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V-ATPase-ENERGIZED EPITHELIA AND BIOLOGICAL INSECT CONTROL

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Summary

Background is provided for the experimentally detailed contributions concerning the structure, distribution and function of V-ATPase-based ion pumps in insect epithelia. The mode of action of an insecticidal bacterial protein, which is dependent upon the V-ATPase-energized state in larval lepidopteran midgut for activity, is discussed.

The nature of the insect electrogenic cation pump

Transport studies on insect epithelia led to an early rejection of the vertebrate paradigm of a Na^+/K^+-ATPase as the primary transducer of energy from chemical bonds to electrochemical ion gradients. More than twenty years ago Keynes (1969) proposed that a unique ion pump existed in insect gastrointestinal epithelia, and possibly in the stria vascularis as well as a few other highly specialized vertebrate tissues, which transported potassium ions electrogenically out of the cells, causing the output side to become electrically positive. The pump was found in the typical columnar epithelial cells of Malpighian tubules as well as in more specialized secretory cells of salivary glands and midgut. The only distinguishing features of cells that contained this pump seemed to be the presence of F_1-ATPase-like projections, later named portasomes (Harvey, 1980), on the cytoplasmic side of their apical plasma membrane. In many cases there was a very close association between the portasomes and mitochondria (Anderson and Harvey, 1966; Gupta and Berridge, 1966). Although it soon became apparent that this pump could transport not only potassium but all other alkali metal ions and possibly chloride (Harvey and Zerahn, 1972), it continued to be called a potassium pump.

At the time of the proposal that ion transport in insect epithelia was due to a unique electrogenic potassium pump (Keynes, 1969), it was already known that the biochemical correlate of the vertebrate sodium pump was a sodium- and potassium-stimulated Mg^{2+}-ATPase and the results of the first unsuccessful attempt to find a potassium-stimulated ATPase in insect ion-transporting epithelia had already been reported (Turbeck *et al.* 1968). Early failures to find potassium-sensitive ATPase activity in insect ion-transporting tissues coupled with observations of very rapid inhibition of active potassium transport by anoxia, and of the presence of high concentrations of extramitochondrial cytochrome b_5 in larval silkworm midgut, prompted the suggestion that at least in the midgut a redox pump might underlie the potassium transport system (Haskell *et al.* 1968). This suggestion was rejected by Mandel *et al.* (1975) on the basis of

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their observations that, during recovery of larval midguts from anoxia, both oxidation of mitochondrial cytochromes and restoration of active potassium transport occurred much more rapidly than oxidation of cytochrome b_5. Further studies of the kinetics of cytochrome reduction, tissue ATP depletion and inhibition of active potassium transport as midguts became anoxic suggested the presence of an ATP pool between respiration and potassium transport (Mandel *et al.* 1980).

At about the same time that evidence strongly favoring an ATPase underlying the midgut potassium pump was published, an abstract appeared claiming discovery of a potassium-modulated ATPase in a subcellular fraction from larval lepidopteran midgut that was enriched in plasma membranes and depleted of mitochondria (Wolfersberger, 1979). This ouabain- and oligomycin-insensitive potassium-stimulated enzyme appeared to be responsible for less than 15% of the ATP hydrolyzing activity in midgut homogenates (Wolfersberger *et al.* 1982). It was therefore practically undetectable in midgut homogenates unless the much more abundant F-ATPase was inhibited. Contamination with mitochondrial enzyme must surely have hindered earlier attempts to demonstrate the presence of potassium-stimulated ATPase activity in this and other insect epithelia containing potassium pumps. In many of these epithelia, mitochondria are very closely associated with the portasome-studded plasma membrane. The observation that this intimate association between mitochondria and the plasma membrane segment presumed to contain the potassium pump did not exist in the posterior portion of larval *Manduca sexta* midgut (Cioffi, 1979) provided the key both to the initial demonstration of potassium-modulated ATPase activity and to the subsequent purification of the enzyme from this tissue.

Since the invaginated apical membrane of the less abundant goblet cells was the only plasma membrane segment in larval lepidopteran midgut studded with portasomes, this membrane was postulated to be the site of the electrogenic potassium pump (Anderson and Harvey, 1966). This prediction was confirmed finally by X-ray microanalysis of potassium at various intracellular and extracellular sites in fully oxygenated and anoxic midguts (Dow *et al.* 1984). Therefore, efforts to correlate midgut potassium-sensitive ATPase activity further with the potassium pump focused on localization of the enzyme to the pump-containing membrane. A subcellular fraction highly enriched in goblet cell apical membranes was isolated from the posterior portion of larval *M. sexta* midguts (Cioffi and Wolfersberger, 1983). Cioffi and Wolfersberger (1983) also devised methods for isolating subcellular fractions highly enriched in other segments of plasma membranes from both major types of midgut epithelial cells. When all fractions were tested for potassium-stimulated ATPase activity, it was clear that this enzyme resided in the potassium-pump-containing goblet cell apical membrane (Wieczorek *et al.* 1986).

Further characterization of the midgut goblet cell apical membrane ATPase showed it to be stimulated about equally by KCl and RbCl. There was also lesser, but significant, stimulation by LiCl and NaCl. Approximately equal stimulation of ATPase activity by a variety of potassium salts, other than KNO_3, which strongly inhibited the enzyme, showed that the midgut goblet cell apical membrane ATPase was a cation-stimulated rather than an anion-stimulated enzyme. Furthermore, the order of effectiveness of cations in stimulating the enzyme was the same as the order of effectiveness of the alkali

metal ions as substrates for the midgut pump (Harvey and Zerahn, 1971). In addition to having the same subcellular location and cation selectivity, the midgut cation-stimulated ATPase and the midgut cation pump were both insensitive to ouabain and had essentially the same affinity for potassium and the same pH optimum. In view of these numerous shared characteristics, Wieczorek *et al.* (1986) proposed that the goblet cell apical membrane ATPase is a key component of the midgut electrogenic alkali metal ion pump.

Initial studies of midgut goblet cell apical membrane ATPase revealed several characteristics that, although not useful in correlating the enzyme with the ion pump, provided information about the nature of the enzyme (Wieczorek *et al.* 1986). As mentioned above, this ATPase was insensitive to ouabain and fluoride but inhibited strongly by nitrate. Furthermore, the enzyme was essentially insensitive to azide, oligomycin, orthovanadate and low concentrations of dicyclohexylcarbodiimide. However, $150\,\mu mol\,l^{-1}$ dicyclohexylcarbodiimide inhibited the ATPase almost completely. Today these inhibitor sensitivities would presumptively identify the midgut goblet cell apical membrane ATPase as a V-type ATPase. In 1986 they virtually eliminated the possibility that it was either a typical P-type or F-type ATPase. Further studies, described elsewhere in this volume (Wieczorek, 1992), have not only firmly established the midgut goblet cell apical membrane ATPase as a V-ATPase and a key component of the midgut potassium pump but have also addressed the interesting question of how one constructs an electrogenic potassium pump beginning with a vacuolar-type proton-motive ATPase.

The existence of a potassium pump in an insect epithelium was first deduced by Ramsay (1953) from *in vivo* measurements on Malpighian tubules and later demonstrated by Harvey and Nedergaard (1964) in isolated, short-circuited larval lepidopteran midgut. Evidence for a similar pump in salivary and labial glands followed (Berridge and Patel, 1968; Kafatos, 1968). The electrogenic nature and apical location of the midgut potassium pump were deduced from microelectrode measurements (Wood *et al.* 1969). More recently, evidence has been presented that a similar potassium pump is responsible for generating the receptor current in a variety of insect sensilla (Kueppers and Thurm, 1979; Wieczorek, 1982). The potassium pumps in all of these epithelia share many similar characteristics (see reviews by Thurm and Kueppers, 1980; Harvey, 1982) and are therefore tacitly assumed to be similar on a molecular level. Evidence for or against this assumption has been difficult to obtain. Some recent work suggests that bafilomycin A_1 may be useful in further establishing a relationship among the electrogenic potassium pumps in various insect epithelia (Bertram *et al.* 1991; Wieczorek *et al.* 1991). However, it is the purification and molecular characterization of the ATPase component of the midgut potassium pump that promises to provide the means for establishing the relatedness of potassium pumps in less readily accessible insect tissues. Elsewhere in this volume Klein (1992) describes the use of antibodies to *M. sexta* midgut V-ATPase in probing tissue sections and extracts for related antigens.

The functions of the electrogenic cation pump in midgut

For many years the major function of the midgut potassium pump in phytophagous

lepidopteran larvae was thought to be to remove excess potassium that diffused into the blood or midgut cells from the midgut lumen. This potassium secretion was thought to relieve the Malpighian tubules and rectum of a major role in regulating blood potassium concentration (Harvey, 1980). Dow (1986) found several well-known aspects of midgut structure and physiology difficult to reconcile with this proposed major pump function. The invaginated goblet cell apical membrane seemed to be a much less favorable location than the columnar cell apical membrane for a pump with the major function of secreting potassium into the midgut lumen. The electrical gradient (lumen >100 mV positive with respect to blood) and pH gradient (lumen pH 3 units higher than blood pH) were both much greater than the potassium concentration gradient across the midgut. Dow (1984) proposed a model in which the electrical potential generated by the pump energized potassium carbonate secretion. In this model, later refined (Dow and Harvey, 1988) and supported by additional experimental evidence (Dow and O'Donnell, 1990), generation of a large transepithelial electrical potential is the primary function of the midgut potassium pump. It is mainly the electrical potential difference created by the electrogenic pump that energizes nutrient uptake by midgut cells, regulates midgut lumen pH and determines the potassium concentration in blood, epithelial cells and midgut lumen (see Harvey, 1992).

Exploiting the midgut steady state for insect control

The magnitude of electrical potential and pH differences across various membranes defining the separate compartments within the midgut epithelium as well as the ion channels present in these membranes are discussed in detail elsewhere in this volume (Moffett and Koch, 1992; Zeiske, 1992). The maintenance of a large electrical potential difference across the apical membrane of both major midgut cell types implies a lack of potassium channels, or at least a lack of open potassium channels, in these membranes. This implication is supported by the observation that, within a few minutes after being added to the solution bathing the basal side of an isolated midgut, radioactive potassium equilibrates with tissue potassium whereas radioactive potassium added to the apical side solution fails to equilibrate with tissue potassium during several hours of incubation (Harvey *et al.* 1986). Plasma membranes without potassium channels seem to be quite rare. Most cells contain a variety of potassium channels and the distribution of potassium ions between their cytoplasm and the extracellular fluid is determined by active transport of other ions with more closely regulated access to the cytoplasm (Hille, 1992). This rare absence of potassium channels in midgut cell apical membranes contributes to their unique susceptibility to the parasporal proteins produced by *Bacillus thuringiensis*.

Bacillus thuringiensis is a spore-forming bacterium which is distinguished from *Bacillus cerus* by the formation of a parasporal inclusion body during sporulation. *B. thuringiensis* was first isolated from diseased silkworms (Ishiwata, 1901). It was demonstrated that only sporulated cultures of this previously undescribed bacillus were toxic to silkworm larvae and the presence of a parasporal inclusion was noted. As early as 1915 the possibility of using this natural insect pathogen in insect pest control was discussed. However, with the emergence of numerous cheap and effective synthetic

chemical insecticides, the use of *B. thuringiensis* as an insect control agent remained mainly a possibility for many years. During these years new isolates of *B. thuringiensis* were obtained from sites all over the world. All were found to be toxic only to larvae of certain insects of the order Lepidoptera. This narrow target range was considered a further liability of *B. thuringiensis* as a commercial insecticide (see Luethy *et al.* 1982, for a discussion of the history of *B. thuringiensis*). In more recent years the realization of the magnitude of their environmental damage and their decreasing effectiveness in controlling insect pests have greatly decreased user and producer as well as public confidence in synthetic chemical pesticides. New *B. thuringiensis* strains toxic to dipteran and coleopteran as well as lepidopteran insects have been isolated (see Hoefte and Whiteley, 1989, for a recent review of *B. thuringiensis* toxins). The advent of recombinant DNA technologies has sparked new interest in developing microbial products for commercial applications. These factors have come together to ignite new interest in *B. thuringiensis* as an insect control agent.

The insecticidal activity of *B. thuringiensis* has been shown to be due to its characteristic proteinaceous parasporal inclusion (Angus, 1954). These parasporal bodies did not dissolve in physiological buffers but appeared to dissolve rapidly in the midguts of susceptible insects. When heated in the presence of detergent plus reducing agent and separated by SDS–PAGE, parasporal bodies toxic to lepidopteran larvae typically yielded one or two bands of approximately $130\times10^3 M_r$. When isolated inclusion bodies were incubated with larval gut contents, the size of the principal bacterial protein decreased with incubation time until a stable product about half the size of the primary inclusion body protein was formed (Huber and Luethy, 1981). This sequence of events could be reproduced *in vitro* by dissolving the parasporal bodies in a pH 10.5 buffer, or a pH 9.5 buffer containing a reducing agent, and then incubating the resulting parasporal body protein solution with trypsin. Thus, it appeared that susceptibility to *B. thuringiensis* was limited to insects with a high pH and trypsin-like enzymes in their gut (Jaquet *et al.* 1987).

Numerous histopathological studies of lepidopteran larvae fed *B. thuringiensis* inclusion bodies (reviewed by Luethy and Ebersold, 1981) revealed initial swelling and distortion of midgut columnar cell microvilli followed by distortions in the shape of cell organelles and finally lysis of the cells. In a prophetic publication, Angus (1968) noted that essentially this same progression of symptoms could be produced by feeding larvae the potassium ionophore valinomycin. However, valinomycin had neither the selectivity nor the specific larvicidal activity of *B. thuringiensis* inclusion body proteins.

The high selectivity and specific larvicidal activity of *B. thuringiensis* parasporal body proteins suggested the presence of a protein–protein recognition step in their mode of action (Luethy *et al.* 1986). This prediction was fulfilled by the demonstration of high-affinity saturable binding between the trypsin-stable portion of a *B. thuringiensis* parasporal body protein (toxin) and brush-border membrane vesicles (BBMV) from larval *Pieris brassicae* midgut (Hofmann *et al.* 1988). This initial report of specific binding between a *B. thuringiensis* toxin and BBMV from a target insect midgut was followed by a series of reports of similar studies with different insects and toxins in which a direct correlation was usually found between larvicidal activity and the concentration and affinity of binding sites (Van Rie *et al.* 1990). However, this correlation was not

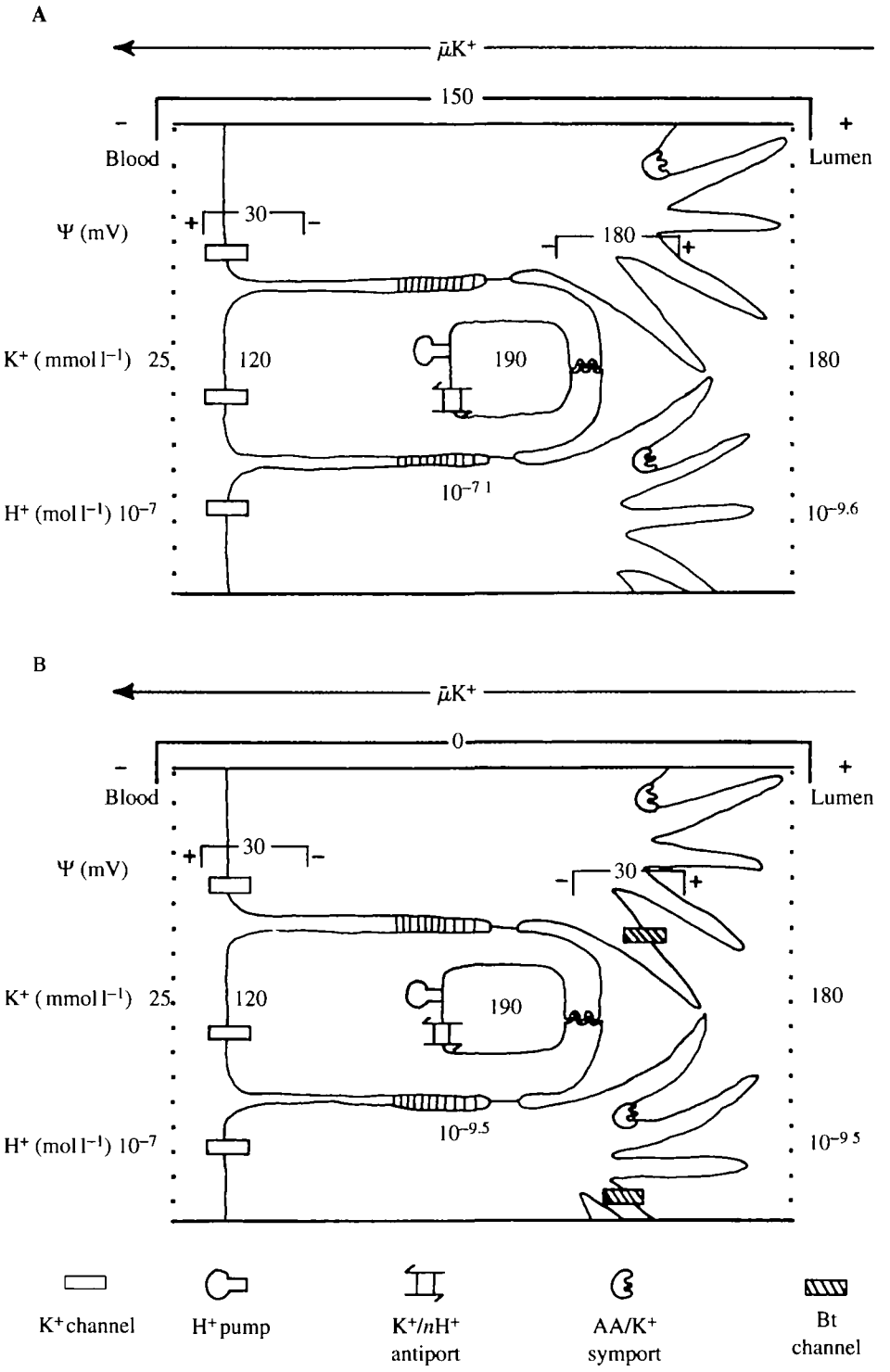
A
$\bar{\mu}K^+$
150
Blood
Lumen
Ψ (mV)
30
180
K^+ (mmol l^{-1}) 25
120
190
180
$10^{-7.1}$
H^+ (mol l^{-1}) 10^{-7}
$10^{-9.6}$
B
$\bar{\mu}K^+$
0
Blood
Lumen
Ψ (mV)
30
30
K^+ (mmol l^{-1}) 25
120
190
180
$10^{-9.5}$
H^+ (mol l^{-1}) 10^{-7}
$10^{-9.5}$
K^+ channel
H^+ pump
K^+/nH^+ antiport
AA/K^+ symport
Bt channel

Fig. 1

Fig. 1. Electrochemical gradient ($\bar{\mu}K^+$), electrical potential differences (Ψ), K^+ concentration and H^+ activity in larval lepidopteran midgut in the normal steady state (A) and after treatment with *Bacillus thuringiensis* toxin (Bt) (B). AA, amino acid.

quantitative (Wolfersberger, 1990). Furthermore, the binding was found to be essentially irreversible (Van Rie *et al.* 1989).

The irreversibility of the toxin–BBMV interaction was expected from previous studies. Wolfersberger *et al.* (1986) were unable to detect toxin on the outer surface or in the inner space of BBMV that had been incubated with toxin for more than 10 min. However, when BBMV that had been incubated with toxin for several minutes to several hours were denatured in the presence of detergent, intact toxin molecules were found among the components of their membranes. On the basis of these results, they proposed that, soon after binding to a cell membrane component, the toxin molecule became integrated into the cell membrane. Hydropathy analyses of the primary structures of toxins, predicting the presence of several potential membrane-spanning helices (Geiser *et al.* 1986), were consistent with this proposal. Experimental evidence strongly favoring this proposal was supplied by Slatin *et al.* (1990), who showed that *B. thuringiensis* toxins were able to form cation-selective channels in planar phospholipid bilayers. These results, together with the results of two earlier studies demonstrating (1) that the primary effect of *B. thuringiensis* toxin on isolated larval *M. sexta* midgut is to increase the passive leak of potassium from lumen to blood rather than to interfere with the active transport of potassium from blood to lumen (Harvey and Wolfersberger, 1979) and (2) that *B. thuringiensis* toxins inhibit amino acid/potassium cotransport by creating an alternative lower-resistance pathway for potassium to cross the midgut columnar cell apical membrane (Sacchi *et al.* 1986), combined with our knowledge of steady-state conditions in larval lepidopteran midgut, allow us to arrive at a mechanism for the larvicidal action of *B. thuringiensis* toxin.

Parasporal bodies ingested by a larva dissolve in the alkaline midgut lumen and are cleaved by trypsin-like enzymes to trypsin-resistant toxin molecules. The toxin diffuses across the peritrophic membrane and binds to specific proteins of the brush-border membrane. Toxin molecules insert into the membrane and form cation-conducting pores. The cation permeability of the membrane increases greatly. The large electrical potential difference (PD) normally maintained across this membrane collapses. The collapse of the transmembrane PD removes the driving force for amino acid uptake by midgut cells and allows for redistribution of cations between the midgut lumen and cell cytoplasm. However, by far the most devastating consequence of the collapse is loss of the force energizing maintenance of the 1000-fold H^+ activity gradient across the apical membrane of midgut cells. The loss of membrane PD is predicted (Fig. 1) to give rise to an increase in cytoplasmic pH large enough to disrupt normal cell metabolism to an extent sufficient to produce the symptoms seen in histopathological studies and lead to the eventual death of the larva.

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