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Delta Endotoxin Inhibits Rb^+ Uptake, Lowers Cytoplasmic pH and Inhibits a K^+-ATPase in *Manduca sexta* CHE Cells

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Summary. Delta endotoxin, a 68 kilodalton protein isolated from *Bacillus thuringiensis* spp. *Kurstaki*, is a potent entomocidal agent that alters a K^+ current across midgut tissue of many phytophagous insects. This toxin completely inhibited the vanadate-sensitive $^{86}Rb^+$ uptake and mimicked the vanadate-induced decrease in cytosolic pH in a cell line (CHE) originating from *Manduca sexta* embryonic tissue. The toxin also inhibited a K^+-sensitive-ATPase in the plasma membranes isolated from these cells. Using the K^+-sensitive-ATPase substrate *p*-nitrophenyl phosphate, delta endotoxin was found to have a K_i of 0.4 μM. These data suggest that the toxin inhibits a K^+-ATPase responsible for $^{86}Rb^+$ uptake in the CHE cells. The relationship between the toxin inhibition of K^+-ATPase and toxin-altered K^+ current is discussed.

Key Words delta-endotoxin · *Manduca sexta* CHE cells · insect cells · *Bacillus thuringiensis* · K^+,H^+-ATPase · intracellular pH and K^+

Introduction

Delta endotoxin (DET) produced by *Bacillus thuringiensis* ssp. *Kurstaki* is the entomocidal agent of a commercially produced crystalline pesticide widely used in insect control. The crystal is ingested by the insect and dissolves in the insect midgut. Following base solubilization and activation by partial proteolytic digestion, the activated DET, a 68,000 dalton protein, or a subfragment of this protein, alters a K^+-dependent current across the midgut eventually resulting in cell death (Greigo et al., 1979; Harvey & Wolfersberger, 1979).

Cell cultures have recently been used to elucidate the predominant mechanisms of ion transport in insect cells (English & Cantly, 1984) and to study DET toxicity (Johnson & Davidson, 1984). In this investigation we used the *Manduca sexta* embryonic cell line CHE to elucidate further the mode of action of DET. This particular insect cell line resembles insect epidermal cells in its ability to produce chitin (Marks et al., 1984) and in its response to 20-hydroxyecdysone (English et al., 1984).

CHE cells have vanadate-inhibitable $^{86}Rb^+$ uptake that is coincident with a vanadate-inhibitable cellular pH regulatory system, possibly resulting from coupled K^+,H^+-ATPase (English & Cantley, 1984). The cells maintain a small Na^+ gradient possibly due to a ouabain-sensitive ATPase implied by ouabain-sensitive β-[2-furyl] acryloyl phosphatase activity in plasma membranes (English & Marks, 1981). Also, CHE cells have two distinct HCO_3^- regulatory systems: a Cl^-/HCO_3^- exchanger sensitive to 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS) and a furosemide and DIDS-sensitive KCl/H^+, HCO_3^- exchange system (English & Cantley, 1984).

This communication relates that DET inhibits the vanadate-sensitive $^{86}Rb^+$ uptake and induces a reduction in cytoplasmic pH in whole CHE cells. DET also inhibits the K^+-ATPase found in the purified plasma membranes isolated form the CHE cells.

Abbreviations

DET, delta endotoxin; ATPase, adenosine triphosphatase; NADH, β nicotinamide adenine dinucleotide phosphate; HEPES, [4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid]; EGTA, ethylene glycol-bis (β-amino-ethyl ether) N,N′-tetra-acetic acid; pNPPase, para nitrophenyl phosphatase; DIDS, 4,4′-diisothiocyano-2,2′ disulfonic acid stilbene; PMSF, phenylmethyl-sulfonyl floride; 6CFD, 6-carboxyfluorescein diacetate; 6CF, 6-carboxyfluorescein.

Materials and Methods

Materials

CHE cells were provided by E.P. Marks, U.S. Dept. of Agriculture, Metabolism Radiation Research Lab., Fargo, N.D. The cells were grown in Grace's insect tissue culture medium (GIBCO, Grand Island, N.Y.) as modified by Yunker et al. (1967). *Bacillus thuringiensis* spp. *Kurstaki* parasporal crystals

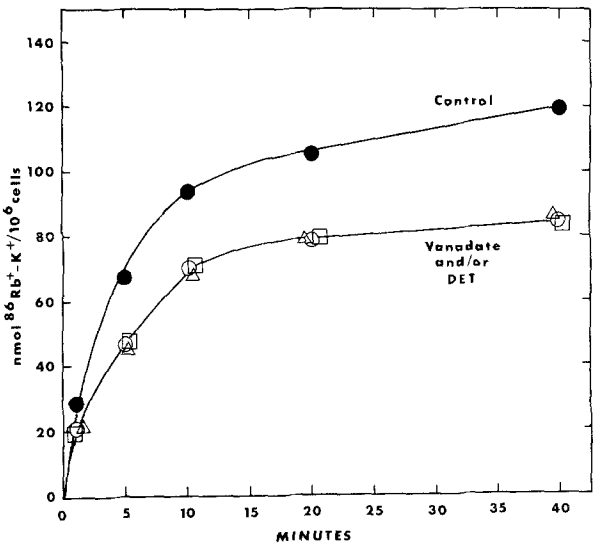

Fig. 1. $^{86}Rb^+$-K^+ uptake into CHE cells. Cells were harvested and resuspended in assay buffer as described in Materials and Methods and treated with either 1 μM DET (○), 200 μM vanadate (△), or 1 μM DET and 200 μM vanadate (□); or neither (●). After a 40-min incubation, the assay was initiated by the addition of $^{86}Rb^+$. Similar rates of $^{86}Rb^+$ uptake were observed for three experiments. Error on each point was typically ≤5 nmol $^{86}Rb^+$-K^+/10^6 cells

were provided by D.E. Johnson, U.S. Dept. of Agriculture Grain Marketing Research Lab., Manhattan, Kan. DET was isolated and purified according to the method of Bulla et al. (1981) and resuspended in H_2O to a final concentration of 0.3 μg/μl.

CHE Cell Membrane Isolation

Plasma membranes were isolated from the CHE cells by the method of Brunette and Till (1971) with the following modifications. *p*-Methylsulfonylfloride (100 μM) was added to the 1 mM $ZnCl_2$ lysis solution and the plasma membranes were resuspended in 320 mM sucrose 10 mM ethylenediaminetetraacetic acid, pH 7.4. Plasma membranes were characterized by examining the activity of 5′ nucleotidase, a plasma membrane marker, assayed according to the method of Heppel and Hilmoe (1955). Assays were conducted in 0.05% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) to eliminate membrane vesicles.

^{86}Rb Uptake

K^+ uptake was estimated using $^{86}Rb^+$ as the tracer. We previously demonstrated that Rb^+ adequately substituted for K^+ in these insect cells (English & Cantley, 1984). Uptake assays were performed according to the method described by Smith et al. (1982). Cells were harvested from the growth medium, washed and resuspended in assay buffer containing (in mM) 55 KCl, 4 $NaHCO_3$, 7 NaH_2PO_4, 23.6 $MgCl_2$, 500 μM $CaCl_2$, 50 glucose, pH 6.5, with Tris. Prior to the assay, portions of the cell suspension were treated with either DET (1 μM) for 40 min or vanadate (200 μM NH_4VO_3) for 20 min, or no treatment. The assay was initiated by the addition of $^{86}RbCl$ (1 to 3 μCi/treatment) suspended in 10 μl of the assay buffer described above. The assay was terminated by centrifuging the cells through 800 μl of ice-cold assay buffer and 300 μl of a 1 : 1 solution of silicone oil (Aldrich Chemical Co., Milwaukee, Wis.): dinonyl phthalate (ICN Pharmaceuticals, Plainview, N.Y.). Cellular volume was monitored using ^3H_2O to label internal cellular water and trapped external water and then subtracting the value for bound [^{14}C]-inulin which labeled only trapped water (Levenson et al., 1980). These measurements were made at 0°C in the presence of excess nonlabeled inulin (24 mM).

Cytoplasmic pH Measurements

Internal cellular pH was measured using the pH-sensitive dye, 6-carboxy-fluorescein diacetate (6CFD). This dye is permeant to the cell but is hydrolyzed internally by cellular esterases to yield impermeant pH-sensitive 6-carboxy-fluorescein (6CF) (Thomas et al., 1979). A total of 2×10^6 cells were harvested, washed and suspended in 10 ml of assay buffer consisting of 500 μM $CaCl_2$, 55 mM KCl, 23.6 mM $MgCl_2$, 4 mM $NaHCO_3$, 7 mM NaH_2PO_4, 25 mM glucose, 40 mM 6CFD, pH 6.5, with Tris. After 40 min the cells were concentrated by centrifugation and resuspended in the same buffer without 6CFD. Measurements of relative fluorescence were made on an SLM 4800 spectrofluorometer (SLM Instruments, Inc. Urbana Champaign, Ill.) with emission at 520 nm and excitation at 465 or 490 nm. The ratio of the 490/465 excitation was used to estimate cytoplasmic pH. A standard curve of 490/465 ratio versus cytoplasmic pH was referenced for cytoplasmic pH estimation. Preparation of the standard curve for the CHE cells is described by English and Cantley (1984).

ATPase and pNPPase Assays

Total membrane ATPase was assayed spectrophotometrically at 340 nm using the continuous pyruvate kinase lactate dehydrogenase, β nicotinamide adenine dinucleotide phosphate (NADH)-coupled system in the presence of 100 mM choline chloride, 20 mM KCl, 2.5 mM ATP, 5 mM $MgCl_2$, 1 mM ethylene glycol-bis (β-amino-ethyl ether) N,N′-tetraacetic acid (EGTA), 20 mM [4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid] (HEPES), 1.4 mM phosphoenol pyruvate, 0.26 mM NADH, 10 μg/ml pyruvate kinase, 10 μg/ml lactate dehydrogenase, 0.05% Nonidet P-40, and 1 mM phenymethy-sulfonyl floride (PMSF), pH 7.4 at 37°C according to the method of Cantley et al. (1978*b*). Since K^+ is required for the coupled assay, K^+ ATPase was measured by [γ-^{32}P]ATP hydrolysis (Nelson et al., 1972) in insect cell saline buffer (55 mM KCl, 11 mM NaCl, 10 mM $MgCl_2$, 100 mM 2-(N-morpholino) ethane sulfonic acid, 2 mM ATP, 0.05% Nonidet P-40, and 1 mM PMSF, pH 6.5). K^+-ATPase was defined as the difference between ATP hydrolysis in the presence or absence of K^+. For control experiments the KCl was replaced by choline chloride. K^+-*p*-nitrophenyl-phosphatase (K^+-pNPPase) was defined as the difference between pNPPase activity in the presence or absence of K^+. This activity was monitored spectrophotometrically at 410 nm as previously described (Robinson, 1976) in the presence of 20 mM HEPES, 3 mM $MgCl_2$, 50 μM dithiothreitol, and 25 mM KCl or 25 mM choline chloride, 0.05% Nonidet P-40, and 1 mM PMSF, pH 7.4. Mg^{2+}-pNPPase activity was determined using the same assay substituting 25 mM choline chloride for KCl. K^+ contamination from the pH electrode was avoided by using pH-sensitive paper (Color pHast, MCB Reagents, Gibbstown, N.J.) to evaluate the buffer pH. This

activity was subtracted from total pNPPase activity to calculate K^+-pNPPase activity. The K_i for DET was determined in the presence of 1 mM ouabain. 50 μM epinephrine was added in the absence of vanadate treatment to chelate contaminating vanadate (Cantley et al., 1978*a*).

Results

DET and Vanadate Inhibit K^+ Transport

DET inhibited a vanadate-sensitive component of $^{86}Rb^+$ uptake into CHE cells. $^{86}Rb^+$ uptake has previously been shown to be a good indicator of K^+ transport in CHE cells (English & Cantley, 1984). Approximately 20% of the initial rate of $^{86}Rb^+$ uptake was previously shown to be vanadate inhibitable suggesting an active K^+ transport system (English & Cantley, 1984). Figure 1 shows that the vanadate-inhibitable component of $^{86}Rb^+$ uptake was also inhibited by DET (1 μM). No additional inhibition was observed when both vanadate and DET were added simultaneously. No ouabain-sensitive (1 mM ouabain) $^{86}Rb^+$ uptake was observed as previously shown (English & Cantley, 1984) and no change in cell volume as judged by ^3H_2O, ^{14}C-inulin procedure could be detected after one hour in DET and/or vanadate (*data not presented*). These results suggest that DET may be inhibiting an active K^+ transport system. The vanadate inhibition suggests the presence of a K^+,H^+-ATPase similar to that characterized in mammalian stomach (Faller et al., 1983).

Cytoplasmic pH Reduced by DET and Vanadate

Both DET and vanadate induce a decrease in cytoplasmic pH indicated by a decrease in relative fluorescence of the 6CF-labeled CHE cells (Table 1). The degree of reduction in cytoplasmic pH was the same for both DET and vanadate treatments, approximately 0.1 pH unit. When cells were treated simultaneously with DET and vanadate, the same reduction in cytoplasmic pH was observed. Therefore, while both DET and vanadate induced a decrease in cytoplasmic pH, the two treatments were not additive. These results further suggest that DET and vanadate inhibited a system that regulates both K^+ and H^+ transport.

Effect of DET and Vanadate on ATPase Activity

A plasma membrane-enriched fraction of cell homogenate was isolated from CHE cells for further investigation of the putative vanadate and DET-sensitive ATPase. The isolated material was found to be enriched 12-fold in plasma membranes over cell homogenates as indicated by the level of 5′-nucleotidase (148 nmol/mg min) an enzyme associated with the plasma membranes. When the plasma membrane-enriched material was assayed for total ATPase activity using the NADH-linked assay, 30% of the total activity was inhibited by 1 μM DET (Table 2). Vanadate (10 μM) inhibited 50 to 60% of the total ATPase activity and DET caused no further inhibition when added in the presence of vanadate. Ouabain had no effect on ATPase activity. Thus approximately half of the vanadate-sensitive ATPase activity from CHE cell plasma membranes was also inhibited by DET. The K^+ dependence of the DET-sensitive ATPase could not be investigated using the NADH-linked assay because of the K^+ requirement for the coupling enzymes. Using somewhat different assay conditions employing [γ-^{32}P]-ATP (Materials and Methods) a specific ATPase activity of 120 nmol/min mg was observed for plasma membranes. 10% of the ATPase activity was inhibited by 1 μM DET and elimination of K^+ from the assay reduced the specific activity by 10% and completely abolished the DET-inhibitable ATPase activity (*data not shown*). Replacement of NaCl with choline chloride or addition of ouabain (1 mM) had no detectable effect on the ATPase activity measured by this technique. Thus, although total ATPase activities measured by the two techniques differed, the DET-inhibitable activity was K^+ dependent and vanadate inhibitable.

Further evidence for a K^+-dependent, DET-inhibitable ATPase was provided using the substrate *p*-nitrophenylphosphate. This molecule is hydrolyzed by a variety of transport ATPases including the Na,K-ATPase (Robinson, 1976). DET inhibited 40% of the total pNPPase activity (Table 2). This fraction was identical to the K^+-dependent fraction determined by subtraction of the pNPPase activity

Table 1. Relative fluorescence of 6-carboxyfluorescein-loaded CHE cells and estimated cytoplasmic pH

Treatment	Ratio of excitation at 490/465 nm	Cytoplasmic pH
Control	1.95 ± 0.01	6.90
Vanadate (200 μM)[a]	1.92 ± 0.01	6.80
DET (1 μM)	1.92 ± 0.01	6.80
Vanadate (200 μM) and DET (1 μM)	1.92 ± 0.01	6.80

[a] Excitation ratio equal to 1.90 and corresponding cytoplasmic pH equal to 6.65 have been obtained for this treatment from other batches of CHE cells (English & Cantley, 1984).

Table 2. Representative ATPase and pNPPase activities of CHE cell plasma membranes (62 μg/ml)[a]

Treatment	Total ATPase[b]	K^+-pNPPase[c] (nmol/min · mg)	Mg^{2-}-pNPPase[d]
Control	30	13	19
Ouabain (1 mM)	30	10	19
DET (1 μM)	21	1	19
Ouabain (1 mM) + DET (1 μM)	21	0	19
Vanadate (10 μM)	10		1
Vanadate (10 μM) + DET (1 μM)	10	0	1
[e]DET (0.6 μM) + Trypsin + Trypsin inhibitor	N.D.[g]	3	19
[f]DET (0.6 μM) + Trypsin digested	N.D.[g]	10	19

[a] Error for five assays from membranes prepared from the same batch of cells was less than 5%.
[b] ATPase activity in the presence of 100 mM choline chloride, 20 mM KCl, 5 mM $MgCl_2$.
[c] Difference between pNPPase assayed in the presence or absence of 25 mM KCl in which 25 choline chloride was used to correct for ionic strength.
[d] pNPPase activity in the presence of 25 mM choline chloride and 3 mM $MgCl_2$.
[e] DET (0.6 μM) was preincubated at 37°C for 3 hr with trypsin (1 unit) and trypsin inhibitor (1 unit) in 25 μl 100 mM imidazole, 5 mM $MgCl_2$, pH 7.4.
[f] DET (0.6 μM) was preincubated at 37°C for 3 hr with trypsin (1 unit) in 25 μl 100 mM imidazole, 5 mM $MgCl_2$, pH 7.4, followed by trypsin inhibitor (1 unit).
[g] Treatment not determined in this assay.

after substitution of 25 mM choline chloride for KCl in the assay. No DET-inhibitable pNPPase activity was observed in the absence of K^+ (Table 2). K^+ therefore was absolutely necessary for the expression of the DET-inhibitable pNPPase as well as the DET-inhibitable ATPase. Vanadate almost completely inhibited both the K^+-dependent and K^+-independent pNPPase activity. Thus, in agreement with the ATPase activities, DET inhibits a K^+-dependent and vanadate-sensitive phosphatase activity in CHE membranes.

DET inhibition of this pNPPase activity was not the result of a nonprotein contaminant in the DET. Following incubation of 0.6 μM DET with 1 unit trypsin for 3 hr at 37°C most of the inhibitory activity was removed. When trypsin inhibitor was included during the trypsin treatment, DET retained its inhibitory activity. Apparently, extensive proteolytic cleavage by trypsin hydrolyzed the peptide essential for DET inhibitory activity.

Affinity of K^+pNPPase for DET

A titration curve of K^+-pNPPase activity versus DET concentration indicates a hyperbolic saturation to 100% inhibition with a K_i of 0.4 μM (Fig. 2). Similar results were obtained using the Na^+-free ATPase assay; however, the lower level of relative K^+-ATPase activity made these data less reliable (*data not shown*).

Discussion

In this communication we report that DET inhibits $^{86}Rb^+$ uptake and induces a decrease in cytoplasmic pH in CHE cells. We also show that DET inhibits a K^+-ATPase in plasma membranes isolated from these cells. These data suggest that DET-sensitive ATPase in the plasma membranes was responsible for DET-sensitive $^{86}Rb^+$ uptake into CHE cells and partially responsible for cytoplasmic pH regulation. However, verification of this will require isolation, purification, and reconstitution of this ATPase. DET-sensitive $^{86}Rb^+$ uptake, K^+-ATPase inhibition and cytoplasmic pH regulation also appear to correspond to the vanadate-sensitive $^{86}Rb^+$ uptake, K^+-ATPase activity and cytoplasmic pH regulation in CHE cells. If these activities are catalyzed by the same enzyme, then the DET-sensitive $^{86}Rb^+$ uptake and ATPase detected here may be due to an ATP-driven K^+/H^+ exchange system similar to that detected in mammalian gastric mucosa (Sachs et al., 1976). In the mammalian gastric mucosa, the K^+,H^+-ATPase binds K^+ on the lumen-side and pumps it into the cell in exchange for H^+ resulting in

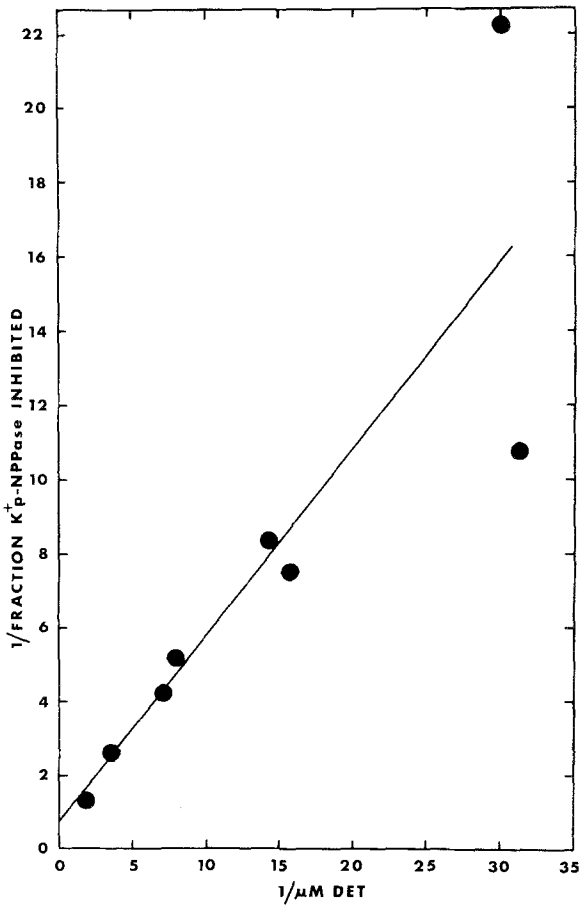

Fig. 2. Double-reciprocal plot of inhibited fraction of K^+-pNPPase *vs.* DET concentration (μM). 20 μg CHE plasma membranes were preincubated with DET at 25°C for 40 min in 100 mM imidazole, 5 mM $MgCl_2$, pH 7.4. The assay was initiated by the addition of pNPPase assay buffer at 37°C and followed spectrophotometrically at 410 nm. K_i was estimated to be 0.4 μM by linear least-squares analysis, assuming one class of sites causing 100% inhibition

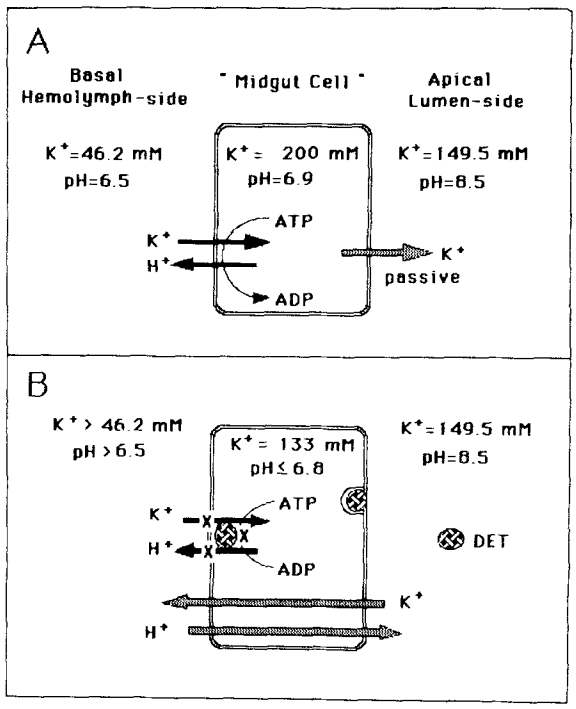

Fig. 3. K^+ concentrations, pH, and proposed K^+,H^+-ATPase in the midgut. (*A*) Intracellular K^+ concentration, pH and proposed (K^+,H^+)-ATPase in this model of a "Midgut Cell" were taken from research on the CHE cell by English and Cantley (1984) or data presented in this manuscript. K^+ concentrations in the midgut lumen and hemolymph were determined by Giordana and Sacchi (1978). Hemolymph and lumen pH were taken from Wigglesworth (1972) and House (1974), respectively. (*B*) Following DET treatment, the toxin is taken up into the cell and inhibits the K^+,H^+-ATPase blocking K^+ uptake and H^+ efflux from the cell. Initially this will decrease the level of intracellular K^+ and decrease cytoplasmic pH. Eventually K^+ and H^+ concentrations in and around the midgut should approach equilibrium. K^+ will flow from the lumen into the hemolymph (Harvey & Wolfersberger, 1979) and H^+ will flow from the hemolymph into the lumen (Heimpel & Angus, 1959)

acidification of the stomach lumen (Ray et al., 1982). This ATPase forms a phosphorylated intermediate during turnover and is inhibited by vanadate (Wallmark & Mardh, 1979; Faller et al., 1983).

A model explaining DET effects on proton and K^+ fluxes in the midgut is presented in Fig. 3. We propose that the DET-sensitive K^+-ATPase is positioned on the hemolymph side of the midgut cell and pumps K^+ into the cell against a concentration gradient as observed in the CHE cell. We also suggest that this potassium pump, like the mammalian gastric K^+,H^+-ATPase couples K^+ influx to H^+ efflux from the cell. This model predicts that the midgut cellular K^+ concentration and pH are higher than that in the hemolymph. Although the cytosolic pH of the midgut cells has not been reported, the midgut cell K^+ concentration is certainly higher than that in the hemolymph (Giodana & Sacchi, 1978). Coupled K^+ influx and H^+ efflux on the hemolymph side of the midgut cells, as proposed in the model, are consistent with the observation that the midgut lumen is highly alkaline while the hemolymph is acidic and insulated from the lumen pH (Heimpel & Angus, 1959; Wolfersberger et al., 1982).

This model predicts that following DET inhibition of the K^+,H^+-ATPase, cytosolic K^+ will decrease in the midgut cell as K^+ flows into the hemolymph down the concentration gradient (Fig. 3*B*). This has been reported by Harvey and Wolfersberger (1979). Our model also predicts that following inhibition of the K^+,H^+-ATPase, the pH of the midgut cytosol will decrease because H^+ cannot be pumped out of the cell. We observed this in the CHE cells. Without an active H^+ pump to acidify

the hemolymph, the hemolymph pH would eventually increase because of the alkaline conditions of the midgut lumen. This was observed following treatment of the midgut lumen with DET (Heimpel & Angus, 1959).

The proposed model suggests that DET is taken up into the midgut cell on the lumen side and subsequently inhibits the K^+,H^+-ATPase on the hemolymph side. This may occur by endocytosis as suggested for other bacterial toxins (Olsnes & Sandvig, 1985) or perhaps by some other receptor-mediated event. Also, the specificity of DET for the K^+-ATPase has not been established; therefore, different cell types may have different DET-sensitive enzymes. In any case, the use of CHE cells and purified plasma membranes containing the DET-sensitive K^+-ATPase should assist these investigations. An alternative explanation for the effect of DET on K^+ transport and a proposal for a lumen-side location for the K^+-ATPase are discussed by Wolfersberger et al. (1982) and Harvey et al. (1983).

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