysis of the cells and death of the insect.

Since Bt toxin disrupts the PD and K^+ and H^+ gradients by opening K^+ channels in the apical membrane, a K^+ channel-blocking agent should prevent Bt

toxin activity and spare the midgut from intoxication. Ba^{2+}, an almost universal K^+ channel blocker, is known to block existing K^+ channels in basal membranes of *Manduca sexta* midgut (Zeiske, Van Driessche & Zeigler, 1986). Ca^{2+} also blocks certain K^+ channels. Armstrong & Matteson (1986) showed that Ca^{2+} has a strong effect on K^+ channel closing in the squid axon and concluded that 'Ba latches the channel closed in the same way that Ca does, but more strongly'. In squid axon, the K^+ channel reopens when the cell is depolarized in the presence of low external $[K^+]$. In the isolated midgut no depolarization occurs and the external $[K^+]$ is high. Therefore, with respect to the midgut, Ca^{2+} might be expected to keep the Btk-induced K^+ channels permanently closed. We report here experiments which demonstrate Ba^{2+} and Ca^{2+} protection of midgut epithelium from Btk toxin damage. The results support the midgut model and the K^+ channel mechanism for Bt toxin inhibition discussed above. They suggest that the K^+ conductance increase induced in CCAM by Bt toxin proceeds through K^+ channels which are blocked by both Ba^{2+} and Ca^{2+}.

Materials and methods

Midguts of *M. sexta* were isolated and short-circuited as described by Harvey & Spaeth (1988). The midgut was mounted as a flat sheet in a removable aperture, which was inserted into a Lucite chamber equipped with electrodes to measure the transepithelial PD and to correct for the electrical gradient introduced when current is passed to null out this PD (Dow, Harvey, Wolfersberger & Boyes, 1985). The control bathing solutions consisted of 32 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} Tris-chloride and 240 mmol l^{-1} sucrose at pH 8·3, and were identical on both sides of the midgut. Under these conditions the short-circuit current (I_{sc}) is an accurate measure of net transepithelial K^+ transport (Cioffi & Harvey, 1981). Experiments were carried out when the isolated midgut was in a pseudo-steady state with the I_{sc} decaying exponentially at approximately 20 % h^{-1}. P1 protoxin was prepared from purified parasporal crystals of *B. thuringiensis* subsp. *kurstaki* by the method of Huber, Luethy, Ebersold & Cordier (1981). P1 protoxin was added to the lumen half-chamber to yield a final concentration of 50 nmol l^{-1}. Chloride salts of Ba^{2+}, Ca^{2+} or other potential effectors were added to the lumen half-chamber, along with appropriate balancers to the blood half-chamber, to increase final concentrations by 4 mmol l^{-1} unless otherwise stated.

Results

A control time course of the I_{sc} is shown in Fig. 2A (dotted trace). After Btk toxin at a final concentration of 50 nmol l^{-1} had been added to the lumen side at 45 min (Fig. 2A, solid trace), the I_{sc} rapidly dropped to zero. Btk toxin at 5 nmol l^{-1} is adequate to inhibit the I_{sc} completely. The excessive concentration of 50 nmol l^{-1} was used to ensure total saturation of Bt binding sites (Luethy *et al.* 1986) on the CCAM of the isolated midgut. The inhibition by Btk toxin reported

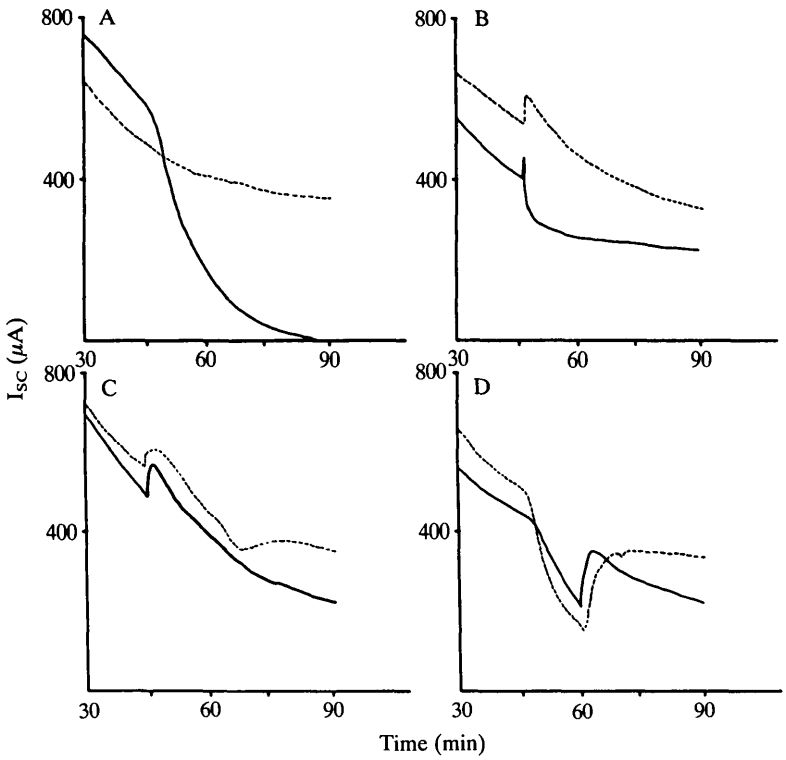

Fig. 2. Experimental traces showing the effects of Btk toxin and cations on the I_{sc} of the isolated *Manduca sexta* larval midgut. (A) (dotted trace) Control time course, no additions; (solid trace) 50 nmol l^{-1} Btk added at 45 min on lumen side. (B) (dotted trace) 4 mmol l^{-1} Ba^{2+} added at 45 min on lumen side; (solid trace) 4 mmol l^{-1} Ba^{2+} added at 45 min on blood side. (C) (dotted trace) 5 mmol l^{-1} Ca^{2+} and (solid trace) 6 mmol l^{-1} Ba^{2+} added on lumen side at 45 min followed by 50 nmol l^{-1} Btk at 60 min on lumen side. (D) 50 nmol l^{-1} Btk added at 45 min on lumen side followed by 5 mmol l^{-1} Ca^{2+} (dotted trace) and 4 mmol l^{-1} Ba^{2+} (solid trace) added at 60 min on lumen side.

here is more rapid and complete than that reported by Harvey & Wolfersberger (1979) who found approximately 60 % inhibition from the lumen side and no inhibition from the blood side. In the present experiments, solubilized P1 protoxin was used in conjunction with automatic voltage-clamping, whereas Harvey & Wolfersberger used alkali-activated spore-crystal powder with manual clamping.

The K^+ channel blocker, Ba^{2+}, at 4 mmol l^{-1} inhibited the I_{sc} from the blood side (Fig. 2B, solid trace; Table 1). The inhibition increased as [Ba^{2+}] was raised from 1 to 20 mmol l^{-1} Ba^{2+} (data not shown) and was quantitatively similar to the inhibition reported by Moffett & Koch (1985) and by Zeiske *et al.* (1986). In contrast, Ba^{2+} at 4 mmol l^{-1} had no effect from the lumen side other than a small, transient increase in I_{sc} (Fig. 2B, dotted trace). However, when Ba^{2+} was added at 6 mmol l^{-1} on the lumen side after 45 min, the I_{sc} was protected from Btk toxin added at a final concentration of 50 nmol l^{-1} at 60 min (Fig. 2C, solid trace). If

5 mmol l^{-1} Ca^{2+} was present instead of Ba^{2+} then a similar protection was observed after a small toxin-induced inhibition (Fig. 2C, dotted trace).

The remarkable ability of Ba^{2+} (solid trace) and Ca^{2+} (dotted trace) to reverse the Btk toxin inhibition of the I_{sc} is shown in Fig. 2D. After a sharp decline of the I_{sc} due to the presence of 50 nmol l^{-1} Btk toxin added at 45 min, 4 mmol l^{-1} Ba^{2+} or 5 mmol l^{-1} Ca^{2+} added at 60 min rapidly restored the I_{sc} to pseudo-steady-state levels.

The concentration of Ba^{2+} necessary to protect or reverse Btk inhibition of the I_{sc} was a dose-dependent function of the amount of toxin present and the time into the pseudo-steady state at which the toxin had been added. If Btk toxin (50 nmol l^{-1}) was present at 45 min, then complete protection or recovery was not always found even if the [Ba^{2+}] added at 60 min was increased to 6 mmol l^{-1} (Table 1). When the toxin was added after 60 min, 4 mmol l^{-1} Ba^{2+} was sufficient for complete protection and reversal (data not shown).

Each of the experiments described above was repeated at least three times. The results, along with appropriate controls, are summarized in Table 1. The first 45 min of each run was essentially a control, since no additions were made prior to that time. The mean I_{sc} at 45 min was 476±19 μA cm^{-2} (S.E.M.). The mean I_{sc} of the six control guts dropped from 524 μA cm^{-2} at 45 min to 449 μA cm^{-2} at 60 min to 400 μA cm^{-2} at 75 min, providing a base with which to compare experimental effects. When 50 nmol l^{-1} Btk toxin was added to the lumen side after 45 min, the mean I_{sc} dropped to 12 μA at 75 min. However, with 4 mmol l^{-1} Ba^{2+} or 5 mmol l^{-1} Ca^{2+} on the lumen side the I_{sc} was protected from the toxin inhibition; the I_{sc} values were 303 and 277 μA cm^{-2}, respectively, at 75 min. Reversal of Btk inhibition is equally clear from Table 1. With the I_{sc} inhibited by toxin added at 45 min, bringing the [Ba^{2+}] to 4 or 6 mmol l^{-1} or the [Ca^{2+}] to 5 mmol l^{-1} on the lumen side led to I_{sc} recovery at 75 min. Protection of the I_{sc} from Btk toxin appeared to be specific to Ba^{2+} and Ca^{2+}; 5 mmol l^{-1} Mg^{2+}, 4 mmol l^{-1} Na^+ or 8 mmol l^{-1} $choline^+$ had no effect on the inhibitory action of Btk toxin on the I_{sc}. Ba^{2+} or Na^+ at 4 mmol l^{-1}, Ca^{2+} or Mg^{2+} at 5 mmol l^{-1}, or $choline^+$ at 8 mmol l^{-1} in the absence of Btk did not affect the time course of the I_{sc} (Table 1).

Discussion

The results shed light on three interrelated topics: (1) the K^+ pump–leak pathway in lepidopteran midgut; (2) the mechanism of action of lepidopteran-specific Bt δ-endotoxin; and (3) Ba^{2+} or Ca^{2+} blockage of K^+ channels. The widely accepted model for midgut K^+ homeostasis shown in Fig. 1 states that an electrical PD of >180 mV combines with a small K^+ activity gradient to yield an electrochemical gradient (μ_K) of approximately 190 mV driving K^+ from the lumen across the CCAM towards the columnar cell cytoplasm. The CCAM is impermeable to K^+ except *via* the amino acid/K^+ symports. The pathway for the limited K^+ movement from lumen to blood, measured in flux experiments, is thought to be *via* the amino acid/K^+ symports, between the cells, or back through the K^+

Table 1. *Effects of Btk toxin, Ba^{2+}, Ca^{2+} and control substances on the short-circuit current of isolated* Manduca sexta *larval midgut*

		I_{sc} ($\mu A\,cm^{-2}$)		
Effectors	*N*	at 45 min	at 60 min	at 75 min
Control	6	524 ± 82	449 ± 85	400 ± 80
50 nmol l^{-1} Btk (L, 45 min)	3	423 ± 110	101 ± 43*	12 ± 15*
4 mmol l^{-1} Ba^{2+} (L, 45 min) 50 nmol l^{-1} Btk (L, 60 min)	4	470 ± 21	429 ± 24	303 ± 19
5 mmol l^{-1} Ca^{2+} (L, 45 min) 50 nmol l^{-1} Btk (L, 60 min)	3	430 ± 81	371 ± 43	277 ± 75
50 nmol l^{-1} Btk (L, 45 min) 4 mmol l^{-1} Ba^{2+} (L, 60 min)	7	426 ± 75	116 ± 47*	218 ± 35
50 nmol l^{-1} Btk (L, 45 min) 6 mmol l^{-1} Ba^{2+} (L, 60 min)	3	480 ± 40	194 ± 53*	267 ± 18
50 nmol l^{-1} Btk (L, 45 min) 5 mmol l^{-1} Ca^{2+} (L, 60 min)	4	535 ± 91	232 ± 54*	389 ± 32
50 nmol l^{-1} Btk (L, 45 min) 5 mmol l^{-1} Mg^{2+} (L, 60 min)	3	418 ± 129	118 ± 77*	59 ± 41*
50 nmol l^{-1} Btk (L, 45 min) 8 mmol l^{-1} $choline^{+}$ (L, 60 min)	3	405 ± 105	123 ± 120*	69 ± 85*
50 nmol l^{-1} Btk (L, 45 min) 4 mmol l^{-1} Na^{+} (L, 60 min)	4	538 ± 15	276 ± 39*	194 ± 49*
4 mmol l^{-1} Ba^{2+} (B, 45 min)	5	523 ± 73	361 ± 53	344 ± 46
4 mmol l^{-1} Ba^{2+} (L, 45 min)	3	491 ± 53	419 ± 16	359 ± 17
5 mmol l^{-1} Ca^{2+} (L, 45 min)	3	547 ± 36	406 ± 44	313 ± 55
5 mmol l^{-1} Mg^{2+} (L, 45 min)	3	530 ± 109	475 ± 123	433 ± 129
8 mmol l^{-1} $choline^{+}$ (L, 45 min)	3	525 ± 149	493 ± 145	470 ± 141
4 mmol l^{-1} Na^{+} (L, 45 min)	4	404 ± 88	349 ± 93	304 ± 95

* Significantly different from control value at the 1 % probability level using *t*-tests.
Values are means ± S.E.M.
L, applied to lumen; B, applied to blood side.
All salts were chlorides. NaCl was added to the opposite side of the chamber to balance the electrical effects of added salts.

pump. K^+ can diffuse between the electrically coupled columnar and goblet cells across the lateral membranes (Moffett, Hudson, Moffett & Ridgway, 1982). The basal membrane (BM) of both columnar and goblet cells is permeable to K^+ *via* channels susceptible to Ba^{2+} blockage (Moffett & Koch, 1985; Zeiske *et al.* 1986). K^+ can diffuse freely in the basal extracellular space of both columnar and goblet cells. The K^+ pump–leak pathway can be deduced from these results. K^+ from columnar cytoplasm, K^+ passing between cells from the lumen, and K^+ from the blood enters the goblet cells and diffuses to the GCAM. Here it is pumped to the

goblet cavity (GCv) and diffuses across the goblet cell apical space and columnar cell apical space to the lumen. The apical PD of >180 mV (lumen positive) is thought to sustain the alkalinity (pH 10–11) of the lumen through a mechanism using carbonate (Dow, 1984).

According to the Wolfersberger *et al.* (1986*a*) model, the primary action of lepidopteran-active Bt δ-endotoxin, after binding to receptors on the CCAM, is either to open pre-existing K^+ channels or to form new ones. When Bt toxin provides K^+ channels in the CCAM the K^+ homeostasis is disrupted. K^+ enters the columnar cells by the toxin channels and shunts the electrogenic pump PD. The transepithelial I_{sc} falls sharply but the K^+ pump in the GCAM is initially unaffected. Only 20 % of the drop in I_{sc} is attributable to increased flux of labelled K^+ from lumen to blood (Harvey & Wolfersberger, 1979). The remaining 80 % of the drop must be due to a futile K^+ cycle in which K^+ enters the columnar cell cytoplasm across the CCAM from the lumen, mixes with labelled K^+ from the basal extracellular space, diffuses to the goblet cell cytoplasm either across intercellular junctions or *via* the basal extracellular space, and is pumped across the GCAM back to the lumen. Such cycling K^+ would appear as a continued K^+ flux to the lumen but would carry no current. When the Btk toxin-induced K^+ channels are blocked by Ba^{2+} or Ca^{2+}, the K^+ homeostasis is immediately restored and the I_{sc} returns to its normal level. Similarly, if Ba^{2+} or Ca^{2+} is present at the time when Btk toxin is added, then the toxin-induced K^+ channels are blocked immediately and no inhibition of the I_{sc} or disruption of K^+ homeostasis is observed.

Whether Btk toxin itself forms the K^+ channels or opens pre-existing channels is not clear from these experiments. Antibodies to *B. thuringiensis* subsp. *thuringiensis* toxin no longer react with toxin which has been bound by CCAM even though toxin protein is extractable from solubilized membranes (Wolfersberger *et al.* 1986*a*). This evidence suggests that Bt toxin itself may be forming K^+ channels. However, almost all biological membranes possess K^+ channels. Such channels might be nonfunctional in normal midgut CCAM but restored in Bt toxin-treated CCAM. The K^+ channels induced in CCAM by Btk toxin are blocked by either Ba^{2+} or Ca^{2+}, unlike the naturally occurring basal K^+ channels which are blocked only by Ba^{2+}.

The Ca^{2+} blockage of K^+ channels implied by these results deserves special comment. Of the large number of K^+ channels reported in the literature many are activated by Ca^{2+}, but blockage by Ca^{2+} had not been reported as late as the review by Latorre & Miller (1983). However, Armstrong & Matteson (1986) described a strong effect of Ca^{2+} on the closing of K^+ channels in squid axon. They discussed reasons why 'the fundamental action of Ca^{2+} on K^+ channels that is postulated here can have escaped detection so long'. We appear to have indirect evidence for another fundamental action of Ca^{2+}, the blocking of Btk-induced K^+ channels in an epithelial tissue.

Our findings regarding Ca^{2+} have practical value because the 5 mmol l^{-1} concentration of Ca^{2+} required for protection from Bt toxin is well within the

levels encountered in nature. Naturally high [Ca^{2+}] in larval midguts may explain why some insects are not susceptible to Bt intoxication. Our results imply that the [Ca^{2+}] should be kept as low as possible in commercial Bt formulations.

That Btk toxin induces a futile K^+ cycle explains why Fast & Morrison (1972) found no change in [K^+] of midgut tissue during Bt toxin inhibition. With the K^+ pump in the GCAM still running and K^+ moving in a futile cycle, no change in midgut [K^+] is expected *in vivo*. A small drop in columnar cell elemental potassium concentration is found by X-ray microanalysis of midguts *in vitro* under short-circuit conditions in which there is a small electrochemical gradient driving K^+ out of the cells (Gupta, Dow, Hall & Harvey, 1985). The drop in goblet cell cavity elemental potassium concentration revealed by X-ray analysis, after Btk intoxication, is presumably an effect of delayed shut-down of the K^+ pump as cellular alkalinity rises owing to the loss of the transepithelial PD.

The amount of Ba^{2+} ($4\,mmol\,l^{-1}$) or Ca^{2+} ($5\,mmol\,l^{-1}$) required to prevent inhibition of the I_{sc} is roughly equal to 80000 times the amount of toxin present ($50\,nmol\,l^{-1}$). The Btk-induced apical channels are more sensitive to Ba^{2+} than the naturally occurring basal channels; $4\,mmol\,l^{-1}$ Ba^{2+} is sufficient to block completely the apical channels (with a 10-fold excess of Btk toxin), whereas $20\,mmol\,l^{-1}$ Ba^{2+} is insufficient to block completely the basal channels (Moffett & Koch, 1985; Zeiske *et al.* 1986). Increasing [Ba^{2+}] or [Ca^{2+}] above $4\,mmol\,l^{-1}$ does not enhance protection or recovery from Btk intoxication. That Ba^{2+} and Ca^{2+} specifically protect from Btk toxin inhibition is clear; Mg^{2+}, choline$^+$ and Na^+ are ineffective.

The rapidity of the onset of Ba^{2+} or Ca^{2+} blockage of Bt toxin inhibition provides a new tool for studying the interaction between the δ-endotoxin and midgut membranes. For the first time the binding and primary action of the endotoxin can be separated from its secondary action. In particular, the time course of Bt toxin-induced cell damage can now be determined because the damaging K^+ channel can be blocked at will by barium or calcium.

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