

# THE INSECT V-ATPase, A PLASMA MEMBRANE PROTON PUMP ENERGIZING SECONDARY ACTIVE TRANSPORT: MOLECULAR ANALYSIS OF ELECTROGENIC POTASSIUM TRANSPORT IN THE TOBACCO HORNWORM MIDGUT

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## Summary

Goblet cell apical membranes in the larval midgut of *Manduca sexta* are the site of active and electrogenic  $K^+$  secretion. They possess a vacuolar-type ATPase which, in its immunopurified form, consists of at least nine polypeptides. cDNAs for the *A* and *B* subunits screened by monoclonal antibodies to the *A* subunit of the *Manduca* V-ATPase or by hybridisation with a cDNA probe for a plant V-ATPase *B* subunit have been cloned and sequenced. There is a high degree of identity to the sequences of the respective subunits of other V-ATPases.

The *M. sexta* plasma membrane V-ATPase is an electrogenic proton pump which energizes, by the electrical component of the proton-motive force, electrogenic  $K^+/nH^+$  antiport, resulting in net electrogenic  $K^+$  secretion. Since the midgut lacks a  $Na^+/K^+$ -ATPase, all solute fluxes in this epithelium seem to be energized by the V-ATPase. Thus, the midgut provides an alternative to the classical concept of animal plasma membrane energization by the  $Na^+$ -motive force generated by the  $Na^+/K^+$ -ATPase.

## Introduction

Insect ion-transporting epithelia contain a unique alkali metal ion pump (Harvey *et al.* 1983a; see Klein, 1992) which is insensitive to ouabain, resides in the apical plasma membrane and pumps ions actively and electrogenically out of cells. Since  $K^+$  is the ion usually pumped under physiological conditions, this pump has been designated simply as the potassium pump. The midgut of phytophagous lepidopteran larvae possesses a potassium pump in the apical membrane of the goblet cells (Dow *et al.* 1984), one of the two main cell types in its monolayered epithelium. As the midgut lacks a  $Na^+/K^+$ -ATPase, all solute fluxes seem to be energized by the potassium pump (Harvey *et al.* 1983a). The demonstration that the activity of the potassium pump is directly correlated with the intracellular ATP concentration (Mandel *et al.* 1980), together with the observation that the cytoplasmic surface of goblet cell apical membranes is studded by 10-nm  $F_1$ -like particles, led to the suggestion that the potassium pump is, like F-ATPases, an ion-transporting ATPase, pumping  $K^+$  instead of protons (Harvey *et al.* 1981). Indeed, studies with partially purified membranes from the midgut of the lepidopteran larva of *Manduca sexta*, the tobacco hornworm, gave the first indications of the existence of a  $K^+$ -

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modulated ATPase (Wolfersberger, 1979; Wolfersberger *et al.* 1982; Harvey *et al.* 1983*b*). Results similar to those obtained from tobacco hornworm midgut were reported for partially purified membrane extracts of sensory sensilla of the adult fly *Protophormia terraenovae* (Wieczorek, 1982; Wieczorek and Gnatzy, 1985).

### **Cation-stimulated ATPase activity in the purified goblet cell apical membrane**

The development of ingenious methods for the isolation of highly purified plasma membranes from different regions of tobacco hornworm midgut epithelial cells, including goblet cell apical membranes containing the potassium pump (Cioffi and Wolfersberger, 1983), opened the field for straightforward biochemical investigations (Wieczorek *et al.* 1986). Purified goblet cell apical membranes (GCAM) exhibited K<sup>+</sup>-stimulated Mg<sup>2+</sup>-dependent ATPase activity. GTP was hydrolyzed nearly as effectively as ATP, but CTP, ADP, AMP and *p*-nitrophenyl phosphate could not serve as substrates. The ATPase was sensitive neither to the Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitors ouabain or vanadate, nor to the mitochondrial ATPase inhibitors azide or oligomycin; however, *N,N'*-dicyclohexylcarbodiimide as well as nitrate were effective inhibitors. Taken together, GCAM ATPase activity displayed characteristic features that distinguished it from P-ATPases as well as from F-ATPases (Pedersen and Carafoli, 1987). In its properties it resembled the ATPases from various acidic organelles (e.g. Gluck *et al.* 1982; Bowman, 1983; Cidon and Nelson, 1983; O'Neill *et al.* 1983) which today are well known as constituting the third elementary family of ion-transporting ATPases, the V-ATPases (Nelson and Taiz, 1989; Forgac, 1989).

### **Purification and molecular properties of the GCAM V-ATPase**

For the isolation of the ATPase from the goblet cell apical membrane (Schweickl *et al.* 1989), we used the non-ionic detergent C<sub>12</sub>E<sub>10</sub> to solubilize the enzyme from a partially purified membrane fraction. The supernatant of the 100 000 *g* fraction was layered on a discontinuous sucrose gradient; after centrifugation at 220 000 *g* for 1 h, nearly all of the azide- and vanadate-insensitive ATPase activity was found in the 30% sucrose fraction uncontaminated by azide- or vanadate-sensitive ATPases. This solubilized and purified ATPase activity showed the same substrate and inhibitor sensitivity as the membrane-bound GCAM ATPase. It was, like the membrane-bound form, sensitive to submicromolar bafilomycin A<sub>1</sub> concentrations (Wieczorek *et al.* 1991) and thus undoubtedly belongs to the family of V-ATPases.

Analysis of western blots, after denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), showed that the purified ATPase consisted of five major subunits with relative molecular masses (*M<sub>r</sub>*) of 67 000, 56 000, 43 000, 28 000 and 16 000 (Fig. 1A; Wieczorek *et al.* 1990). The same result was observed when we used highly purified GCAM instead of the partially purified membrane fraction for the solubilization and purification procedure (Fig. 1B; A. Lepier and H. Wieczorek, unpublished results). Analysis of all polypeptides occurring in the highly purified goblet cell apical membrane revealed that the ATPase is a major constituent of GCAM (Fig. 1C; Wieczorek *et al.*

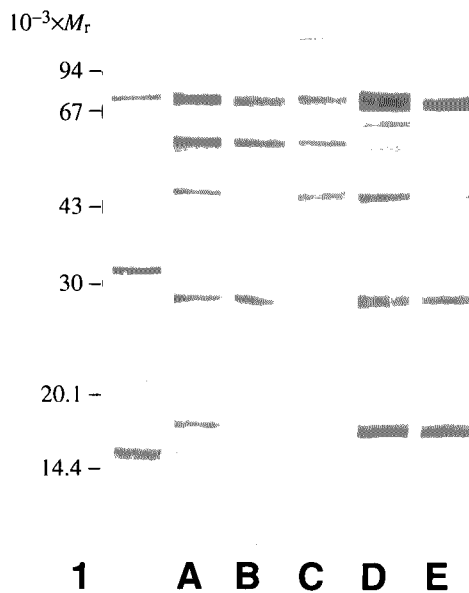


Fig. 1. Polypeptide pattern of GCAM ATPase and of highly purified GCAM. (A,D) ATPase, prepared according to Schweickl *et al.* (1989) or Wieczorek *et al.* (1990). (B,E) ATPase, prepared as for A and D, but from the highly purified GCAM. (C) GCAM, prepared according to Cioffi and Wolfersberger (1983) or Wieczorek *et al.* (1990). Western blots after SDS-PAGE stained with Amido Black (A–C) or with immune serum directed to the purified ATPase, visualized by alkaline phosphatase-conjugated anti-rabbit antibodies (D and E); methods according to Wieczorek *et al.* (1991). Left lane:  $M_r$  standards (94 000, 67 000, 43 000, 30 000, 20 100, 14 400).

1991). Immunostaining with a polyclonal antiserum to the purified ATPase exhibited, in addition to the five major bands, several bands between  $M_r$  67 000 and 43 000 and two glycosylated bands at  $M_r$  40 000 and 20 000 (Fig. 1D,E; Wieczorek *et al.* 1991; U. Klein, A. Lepier, B. Förg-Brey and H. Wieczorek, unpublished results). In the range below  $M_r$  20 000, two minor bands occurred in addition to the major  $M_r$  16 000 band, one at  $M_r$  14 000, and one at  $M_r$  17 000. The latter, which is not visible in Fig. 1, binds  $10 \mu\text{mol l}^{-1}$  *N,N'*-dicyclohexylcarbodiimide (A. Lepier and H. Wieczorek, unpublished results) and may therefore represent the proton-conducting proteolipid. To check for constitutional components of the ATPase, four monoclonal antibodies to the ATPase, which bind to defined subunits of  $M_r$  67 000, 28 000 or 16 000 in the SDS-denatured state, were used for affinity chromatography of the purified ATPase on antibody-saturated Protein G-Sepharose (U. Klein, A. Lepier, B. Förg-Brey and H. Wieczorek, unpublished results). The polypeptide pattern of the immunopurified ATPase as revealed by staining with the polyclonal antiserum was independent of the antibody binding sites. Apart from slight variations regarding some of the minor bands, it was not altered essentially by this purification step. According to our present experimental evidence, the insect plasma membrane V-ATPase consists of at least nine polypeptides of  $M_r$  67 000, 56 000, 43 000,

40 000, 28 000, 20 000, 17 000, 16 000 and 14 000. This high number of subunits corresponds to the high subunit number found in other V-ATPases.

We prepared a cDNA library from larval midgut of *Manduca sexta* (Gräf *et al.* 1992), and screened it with two monoclonal antibodies to the 67 000  $M_r$  subunit (subunit A) of the GCAM ATPase, both of which also had been used for affinity purification of the holoenzyme. The primary structure of this first invertebrate subunit A, as predicted from the cDNA sequence, resembled that of the hitherto published eukaryotic subunits A (*Neurospora crassa*: Bowman *et al.* 1988; carrot: Zimniak *et al.* 1988; yeast: Hirata *et al.* 1990; *Bos bovis*: Pan *et al.* 1991; Puopolo *et al.* 1991). It exhibited the highest degree of similarity to the bovine amino acid sequences with an identity of more than 80%. Although we cannot be sure whether we have cloned the subunit A of the GCAM ATPase instead of that of an endomembrane V-ATPase, several lines of evidence favour the assumption that our published cDNA sequence is that of the plasma membrane V-ATPase (Gräf *et al.* 1992). A cDNA clone encoding midgut subunit B of *Manduca sexta* midgut V-ATPase was obtained by hybridization with a labelled cDNA encoding subunit B of plant tonoplast V-ATPase (Novak *et al.* 1992). The deduced amino acid sequence of *Manduca sexta* subunit B was more than 98% identical with that of a V-ATPase subunit B from the midgut and Malpighian tubules of the lepidopteran *Helicoverpa virescens* (Gill and Ross, 1991) and showed a high degree of identity to all eukaryotic subunits B of V-ATPases published so far.

### ATP-dependent proton transport and $K^+/nH^+$ antiport

Having established that the goblet cell apical membrane of tobacco hornworm midgut possesses a V-ATPase, one problem became evident: all V-ATPases known so far are proton pumps, but what we had been expecting in this  $K^+$ -transporting membrane was a potassium pump. We therefore investigated vesicle preparations enriched with or derived from the highly purified GCAM and demonstrated that they exhibited ATP-dependent and electrogenic proton transport as monitored with the fluorescent probes Acridine Orange and Oxonol V (Wieczorek *et al.* 1989, 1991). The substrate specificity and inhibitor sensitivity of the proton transport were identical to those of the GCAM ATPase. However, unlike the ATPase activity, ATP-dependent proton transport was not stimulated by  $K^+$ . In the presence of  $20 \text{ mmol l}^{-1} K^+$  no pH gradient developed, and the addition of  $K^+$  to the vesicle suspension dissipated an established pH gradient (Fig. 2A). Because the salt specificity of this counteraction was identical with the stimulating capacity of salts on the membrane-bound ATPase activity, we made the hypothesis that a  $K^+/H^+$  antiport exists across the goblet cell apical membrane. This hypothesis was proved to be correct by our demonstration that vesicles derived from highly purified GCAM, which had been preloaded with  $20 \text{ mmol l}^{-1} K^+$ , exhibited an ATP-independent development of a pH gradient in a medium containing less than  $1 \text{ mmol l}^{-1} K^+$  (Fig. 2B). A polyclonal antiserum to the purified GCAM ATPase inhibited ATP-dependent proton transport, but not ATP-independent  $K^+/H^+$  antiport, suggesting that the ATPase and the antiporter are two different molecular entities.

The evidence so far accumulated enabled us to 'construct' a potassium pump, in which

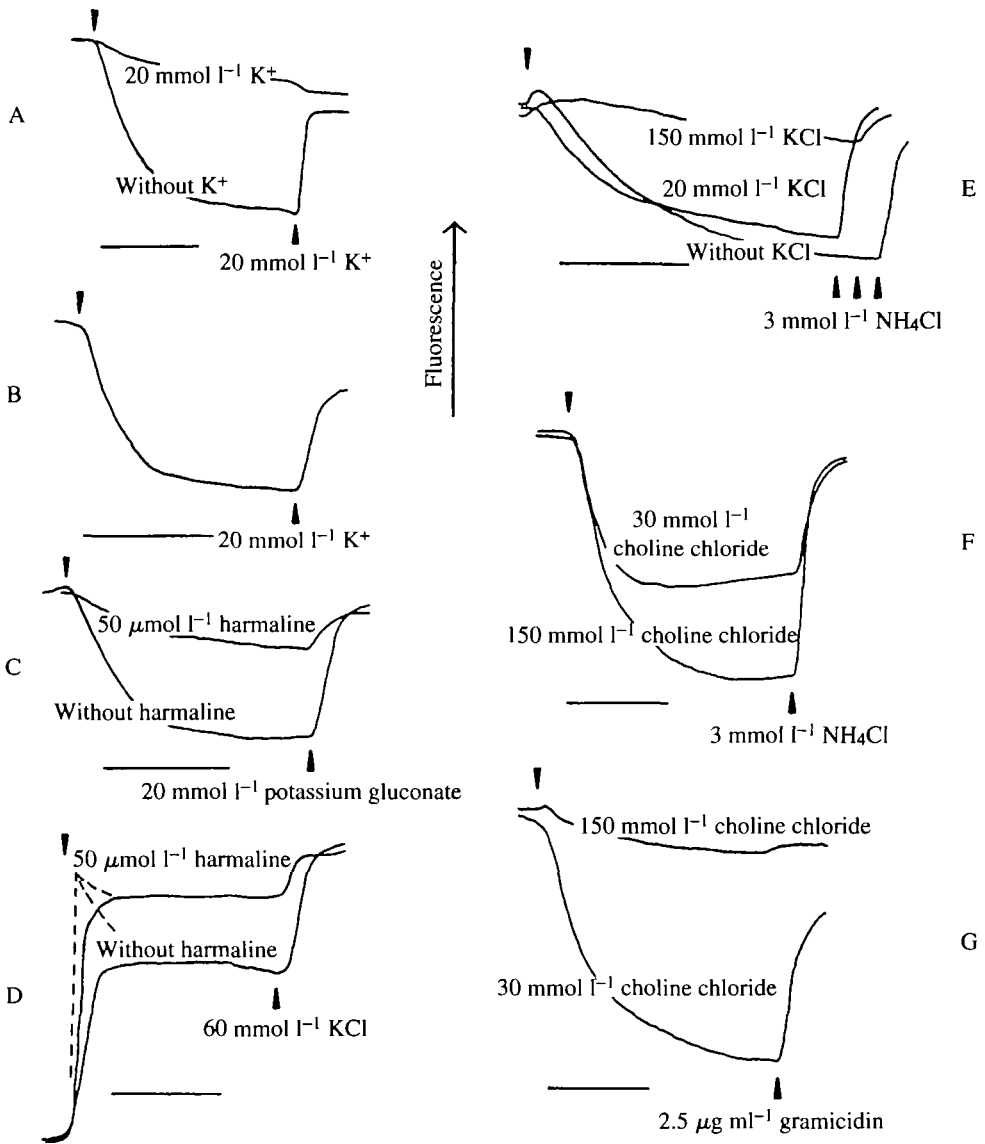


Fig. 2. Proton transport in GCAM vesicles. The fluorescence quench of Acridine Orange (A, B, C, E and F) served as an indicator for the acidification of vesicles, and the fluorescence of Oxonol V (D and G) as an indicator for an intravesicular positive voltage (methods according to Wieczorek *et al.* 1989, 1991). Horizontal bars are time scales indicating 30 s. All experiments were performed with highly purified GCAM, except F and G, for which a partially purified GCAM preparation (according to Wieczorek *et al.* 1989) was used. All experiments except E were started by the addition of a vesicle suspension to the reaction mixture; E was started by the addition of  $\text{Mg}^{2+}$  to the reaction mixture containing the vesicles. Proton or voltage gradients were dissipated as indicated. The dotted lines in D show that the addition of vesicles led to a fast increase in fluorescence superimposed on the initial fluorescence decrease (discussed in Wieczorek *et al.* 1991). Further information is given in the text.

primary electrogenic ATP-dependent proton transport energizes secondary  $K^+/H^+$  antiport. However, the evidence for  $K^+/H^+$  antiport was not identical with the proof that the V-ATPase was an exclusive proton pump. Although all V-ATPases known so far pump protons in the presence of high cytosolic  $K^+$  concentrations, we could not exclude the possibility that the GCAM ATPase is a  $K^+$  pump, transporting protons only in the absence of  $K^+$ . This problem was overcome by experiments in which we inhibited the  $K^+/H^+$  antiport with amiloride and demonstrated that, under this condition, an ATP-dependent pH gradient could be established even in the presence of  $K^+$ . Therefore, we could conclude that the V-ATPase in the goblet cell apical membrane is exclusively a proton pump. These results provided conclusive evidence for our hypothesis that  $K^+$  transport in the tobacco hornworm midgut is the result of the energization of  $K^+/H^+$  antiport by a V-ATPase.

Another problem arose when Chao *et al.* (1991) demonstrated by means of ion-sensitive microelectrodes that, under short-circuit conditions, no pH gradient and no  $K^+$  gradient could be observed across the goblet cell apical membrane. The only possible driving force left was the electrical potential difference of about 100 mV across the goblet cell apical membrane. Therefore  $K^+/H^+$  antiport had to be electrogenic,  $K^+/nH^+$ , with more than one proton exchanged for each  $K^+$  crossing the membrane. Indeed, we had already reported that the fluorescence quench of Oxonol V, indicating the development of a voltage across the vesicle membrane, was lowered by about 15% if ATP-dependent proton transport was monitored in the presence of  $K^+$  (Wieczorek *et al.* 1989). This result can be interpreted as an indication of the electrogenicity of the  $K^+/H^+$  antiport, although the voltage across the vesicle membrane was not influenced dramatically. Our assumption was validated by the direct demonstration that the ATP-independent exchange of  $K^+$  and protons was indeed electrogenic (Wieczorek *et al.* 1991). Fig. 2C,D demonstrates that harmaline, an inhibitor of  $K^+/H^+$  antiport, also inhibited the development of the antiport-dependent voltage. Our present lines of research include the isolation of the antiporter and the determination of the stoichiometry of  $K^+/nH^+$  antiport.

Most of our experiments had been performed at a pH of approximately 8. However, the microelectrode measurements of Chao *et al.* (1991) had shown that the V-ATPase has to operate at a pH of about 7. We therefore measured ATP-dependent proton transport at a pH of 7.2 (H. Wieczorek and K. Dethleffsen, unpublished results). Fig. 2E demonstrates that at this pH the proton gradient established by the V-ATPase was not dissipated by 20 mmol  $l^{-1}$   $K^+$ . Instead, higher concentrations of more than 100 mmol  $l^{-1}$  were required for complete dissipation. This means that *in vivo* the antiporter's properties are tuned to the physiological  $K^+$  concentration.

The goblet cell apical membrane not only houses a V-ATPase and a  $K^+/nH^+$  antiporter, but also anion channels (Wieczorek *et al.* 1989), as can be concluded from Fig. 2F,G: when ATP-dependent proton transport was measured at a high chloride concentration, the pH gradient increased drastically, but the development of a membrane potential was largely suppressed. It is a present matter of discussion (see Harvey, 1992) to what extent these anion channels are open under physiological conditions and whether they are regulated as has been shown, for example, for chloride channels from plasma membranes and from endomembranes (Kunzelmann *et al.* 1991; Mulberg *et al.* 1991).

### Conclusion and perspectives

Evidence accumulated over recent years indicates that active and electrogenic  $K^+$  secretion in the tobacco hornworm midgut results from electrogenic  $K^+/nH^+$  antiport, which is energized by the electrical component of the proton-motive force generated by an electrogenic V-type proton pump (Fig. 3). The ATPase in the goblet cell apical membrane belongs to the increasingly large subfamily of plasma membrane V-ATPases and is the first V-ATPase shown to energize secondary active transport in a eukaryotic plasma membrane. Thus, the midgut may serve as a model epithelium providing an alternative to the classical concept of animal plasma membrane energization by the  $Na^+$ -motive force generated by the  $Na^+/K^+$ -ATPase (see Klein, 1992).

The occurrence of a V-ATPase in the plasma membrane presents a challenge to determine how sorting of the integral as well as of the peripheral subunits is accomplished during biogenesis. Furthermore, we do not know how many genes code for the diverse subunits of the various V-ATPases or how they are regulated. At present, many published sequences for V-ATPase subunits have been identified just with the membranes from which the V-ATPase protein has been isolated, but there is evidence from plants as well as from vertebrates that more than one gene per subunit may exist (e.g. subunit A: Starke *et al.* 1991; Pan *et al.* 1991; Puopolo *et al.* 1991). So far the molecular analysis of the insect plasma membrane V-ATPase lags behind that of the prominent master V-ATPases,

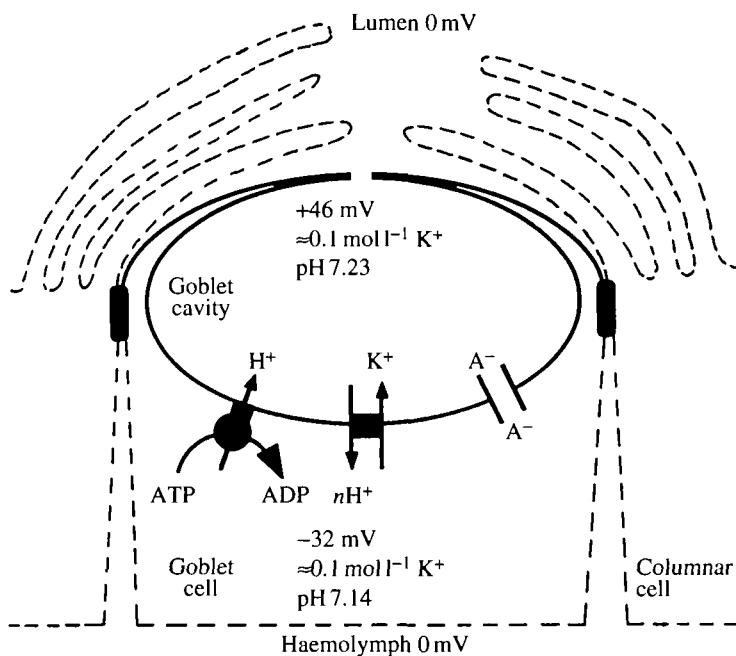


Fig. 3. Functional elements of active and electrogenic  $K^+$  transport across the goblet cell apical membrane (continuous line) of tobacco hornworm midgut. Voltages, pH values and  $K^+$  concentrations are as measured under short-circuit conditions (for further explanation, see Wiczorek *et al.* 1991).

but we hope that somehow and sometime the midgut may be recognized and accepted as a model epithelium not only by physiologists but also by molecular cell biologists.

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