Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 82, Suppl. III: 29-34, 1987
Intern. Symp. on Insects.

THE POTASSIUM IMPERMEABLE APICAL MEMBRANE OF INSECT EPITHELIA: A TARGET FOR DEVELOPMENT OF SAFE PESTICIDES

WILLIAM R. HARVEY*, DWIGHT N. CRAWFORD, NAOMI S. EISEN, VERONICA F. FERNANDES, DANIEL D. SPAETH & MICHAEL G. WOLFERSBERGER

Department of Biology, Temple University, Philadelphia PA, 19122, USA

Columnar cell apical membranes (CCAM) in series with goblet cell apical membranes (GCAM) form an electroosmotic barrier separating the midgut lumen from epithelial cell cytoplasm. A unique K^+ ATPase in GCAM generates three gradients across this barrier. A > 180 mV electrical gradient (lumen positive) drives amino acid uptake through voltage-dependent K^+ symports. A > 1000-fold $[H^+]$ gradient (lumen alkaline) and a > 10-fold $[K^+]$ gradient (lumen concentrated) are adptations to the high tannin and high K^+ content, respectively, in dietary plant material. Agents which act on the apical membrane and disrupt the PD, H^+, or K^+ gradients are potential insecticides. Insect sensory epithelia and mammalian stria vascularis *maintain similar PD and K^+ gradients but would not be exposed to ingested anti-apical membrane insecticides. Following the demonstration by Sacchi et al. that* Bacillus thuringiensis *delta-endotoxin (Bt) induces specifically a K^+ conductance increase in CCAM vesicles, we find that the K^+ channel blocking agent, Ba^{2+}, completely reverses Bt inhibition of the K^+-carried short circuit current in the isolated midgut of* Manduca sexta. *Progress in characterizing the apical membrane includes finding that fluorosulfonylbenzoyladenosine binds specifically to certain GCAM polypeptides and that CCAM vesicles can be mass produced by Ca^{2+} or Mg^{2+} precipitation from* Manduca sexta *midgut.*

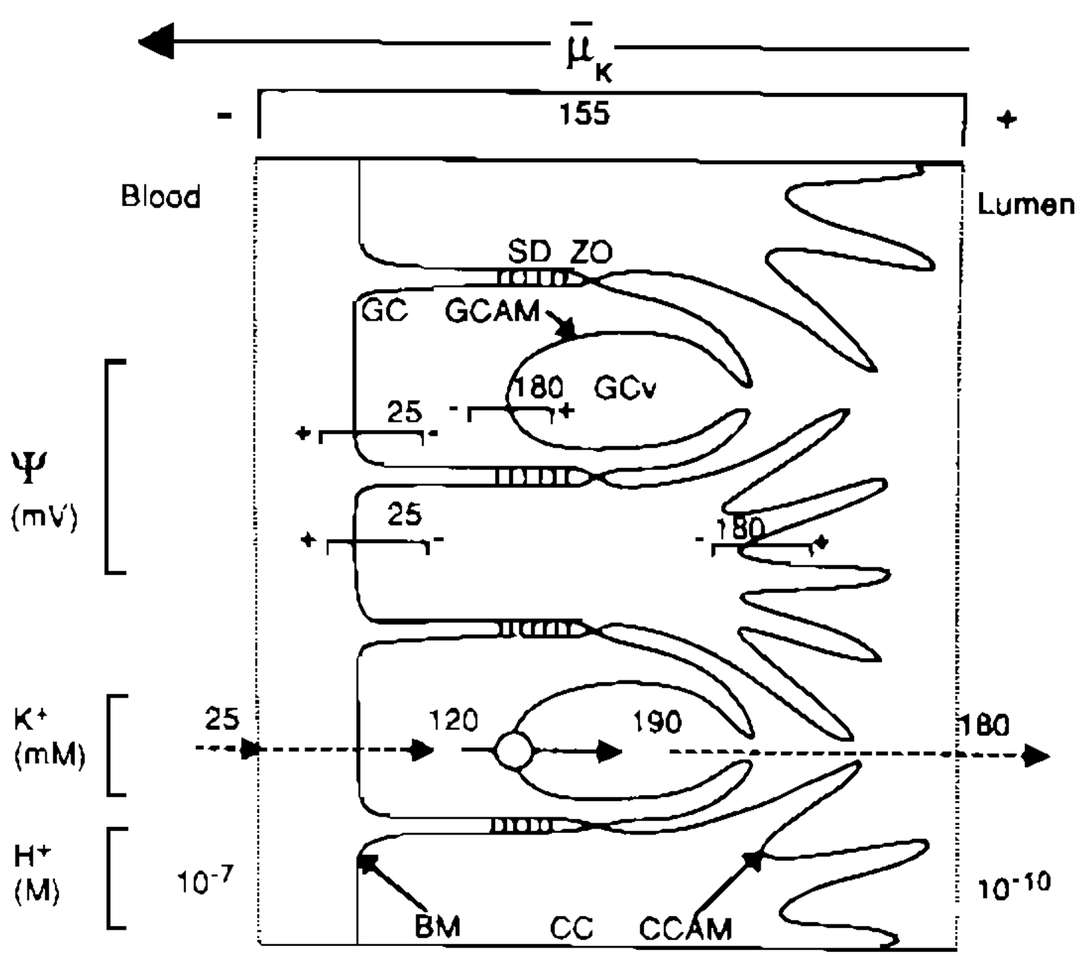
The lepidopteran midgut serves as a model for K^+ homeostasis involving gastrointestinal and sensory epithelia of insects and may be helpful in understanding the K^+ pump-leak steady state involved in mammalian hearing. Four aspects of this midgut have been investigated in detail: the K^+ ATPase in goblet cell apical membrane (GCAM), the AA-K^+ symports in columnar cell apical membrane (CCAM), the development of the electrical and H^+ gradient across GCAM-CCAM, and the K^+ channels in basal membrane (BM). *Bacillus thuringiensis* var *kurstaki* (Btk) delta-endotoxin has been shown to act by forming K^+ channels in CCAM. Btk delta-endotoxin inhibition of the isolated *Manduca* midgut is reversed by a K^+ channel blocking agent, Ba^{2+}, and by Ca^{2+}.

Since active K^+ transport by insect epithelia was first demonstrated in Malpighian tubules (Ramsay, 1953) and in lepidopteran midgut (Harvey & Nedergaard, 1964), insect K^+ homeostasis has been widely studied (Dow, 1986). The large, available, midgut of lepidopteran larvae such as *Manduca sexta* is the object of choice for both biophysical and biochemical studies. Thus, Dow (1986) described the midgut as "the frog skin of insect epithelia". The K^+ pump-leak steady state described in the "midgut model" (Fig.) explains the constancy of blood, cell, and luminal K^+ concentrations. In this model K^+ is actively transported from cells to lumen by the K^+ pump in GCAM. K^+ leaks back to the columnar cells via amino acid-K^+ symports in CCAM. K^+ equilibrates between columnar and goblet cells via gap junctions in the lateral membranes and between blood and cells by K^+ channels in the basal membranes. Although K^+ is the only ion actively transported by the lepidopteran midgut *in vivo* Na^+ is transported by certain Malpighian tubules *in vivo* and all of the alkali metal ions can be transported *in vitro* (see Harvey & Zerahn, 1972).

Overwhelming evidence from several insect epithelia places the K^+ pump on the apical membrane. Combining X-ray microanalysis with K^+ selective microelectrode penetrations, Gupta et al. (1978) showed that the K^+ pump is on apical membranes of salivary gland epithelial cells. Similar X-ray data placed the K^+ pump on GCAM in lepidopteran midgut (Dow et al., 1983). The K^+ ATPase was characterized

*To whom correspondence should be addressed.

Research in our laboratory is supported in part by grants from the National Institute of Health (AI-22444) and the United States Department of Agriculture (87-CRCR-1-2487).



Model for K^+ homeostasis in lepidopteran midgut. K^+ is pumped from goblet cells (GC) across goblet cell apical membrane (GCAM) to goblet cavity (GCv), diffuses to lumen and leaks back into columnar cells (CC) via amino acid-K^+ symports (not shown) in columnar cell apical membrane (CCAM). K^+ equilibrates between CC and CC, and between CC and GC via gap junctions and equilibrates across basal membranes (BM) via K^+ channels. The electrogenic pump PD of 180 mV (lumen positive to cell) is expressed across both GCAM and CCAM. A K^+ diffusion PD of 25 mV (cell negative to blood) is expressed across BM. The cells are joined septate desmosomes (SD); a zonula occludens (ZO) limits paracellular K^+ diffusion. The electrochemical gradient of ca. 200 mV (lumen positive) is mostly due to the electrical gradient of 155 mV especially if the low K^+ activity coefficient of 0.3 (not shown) is considered. The thousand-fold H^+ gradient (lumen alkaline) is thought to be maintained by the K^+ pump PD (Crawford & Harvey, 1988; modified from Harvey et al., 1986).

and shown to be in GCAM of midgut cells (Wolfersberger et al., 1982; Wieczorek et al., 1986). These results confirmed Harvey's (1980) deduction, from ultrastructural evidence, that the K^+ pump is associated with 10 nm particles, designated portasomes, on the cytoplasmic surface of the apical membrane of all K^+ transporting insect epithelia. Dow (1984) showed that carbonate accompanies transported K^+ and renders the lumen alkaline. He argued that the generation of a large PD, rather than K^+ regulation, better explains the location of K^+ pump on the border of the goblet cavity.

The $\cdot K^+$ leak serves diverse functions. In Malpighian tubules K^+ secreted (pumped) into the Malpighian tubule lumen leaks back to the blood (is reabsorbed) through the rectum. All small molecules in the blood pass into the tubule lumen along with water accompanying the secreted K^+. The rectal wall selectively reabsorbs certain solutes and the rest pass out in the urine. Thus the K^+ pump-leak steady state shared by Malpighian tubule and rectum has a kidney-like function in insects (Maddrell, 1980).

Thurm & Kuppers (1980) reviewed both electrical and cytological evidence that electrogenic K^+ transport by portasomes on the apical membrane of trichogen, tormogen, and thecogen cells contributes to the resting potential in insect sensory sensilla. K^+ pumped across these epithelial cell apical membranes to the lymph cavity leaks back to the cells and modulates receptor potentials. Wieczorek (1982) identified a K^+ ATPase similar to midgut GCAM ATPase in dipteran sensilla. That the receptor PD in insect sensory sensilla is modulated from the electrogenic K^+ pump PD is a refreshingly different mechanism that the usual Na^+, K^+ based, Hodgkin-Huxley explanation for bioelectrical activity.

After Nedergaard (1972) showed that amino acid transport across isolated midgut is both voltage and K^+ dependent but K^+ pump-independent, Giordana, Hanozet, Sacchi and collaborators showed that the uptake of both aromatic amino acids (Hanozet et al., 1980) and basic amino acids (Giordana et al., 1985) use separate K^+-amino acid symports located in brush border membranes. The AA-K^+ cotransport is driven by the K^+ pump-generated electrical gradient (lumen positive) and to a lesser extent by the K^+ concentration gradient (lumen concentrated) across the apical membrane.

Moffett et al. (1982) demonstrated that K^+ is essentially at equilibrium across BM between blood and epithelial cells in *M. sexta* midgut. Then Zeiske et al. (1986) showed by noise analysis that this equilibration takes place through K^+ channels. Ba^{2+} at approximately 5 mM interferes with this K^+ equilibration by blocking the K^+ channels.

The midgut model guided Wolfersberger and associates in elucidating the mode of action of Btk delta-endotoxin. Ingested endotoxin crystals dissolve in the alkaline midgut contents and the 130-140 kDa protoxin is digested by midgut proteases to 55-70 kDa protease-resistant toxin. Unlike the large protoxin protein, the toxin diffuses readily across the peritrophic membrane (Wolfersberger et al., 1986b) and interacts with specific receptors on the CCAM of midgut cells (Luethy et al., 1986). The toxin appears to insert into the CCAM of the midgut of susceptible insects (Wolfersberger et al., 1986a), and to increase specifically the K^+ conductance

of this membrane (Sacchi et al., 1986). Providing a K^+ channel in the normally K^+ impermeable CCAM could lead to all of the secondary actions of Bt toxin by disrupting electrical, K^+ and pH gradients (Harvey et al., 1986), Crawford & Harvey (1988) showed that a K^+ channel blocking agent, Ba^{2+}, both prevents and reverses Btk toxin inhibition of the isolated *M. sexta* midgut.

In summary, K^+ homeostasis is used for such diverse functions as K^+ regulation, pH regulation, nutrient uptake, and generation of receptor potentials. In this respect insects differ from vertebrates which rely on the Na^+, K^+ pump, amino acid-Na^+ symports, and Na^+ channels for analagous functions. Even in K^+ transporting vertebrate epithelia such as the gastric mucosa, the K^+ pump and ATPase differ from those of insect epithelia. Only in the vertebrate inner ear is there a K^+ pump-leak steady state analagous to that of these insects. An electrogenic K^+ pump located in the apical membrane of *stria vascularis* cells produces the K^+ rich endolymph (Sellick & Bock, 1974; see Keynes, 1969). Subsequent leakage of K^+ through hair cells is the key step in transduction of sound waves to auditory nerve signals (Hudspeth, 1985). The analogy between this K^+ pump-leak steady state and that in insect gastrointestinal and sensory epithelia is profound.

RESULTS AND DISCUSSION

The apical membrane of K^+ pump-containing midgut cells is directly accessible to any ingested agents which can pass through the peritrophic membrane whereas the pump location in other insect epithelia is accessible only via the blood. Therefore, agents like Btk toxin which disrupt the midgut apical membrane are prime candidates for environmentally safe insecticides. *Bt* var *israelensis* (Bti) toxin is specific for dipteran vectors of tropical diseases. In the same way that the frog skin model assists in understanding Na^+ homeostasis in mammalian intestine, the lepidopteran midgut model may assist in understanding K^+ homeostasis in dipteran larval midguts, in particular those whose highly alkaline midgut contents resemble those of lepidopteran larvae (Dadd, 1975). Results from recent experiments directed at analyzing the properties of the K^+ impermeable apical membrane are reported and discussed here.

1. Distinction of basal and K^+ stimulated ATPase in GCAM

Substrate specificity experiments provided the first clear distinction between the K^+ stimulated ATPase activity and the basal Mg-ATPase activity in GCAM. In the absence of KCl, UTP was hydrolyzed at the same rate as ATP (25 vs 27 μmol/hr-mg). However, in the presence of 30 mM KCl the rate of UTP hydrolysis was unchanged while the rate of ATP hydrolysis increased to 47 μmol/hr-mg. The K^+ stimulated ATPase is more sensitive than the basal ATPase to fluorosulfonylfonylbenzolyladenosine (FSBA); the kinetics of FSBA inhibition of the K^+ stimulated activity are more complex than the uncompetitive inhibition of the basic activity. Freezing GCAM vesicles in the presence of 10% DMSO actually increased their basal ATPase activity but destroyed their K^+ stimulated ATPase activity.

2. Solubilization and purification of K^+ ATPase

GCAM was solubilized with retention of K^+-ATPase activity. The 100,000g supernatants of GCAM solubilized either with octylglucoside or octaethyleneglycol dodecyl ether contained ATPase activity which was stimulated by added KCl. Solubilization of the GCAM by either detergent, at a concentration slightly in excess of the detergent's critical micellular concentration (CMC), was essentially complete; no pellet was visible after centrifugation and the supernatants yielded the same pattern on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as the original membranes. Detergent concentrations less than CMC, as well as glycerol or salt failed to solubilize any measurable GCAM ATPase activity. Although isolated GCAM vesicles retain their ATPase activity for up to one week at 4°C, solubilized GCAM lost approximately 25% of its ATPase activity within 24 hr at the same temperature. The octylglucoside solubilized membranes consistently showed much lower specific ATPase activity than the starting material. However, the octaethyleneglycol dodecyl ether solubilized membranes frequently showed a specific ATPase activity equal to or greater than the starting material. The characteristics of the solubilized and membrane-bound ATPase are similar, they are surely due to the same proteins. Both membrane-bound and solubilized GCAM-ATPases require Mg^{2+} or Mn^{2+}, and are inhibited by nitrate. Both forms have similar substrate specificities but the solubilized enzyme is more sensitive to product inhibition (Wolfersberger et al., 1987).

Purification of the solubilized GCAM ATPase was attempted using ATP-sepharose

affinity chromatography, gel filtration, and high performance ion exhange chromatography. All techniques yielded at least some fractionation of the GCAM proteins. Nearly all the applied protein was eluted in the excluded volume from a Sephacryl 300 gel filtration column. All the ATPase activity applied to the column was in this excluded fraction and its specific activity was only slightly greater than that of the starting material. This result suggests that the holoenzyme-detergent complex is very large. Much of the GCAM ATPase activity but less than 10% of the applied GCAM protein was retained by an ATP-sepharose affinity column. When the retained protein was eluted with ATP containing buffer and the fractions were separated by SDS-PAGE, the retained polypeptides were all in the range of 45 to 70 kDa. ATPase activity was associated with the protein retained by the ATP affinity column but its specific activity was not determined.

Initial attempts to label GCAM proteins with 14-C FSBA were successful to the extent that significant counts from certain portions of SDS-PAGE lanes were detected by liquid scintillation counting. However, the resolution was insufficient to assign the counts to specific protein bands. This was accomplished by electroblotting the polypeptides from the gel onto a nitrocellulose membrane and preparing an autoradiogram of the membrane. Comparison of autoradiograms with stained blots reveals at least two GCAM polypeptides that are both retained by an ATP affinity column and labeled by FSBA. These polypeptides are likely to contain catalytic and/or regulatory sites of GCAM ATPase.

3. Immunodifferences between GCAM and *Neurospora* vacuolar ATPases

GCAM K^+ ATPase is clearly different from both P-type and F-type ATPases (Wieczorek et al., 1986). Additionally, it is not homologous to the vacuolar ATPase of *Neurospora*. Polyclonal antibodies to the 70 kDa polypeptide of *Neurospora* vacuolar ATPase were kindly supplied by Dr. B.J. Bowman of the University of California in Santa Cruz. SDS-PAGE gels of GCAM were blotted onto nitrocellulose and probed with various dilutions of antibody to the 70 kDa *Neurospora* polypeptide. Even at very low dilution, these antibodies failed to react with any GCAM components. The anti-vacuolar ATPase antibodies also failed to inhibit either the basal or KCL-stimulated ATPase activity of GCAM. From both the inhibition and cross reaction experiments we conclude that *M. sexta* GCAM does not contain polypeptides antigenically similar to the 70 kDa polypeptide of *Neurospora* vacuolar ATPase. Therefore, the catalytic site - containing polypeptide of GCAM ATPase is unlikely to be highly homologous to that of *Neurospora* vacuolar ATPase.

4. Lack of Btk toxin inhibition of K^+ ATPase

Purified trypsin - activated Btk delta-endotoxin had no effect on GCAM ATPase activity over a range of toxin concentration from 10^{-13} M to 10^{-8} M. The highest concentration used corresponds to 0.1 mg toxin per mg GCAM protein and must surely correspond to a molar excess of toxin over ATPase. This result is consistent with the explanation that Btk toxin acts by forming K^+ channels in GCAM which increase the passive K^+ permeability of the midgut rather than by inhibiting the K^+ pump.

5. Methods for mass production of CCAM

Methods for rapid insolation of large quantities of CCAM vesicles from *Manduca sexta* larval midgut were developed for use in vesicle transport studies. The transport properties of CCAM vesicles, such as their K^+ permeability, are important for testing our K^+ homeostasis model at the subcellular level. A series of experiments were inititated to compare the properties of CCAM vesicles (BBMV) prepared by easily scalable batch techniques (Wolfersberger et al., 1987) to those prepared by our present sonication method (Cioffi & Wolfersberger, 1983). Preliminary results indicate that Mg^{2+} and Ca^{2+} precipitation, yield preparations similar to those prepared by sonication and that enzymically active CCAM vesicles can be prepared from frozen tissue using these precipitation methods (Eisen et al., 1988).

6. Apical membrane effectors as environmentally safe insecticides

Potential apical membrane effectors were screened for an effect on the short circuit current (SCC) of the isolated *Manduca sexta* midgut using a new "removable aperture" chamber (Dow et al., 1985). Under the conditions used in these experiments this SCC is a valid measure of K^+ transport (Harvey & Spaeth, 1988). Neem tree extract (Margosine), postulated to be an insect gut poison, had no effect at the highest concentration available. Other pesticides, including Carbaryl, Rotenone, Lannate, and Gardonna at high concentrations did not inhibit the *Manduca* midgut.

Trimethyl tin chloride inhibits the potassium current in *Spodoptera littoralis* at 10^{-9} M from the blood side but required 10^{-3} M from the lumen side (Thomas & May, 1984). Strong inhibition of *Manduca sexta* midgut required 10^{-6} M from either lumen side or blood side; at this concentration trimethyl tin chloride is a known mitochondial inhibitor.

7. Sparing of midgut from Btk toxin inhibition

Since the apical membrane sustains PD, K^+, and H^+ gradients and since Btk toxin disrupts these gradients by opening K^+ channels in this membrane, a K^+ channel blocking agent should prevent Bt toxin activity and spare both midgut and insect from Bt intoxication. Ba^{2+} is a near universal K^+ channel blocker known to block existing basal K^+ channels in *M. sexta* midgut (Zeiske et al, 1986). Therefore Crawford & Harvey (1988) studied the effects of Ba^{2+} in preventing Bt toxin inhibition of the SCC. Their results are summarized here.

When Btk delta-endotoxin at a final concentration of 50 nM was added to the lumen side at 45 minutes the SCC rapidly dropped to zero. Ba^{2+}, at 4 mM inhibits the SCC from the blood side but has minimal effects from the lumen side. However when 4 mM Ba^{2+} was present on the lumen side the SCC was protected from Btk toxin. Ca^{2+} was as effective as Ba^{2+}. Furthermore both Ba^{2+} and Ca^{2+} reversed the 50 nM Btk toxin inhibition of the SCC. Both protection of the SCC from Btk toxin and reversal of inhibition appear to be specific to Ba^{2+} and Ca^{2+}. Neither 4 mM Mg^{2+} nor 8 mM Na^+ or 8 mM choline interferred with the inhibitory action of Btk toxin on the SCC. In the absence of Btk toxin 4 mM Ba^{2+}, Ca^{2+}, Mg^{2+} and 8 mM Na^+ or choline did not affect the SCC.

These results are predicted from the Wolfersberger et al. (1986a) model for the primary action of lepidopteran-specific Bt delta-endotoxin. After binding to receptors on columnar cell apical membranes, the toxin either opens preexisting K^+ channels or forms new ones. When Btk toxin provides K^+ channels in CCAM the K^+ homeostasis is disrupted. K^+ enters the columnar cells by the toxin channels and shunts the electrical PD. The transepithelial SCC falls sharply but the K^+ pump in GCAM is initially unaffected (Harvey & Wolfersberger, 1979). Part of the drop in SCC is attributable to increased labeled-K^+ flux from lumen to blood. The rest of the drop must be due to a futile K^+ cycle in which K^+ enters the columnar cell cytoplasm across CCAM from the lumen, mixes with labeled-K^+ from basal space, diffuses across lateral junctions to goblet cell cytoplasm and is pumped across 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+} the K^+ homeostasis is immediately restored and the SCC returns to its normal level. Similarly, when Ba^{2+} is present at the time when Btk toxin is added, the toxin-induced K^+ channels will be blocked immediately upon formation and no SCC inhibition or disruption of K^+ homeostasis will be observed.

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 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 [K] 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 et al., 1985).

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