*J. exp. Biol.* ***196****, 361–373 (1994)*
*Printed in Great Britain © The Company of Biologists Limited 1994*



# $K^+/H^+$ ANTIPORT IN THE TOBACCO HORNWORM MIDGUT: THE $K^+$-TRANSPORTING COMPONENT OF THE $K^+$ PUMP

ALEXANDRA LEPIER[1], MASAAKI AZUMA[2], WILLIAM R. HARVEY[3] AND HELMUT WIECZOREK[1,*]

[1]*Zoologisches Institut der Universität, Luisenstraße14, D-80333 München, Germany,* [2]*Faculty of Agriculture, Tottori University, Tottori 680, Japan* and [3]*Department of Biology, Temple University, Philadelphia PA 19122, USA*

## Summary

The midgut of the tobacco hornworm secretes $K^+$ across the apical plasma membrane of its goblet cells. This secondary $K^+$ transport results from $K^+/H^+$ antiport energized by the proton-motive force generated by a primary, $H^+$-transporting plasma membrane V-ATPase. Thus, the lepidopteran midgut constitutes a well-established example of the emerging concept that the proton-motive force is an alternative to the classical sodium-motive force for the energization of animal plasma membranes.

$K^+/H^+$ antiport in the tobacco hornworm midgut is electrophoretic, exchanging $2H^+$ for $1K^+$. Under physiological conditions, it is energized by the voltage component of the proton-motive force. The strong coupling of electrophoretic $K^+/2H^+$ antiport with the electrogenic V-ATPase provides, in principle, the minimal device for the alkalization of the midgut lumen to pH values higher than 11.

$K^+/H^+$ antiport is insensitive to bafilomycin A1, but is inhibited by amiloride or Concanavalin A. Lectin staining of blots after SDS–PAGE revealed several glycosylated polypeptides in the goblet cell apical membrane which are not part of the V-ATPase and thus are candidates for the antiporter protein. Current efforts are focused on the isolation of the $K^+/H^+$ antiporter.

## The $K^+$ pump in the lepidopteran midgut: an exception to a general rule

It is widely accepted that animal plasma membranes are energized by the $Na^+/K^+$-ATPase which generates a sodium-motive force driving sodium-dependent secondary transport processes (Harold, 1986). The larval lepidopteran midgut is a well-documented exception to this dogma since it lacks a $Na^+/K^+$-ATPase (Jungreis and Vaughan, 1977). Instead, all secondary transport processes across the midgut epithelium, including the absorption of amino acids and the regulation of the high pH in the midgut lumen, appear to be energized by a unique alkali metal ion pump (Harvey *et al.* 1983*a*). As $K^+$ is the ion transported *in vivo*, this pump has been referred to simply as the $K^+$ pump (pump = transport mechanism). It was discovered in the isolated midgut of the *Cecropia*

*To whom reprint requests should be addressed.

Key words: $K^+/H^+$ antiport, plasma membrane, V-ATPase, potassium pump, insects, *Manduca sexta*, midgut.

silkworm 30 years ago (Harvey and Nedergard, 1964), shortly after the discovery of the $Na^+/K^+$-ATPase by Skou (1957), and was characterized as $Na^+$-independent active transport of $K^+$. The $K^+$ pump resides in the apical plasma membrane (Wood *et al.* 1969) of goblet cells (Dow *et al.* 1984; Moffett and Koch, 1988), one of the two types of differentiated cells in the single-layered epithelium. It transports $K^+$ electrogenically into the gut lumen, thereby creating a transmembrane voltage in excess of 250 mV, lumen positive (Dow and Peacock, 1989).

### Active $K^+$ transport is energized by a plasma membrane V-ATPase

Active $K^+$ transport and intracellular ATP concentration decrease in parallel during oxygen deprivation (Mandel *et al.* 1980). Moreover, the cytoplasmic side of the goblet cell apical membrane is studded by $F_1$-like particles (Anderson and Harvey, 1966). Therefore, it was tempting to suggest that, like F-ATPases, the $K^+$ pump is an ion-transporting ATPase, pumping $K^+$ instead of $H^+$ (Harvey *et al.* 1981). Indeed, partially purified membranes from larval *Manduca sexta* (Lepidoptera, Sphingidae) midgut contained a $K^+$-modulated ATPase activity (Wolfersberger, 1979; Wolfersberger *et al.* 1982). Using ingenious methods for the isolation of highly purified midgut plasma membranes (Harvey *et al.* 1981; Cioffi and Wolfersberger, 1983), studies with purified goblet cell apical membranes proved the presence of $K^+$-stimulated ATPase activity (Wieczorek *et al.* 1986). Characterization of this ATPase after solubilization and purification demonstrated that it belongs to the family of V-ATPases (Schweikl *et al.* 1989; Wieczorek *et al.* 1990, 1991; Wieczorek, 1992). A typical representative of these heteromultimeric proteins, the insect plasma membrane V-ATPase is composed of at least seven different subunits (Wieczorek, 1992). Amino acid sequences deduced from cDNAs encoding four of these subunits show substantial similarities to subunits of V-ATPases from other sources (Dow *et al.* 1992; Gräf *et al.* 1992, 1994; Novak *et al.* 1992).

Since the V-ATPase appears to be the only ATP-hydrolyzing enzyme in the goblet cell apical membrane, it necessarily has to energize active $K^+$ transport. However, the insect plasma membrane V-ATPase is, like all V-ATPases, an electrogenic proton pump (Wieczorek *et al.* 1989). In contrast, goblet cell apical membranes do not transport $H^+$ but $K^+$.

### $K^+/H^+$ antiport is the source of $K^+$ transport

Using fluorescent Acridine Orange as a pH indicator in vesicle studies, the ATP-dependent proton transport was shown to have the same substrate and inhibitor specificity as enzyme-mediated ATP hydrolysis (Schweikl *et al.* 1989; Wieczorek *et al.* 1989). Nevertheless, there was a marked difference between the two activities. $K^+$ and other alkali metal ions transported by the $K^+$ pump stimulated ATPase activity (Wieczorek *et al.* 1986), whereas these same ions in vesicle experiments inhibited the development of a pH gradient (Wieczorek *et al.* 1989). Moreover, the addition of $K^+$ and other cations to the extravesicular medium after the generation of an ATP-dependent pH gradient caused its dissipation (Wieczorek *et al.* 1989). Two explanations could account for this

seemingly contradictory behaviour. On the one hand, the insect enzyme could be a $K^+$-ATPase that pumps $H^+$ in the absence of $K^+$ or other alkali metal ions, but pumps cations in their presence; the dissipation of a pH gradient after addition of the ions would, in this case, be due to unspecific proton leaks in the vesicle membrane. On the other hand, the insect enzyme could be a proton pump energizing alkali metal ion/proton antiport in the same membrane; the apparent inhibition of the generation of a pH gradient in the presence of alkali metal ions and its dissipation after their addition would, in that case, be due not only to unspecific proton leaks but also to the presence of a $K^+/H^+$ antiport as a 'specific' proton leak. Two lines of evidence favour the latter hypothesis that the V-ATPase is a proton pump.

First, vesicles derived from the highly purified goblet cell apical membrane exhibit ATP-independent $K^+/H^+$ antiport (Wieczorek *et al.* 1991). Vesicles which had been preloaded with 20 mmol $l^{-1}$ $K^+$ developed a pH gradient, inside acidic, in a medium containing less than 1 mmol $l^{-1}$ $K^+$ (Fig. 1A). The addition of extravesicular $K^+$ caused the dissipation of the $K^+$-generated pH gradient in the same way as it dissipated an artificially generated pH gradient (Fig. 1B). These experiments alone could not exclude the possibility that the driving force for the distribution of protons was a cation diffusion potential established by the influx of cations through a cation channel. However, the existence of a cation channel would be inconsistent with the cation effects on the pH gradient generated by the electrogenic proton pump, since the ATP-dependent membrane potential had been shown to decrease by 15 % in the presence of extravesicular $K^+$ (Wieczorek *et al.* 1989).

Second, the insect V-ATPase was shown to be an exclusive proton pump (Wieczorek *et al.* 1991). Amiloride inhibited $K^+/H^+$ antiport with an $I_{50}$ value of about 0.2 mmol $l^{-1}$, whereas the proton pump was less sensitive, with an $I_{50}$ value of about 0.7 mmol $l^{-1}$. In the presence of 0.7 mmol $l^{-1}$ amiloride, which completely inhibited $K^+/H^+$ antiport but only partially

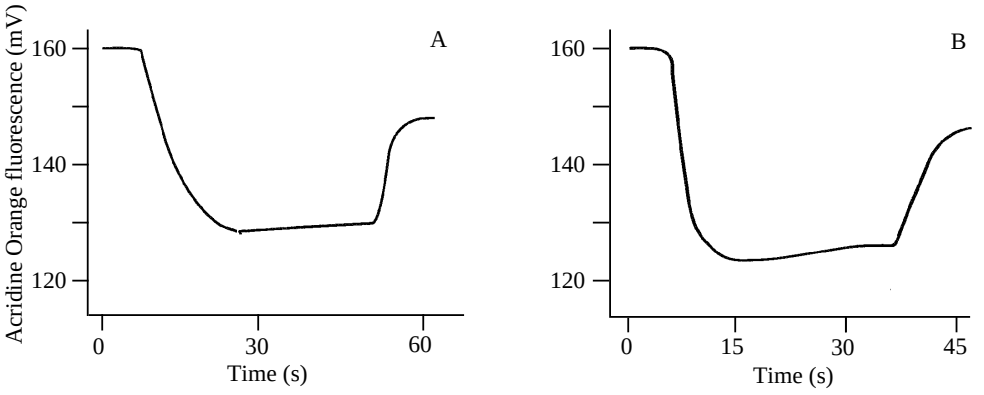


Fig. 1. $K^+/H^+$ antiport determined by the fluorescence quenching of Acridine Orange. Vesicles were preloaded with either 20 mmol $l^{-1}$ $K^+$ (A) or 3 mmol $l^{-1}$ $NH_4^+$ (B). Assays were started by the addition of vesicles (highly purified goblet cell apical membranes) to a $K^+$-free solution. pH gradients were dissipated by the addition of 20 mmol $l^{-1}$ $K^+$ (final concentration). Further experimental conditions according to Wieczorek *et al.* (1991).

inhibited the proton pump, an ATP-dependent pH gradient developed even when extravesicular $K^+$ was present (Fig. 2). Since the fluorescence quench was similar to that obtained in the presence of 0.7 mmol $l^{-1}$ amiloride, but without extravesicular $K^+$, ATP-dependent proton transport in the presence of amiloride could be considered not to be dependent on the extravesicular $K^+$ concentration. Therefore, the V-ATPase in the goblet cell apical membrane appears to be a proton pump, working in parallel with a $K^+/H^+$ antiporter.

### V-ATPase and $K^+/H^+$ antiporter are different proteins

$K^+/H^+$ antiport is not inhibited by bafilomycin $A_1$, in contrast to the V-ATPase in the same membrane (Wieczorek *et al.* 1991). From this result and from the differential sensitivities of both processes to amiloride, one could already deduce that ATP-dependent $H^+$ transport and $K^+/H^+$ antiport are functionally different entities. It was not clear, however, whether both functions were mediated by the multisubunit V-ATPase or whether $K^+/H^+$ antiport was carried out by a separate antiporter protein. The latter possibility was supported by the finding that polyclonal antibodies to the V-ATPase holoenzyme, at a concentration of 0.6 mg $ml^{-1}$ IgG, inhibited ATP-dependent proton transport and ATP hydrolysis completely, but left $K^+/H^+$ antiport unaffected (Wieczorek *et al.* 1991). Even after preincubation of vesicles with up to 6 mg $ml^{-1}$ IgG for up to 5 h at room temperature, no substantial inhibition of $K^+/H^+$ antiport could be observed (A. Lepier and H. Wieczorek, unpublished results). Obviously ATPase and antiporter are two

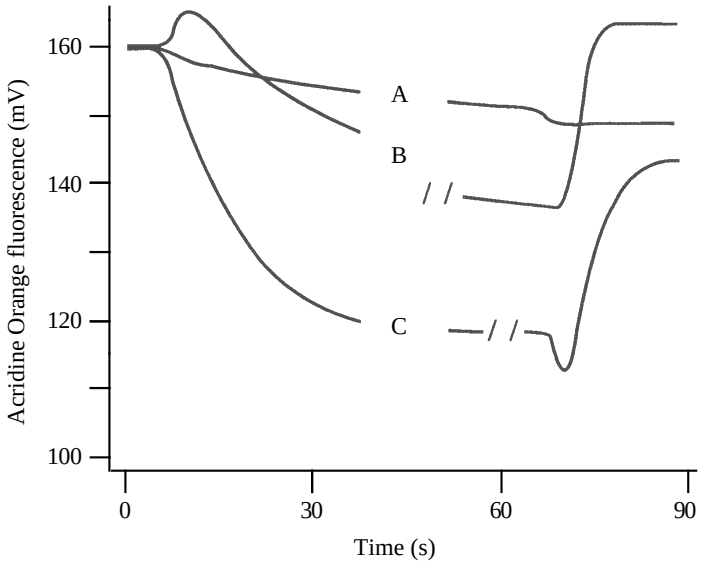


Fig. 2. ATP-dependent proton transport in the presence of $K^+$ determined by the fluorescence quenching of Acridine Orange. Assays were started by the addition of $K^+$-free vesicles (highly purified goblet cell apical membranes) to a solution containing 20 mmol $l^{-1}$ $K^+$ (A), 20 mmol $l^{-1}$ $K^+$ plus 0.7 mmol $l^{-1}$ amiloride (B) or neither $K^+$ nor amiloride (C, control). pH gradients were dissipated by the addition of 20 mmol $l^{-1}$ $K^+$ (A and C, final concentration) or 3 mmol $l^{-1}$ $NH_4^+$ (B, final concentration). Further experimental conditions according to Wieczorek *et al.* (1991).

different molecules. Alternatively, failure to inhibit the antiport could be due to minor antigenicity of antiport epitopes, resulting in an amount of antibody too small to affect $K^+/H^+$ antiport. However, since all major subunits of the purified V-ATPase seen in protein-stained SDS gel blots could also be detected by immunostaining with the polyclonal antibodies, this possibility may be excluded. We cannot exclude completely, however, the possibility that most epitopes were not available in the vesicle experiments, since most of the antiporter moiety may be embedded in the membrane.

Independent evidence that $K^+/H^+$ antiport is not part of the V-ATPase was provided by solubilization of partially purified goblet cell apical membranes with cholate (A. Lepier and H. Wieczorek, unpublished results). After mixing cholate with the membrane suspension and subsequent centrifugation at 100 000 ***g*** for 1 h, the pellets were resuspended in a solution free of cholate and washed twice. Compared with control vesicles, cholate-treated vesicles exhibited a more than twofold higher specific fluorescence quench due to ATP-dependent $H^+$ transport (Fig. 3). This result may be explained by the reorientation of the vesicles during cholate treatment (see Simon and Burckhardt, 1990). However, in contrast to untreated vesicles, the addition of $K^+$ to the extravesicular solution no longer dissipated the established pH gradient. Thus, after cholate treatment, $K^+/H^+$ antiport evidently disappeared while ATP-dependent proton transport remained intact. SDS–PAGE (SDS–polyacrylamide gel electrophoresis) revealed that cholate-treated membranes contain fewer polypeptides than control membranes, but all polypeptides known to be part of the purified V-ATPase are still present (not shown). Evidently the $K^+/H^+$ antiporter was solubilized by cholate and

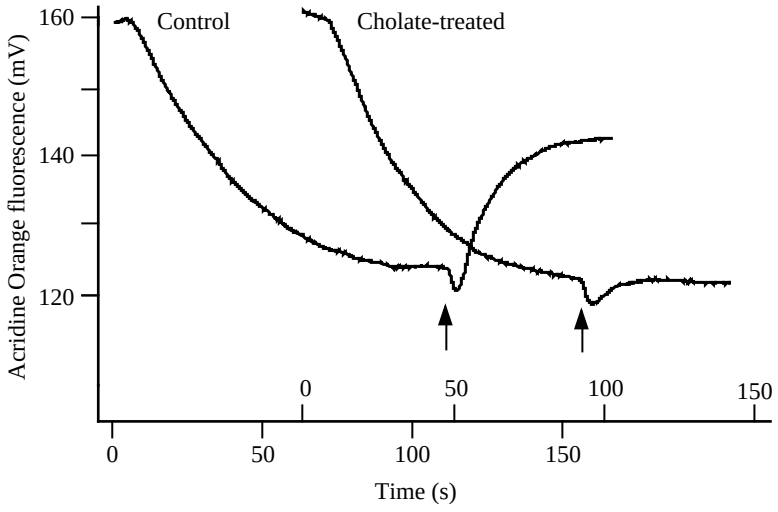


Fig. 3. ATP-dependent proton transport after treatment with 1.6 % sodium cholate determined by the fluorescence quenching of Acridine Orange. Assays were started by the addition of 1 mmol $l^{-1}$ $Mg^{2+}$ to a solution containing vesicles (partially purified goblet cell apical membranes) and 1 mmol $l^{-1}$ ATP. Arrows indicate the addition of 20 mmol $l^{-1}$ $K^+$ (final concentration) to the vesicle suspension. The protein concentration was 15 $\mu$g $ml^{-1}$ for the untreated vesicles and 5.3 $\mu$g $ml^{-1}$ for the vesicles treated with cholate. Further experimental conditions according to Wieczorek *et al.* (1991).

should be found in the supernatant. These results support the hypothesis that the $K^+/H^+$ antiporter is not part of the V-ATPase. Conversely, they provide additional evidence that the V-ATPase is exclusively a proton pump.

## Stoichiometry of $K^+/H^+$ antiport may explain the strong alkalization of the luminal fluid

Vesicle studies, using oxonol V as an indicator of membrane potential, had revealed that $K^+/H^+$ antiport in larval *M. sexta* midgut is electrogenic, exchanging one $K^+$ for more than one $H^+$ (Wieczorek *et al.* 1991). To analyze the antiport stoichiometry in highly purified vesicles from goblet cell apical membranes, we applied the static head method (Turner and Moran, 1982) using fluorescent Acridine Orange to estimate $K^+$-dependent vesicle acidification (M. Azuma, W. R. Harvey and H. Wieczorek, in preparation). Vesicle membranes were clamped at zero voltage by 150 mmol $l^{-1}$ chloride, which activates anion channels that are otherwise closed (Wieczorek *et al.* 1989). Clamped vesicles, preloaded with 150 mmol $l^{-1}$ KCl in a pH 6.5 buffer, were acidified by $K^+/H^+$ antiport when they were injected into a $K^+$-free solution of pH 7.0. After reaching a steady state of the fluorescence quench, different amounts of $K^+$ were added to the reaction mixtures. The addition of sufficient $K^+$ to account for a stoichiometry of $1K^+$ exchanged for $2H^+$ led to a dissipation of the fluorescence quench to a value comparable in size to that when $K^+$-free control vesicles of pH 6.5 were injected into a $K^+$-free solution of pH 7.0; in the latter case, the fluorescence quench was due only to the addition of acidic vesicles, but not to $K^+/H^+$ antiport. Thus, dissipation of the fluorescence quench by $K^+$ under $K^+/2H^+$ conditions met static head conditions in which the driving forces of $K^+$ and of $H^+$ were balanced. Therefore, we concluded that the transport stoichiometry of potassium/proton antiport in the goblet cell apical membrane is $1K^+/2H^+$.

V-ATPase and $K^+/2H^+$ antiport are tightly coupled since extravesicular $K^+$ prevents ATP-dependent vesicle acidification (see above) and since, in transepithelial transport studies, the midgut pumps $K^+$ rather than $H^+$ (Harvey *et al.* 1983*b*; Dow, 1984). One important consequence of the tight coupling is that, in principle, V-ATPase and $K^+/2H^+$ antiporter provide the minimal device for the strong alkalization of the midgut lumen (Fig. 4); the luminal pH values in lepidopteran larvae are the most alkaline that have been measured in a biological system and can exceed 12 in certain species (Dow, 1984). The stoichiometry of $1K^+/2H^+$ may explain how antiport results in electrophoretic exchange of lumen $H^+$ for cellular $K^+$. Since $H^+$, is replaced by a $K^+$, the midgut lumen must necessarily become alkaline. Thus, an antiport of more than one proton for one strong cation that is electrically coupled to a proton pump effectively reverses the direction of pumping so that protons are reabsorbed and the extracellular milieu becomes alkaline. This mechanism, in principle, can apply to any alkalization.

## Attempts to isolate the $K^+/H^+$ antiporter: a rocky road

### *Lectins*

Since the antiporter is a plasma membrane protein, it is likely to be glycosylated. Therefore, a number of lectins with different sugar specificities were tested for inhibition

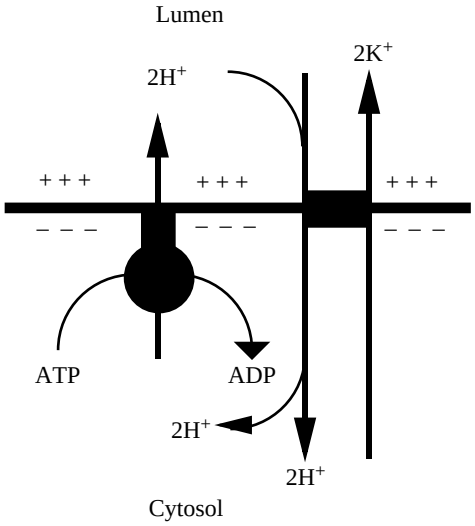


Fig. 4. Minimal alkalizing mechanism consisting of an electrophoretic $K^+/2H^+$ antiport driven by the membrane potential generated by an electrogenic $H^+$-pumping V-ATPase. The model is based on data from lepidopteran midgut. Because of the transmembrane voltage of more than 250 mV produced by the $H^+$ pump, the $H^+$/ATP stoichiometry of the V-ATPase cannot be more than 2; this is in line with stoichiometry values determined for other V-ATPases (Johnson *et al.* 1982; Schmidt and Briskin, 1993). The stoichiometry of the overall $K^+$ pump has been estimated to be $2K^+$ transported per ATP hydrolyzed (Harvey *et al.* 1967). In the living midgut, the free energy of ATP hydrolysis is sufficient to maintain a lumen pH of 11 with $CO_3^{2-}$ as the gegenion (Harvey, 1992). Note that, in general, any strong cation can replace $K^+$. The model depicted in the figure shows the minimal alkalizing mechanism on the basis of identified elements; it does not exclude that further, so far unidentified, processes are also involved in midgut alkalization.

of $K^+/H^+$ antiport in vesicles obtained from highly purified goblet cell apical membranes (A. Lepier and H. Wieczorek, unpublished results). Vesicles were preincubated with lectin for 30 min at room temperature before recording $K^+/H^+$ antiport. Concanavalin A caused a distinct inhibition of $K^+/H^+$ antiport (Fig. 5), whereas wheat germ agglutinin, soybean agglutinin and peanut agglutinin had little or no effect (not shown). Except for peanut agglutinin, the lectins caused visible vesicle agglutination. Since soybean agglutinin also caused agglutination but did not inhibit $K^+/H^+$ antiport, mere agglutination of vesicles cannot be the reason for reduced antiport activity. Therefore, it seems probable that Concanavalin A binds to the $K^+/H^+$ antiporter.

Since cholate appears to have solubilized the $K^+/H^+$ antiporter (see above), we compared Concanavalin A staining in Western blots after SDS–PAGE of pellets and supernatants from cholate-treated membranes and of untreated membranes (Fig. 6). Several Concanavalin-A-binding polypeptides were almost completely solubilized by cholate, the most prominent being a band of approximately 95 kDa. Although it is tempting to suggest that this 95 kDa protein is the antiporter, the result should not be overemphasized. The turnover number of the antiporter may be high and thus it may be

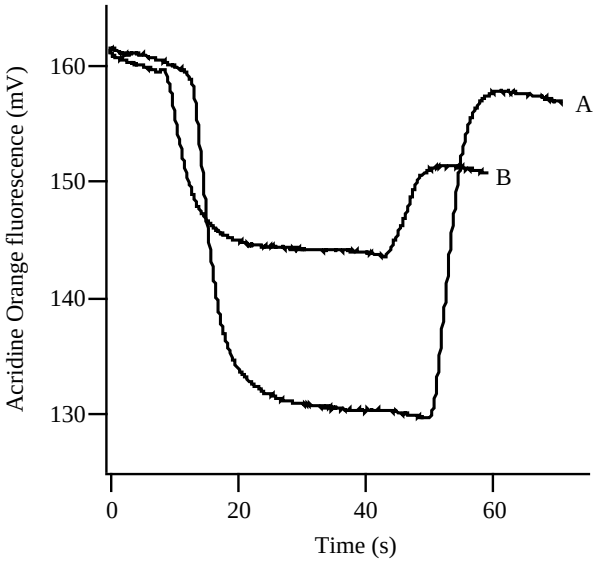


Fig. 5. Influence of Concanavalin A on $K^+/H^+$ antiport determined by the fluorescence quenching of Acridine Orange. Vesicles (highly purified goblet cell apical membranes) were preloaded with 20 mmol $l^{-1}$ $K^+$ and preincubated with a final concentration of 2.5 mg $ml^{-1}$ Concanavalin A for 30 min at room temperature in a pH 8.1 buffer containing 50 mmol $l^{-1}$ tetramethylammonium chloride. Assays were started by the addition of vesicles without Concanavalin A (A) or after preincubation with Concanavalin A (B) to a $K^+$-free solution. pH gradients were dissipated by the addition of 20 mmol $l^{-1}$ $K^+$ (final concentration). Further experimental conditions according to Wieczorek *et al.* (1991).

present in the goblet cell apical membrane only in small amounts. The antiporter could also be one of those weakly stained polypeptides in the supernatant. Current efforts are focused on the clarification of this point.

### *Antibodies*

To obtain information about the antiporter at the DNA level, we raised antibodies to start an approach based on immuno-shotgun screening of a midgut lambda ZAP expression library (Gräf *et al.* 1994). The polyclonal antibodies were produced against a fraction obtained by solubilization of goblet cell apical membranes with $C_{12}E_{10}$ followed by density gradient centrifugation (A. Lepier and H. Wieczorek, unpublished results). The 10 % sucrose fraction (Schweikl *et al.* 1989) contained virtually all the proteins from the goblet cell apical membrane except for the V-ATPase, but included those polypeptides which reacted with Concanavalin A. Unfortunately, the purified antibodies failed to inhibit $K^+/H^+$ antiport. This failure may have the same explanation as the failure of polyclonal antibodies against the V-ATPase to inhibit antiport (see above).

We also tested an inhibiting monoclonal antibody to the luminal $2Na^+/H^+$ antiporter of the lobster hepatopancreas (De Couet *et al.* 1993; kindly provided by Dr G. Ahearn) on immunoblots of SDS gels from goblet cell apical membranes. Although this antibody

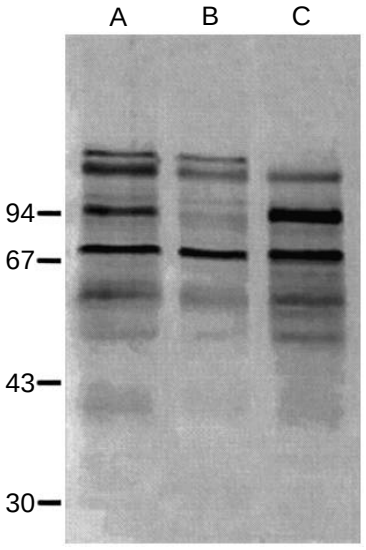


Fig. 6. Western blots of partially purified goblet cell apical membranes before and after treatment with 1.6 % sodium cholate. After SDS–PAGE, blots of untreated membranes (A), of the 100 000 ***g*** pellet of treated membranes (B) and of the supernatant containing solubilized proteins (C) were stained with 10 $\mu$g ml$^{-1}$ Concanavalin A and 20 $\mu$g ml$^{-1}$ horseradish peroxidase (modified according to Clegg, 1982; Hawkes, 1982). Numbers are molecular masses (kDa). Approximately 20 $\mu$g of protein was loaded onto each lane.

showed cross reactions in a variety of crustacean tissues that exhibited $2Na^+/H^+$ exchange (Kimura *et al.* 1994), no bands were recognized in the *M. sexta* preparation (A. Lepier and H. Wieczorek, unpublished results). The antibody was also tested for binding to the native goblet cell apical membranes in a dot-blot experiment. In contrast to the antiserum against the V-ATPase, which was used as a control, no signal could be detected with the monoclonal antibody, even at a concentration that inhibits $2Na^+/H^+$ antiport in hepatopancreatic brush-border membrane vesicles.

*A molecular approach by homology to vertebrate $Na^+/H^+$ antiporters*

A further approach was based on the putative homology of the insect $K^+/H^+$ antiporter to the group of electroneutral $Na^+/H^+$ exchange proteins. Since no invertebrate amino acid sequence data were available, we took advantage of sequences derived from cDNAs encoding mammalian $Na^+/H^+$ antiporters (A. Lepier and H. Wieczorek, unpublished results). All mammalian $Na^+/H^+$ antiporters so far sequenced belong to one of four isoforms (NHE-1 to NHE-4) with remarkable similarities of primary structure, but differing with respect to their membrane localization and their sensitivity to amiloride (Tse *et al.* 1993; Bianchini and Pouyssegur, 1994). A number of degenerate oligonucleotide primers (17–20mers) were selected from suitable amino acid sequence stretches of the 'housekeeping' mammalian $Na^+/H^+$ antiporter isoform NHE-1 (Tse *et al.* 1993) for use in a reverse transcription/polymerase chain reaction (RT–PCR) experiment

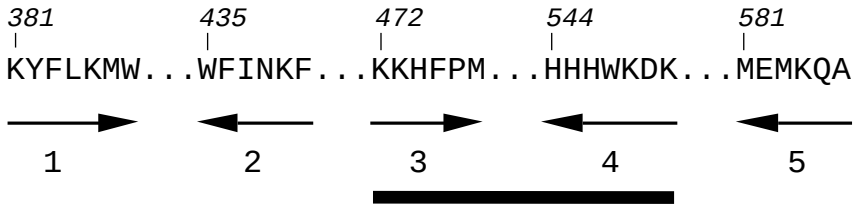


Fig. 7. Amino acid sequence stretches of human NHE-1 (Sardet *et al.* 1989) used for the construction of degenerate polymerase chain reaction primers. Arrows: downstream/upstream primers 1–5 [1, AA(G)TAT(C)TTT(C)T(C)TIAAA(G)ATGTGG; 2, AAT(C)TTA(G)TTIATA(G)AACCA; 3, AAA(G)AAA(G)CAT(C)TTT(C)CCIATGTG; 4, TTA(G)TCT(C)TTCCAA(G)TGA(G)TGA(G)TG; 5,GCT(C)TGT(C)TTCATT(C)TCCAT]. Numbers in italics show positions of amino acids in the protein. The bar marks a 240 base pair fragment sequenced from human blood cDNA (see text).

(Fig. 7). These primers were selected for minimal degeneracy and sufficient spanning fragment length. Sequences of amino acid residues that are conserved in all isoforms but would only allow the construction of highly degenerate primers were not considered. In a first experiment, reverse transcription of poly(A)+ mRNA from *M. sexta* midgut, from Malpighian tubules (which also exhibit $K^+/H^+$ antiport; Grinstein and Wieczorek, 1994) and, as a control, from human blood was primed with primer 5 (Fig. 7), which is specific for NHE-1. PCR amplifications using the cDNA as template were performed at low stringency (annealing temperature 40 °C). Bands within the expected size range were excised from low-melting-point agarose gels and used for reamplification with one or two nested primers. Finally, fragments of about 240 bp (between primers 3 and 4, Fig. 7) from all three sources were cloned and sequenced completely. The sequences from *M. sexta* midgut and Malpighian tubules were identical; they turned out to be new sequences and were not related to any antiporters or other DNA sequences in the GenBank, whereas the sequence amplified from human blood was identical to the corresponding section of the known human $Na^+/H^+$ antiporter NHE-1 isoform (Sardet *et al.* 1989). This result suggests that no protein with sufficient homology to the mammalian NHE-1 isoform to be detected by this method is being expressed in these *M. sexta* tissues. Recently the putative apical localization of NHE-2 and NHE-3 in various mammalian epithelia has been confirmed (Tse *et al.* 1993; Bianchini and Pouyssegur, 1994). RT–PCR studies on mRNA from *M. sexta* midgut and Malpighian tubules using primers specific for NHE-2 are currently under way.

## Conclusion and perspectives

Compelling evidence suggests that a $K^+/H^+$ antiporter is the $K^+$-transporting component of the apical electrogenic $K^+$ pump in larval *M. sexta* midgut. The antiporter is electrophoretic and exchanges $2H^+$ for $1K^+$, driven by the voltage component of the proton-motive force that is generated by the proton-pumping V-ATPase situated in the same plasma membrane. The lepidopteran midgut is the first example from animal tissues

in which the proton-motive force generated by a primary proton pump has been established to be the energy source of secondary transport across the plasma membrane. The $K^+$ pump of *M. sexta* midgut belongs to a group of insect alkali metal ion pumps involved in various physiological functions, such as amino acid absorption, water and ion homeostasis and sensory transduction (Harvey *et al.* 1983*a*). These cation pumps appear to constitute an evolutionarily conserved family of cation pumps consisting of a proton-pumping V-ATPase and a cation/proton antiporter (Grinstein and Wieczorek, 1994). Thus, insect cation/proton antiporters now are a well-established example of the emerging concept that the proton-motive force is an alternative to the sodium-motive force for the energization of animal plasma membranes.

Whereas $K^+/H^+$ antiport is electrophoretic in *M. sexta* midgut, this property seems not to be a general rule in insects; for example, $K^+/H^+$ antiport may be electroneutral in Malpighian tubules (see Grinstein and Wieczorek, 1994). Regardless of its hypothesized involvement in the production of a high luminal pH, the midgut $K^+/2H^+$ antiporter faces an alkaline lumen throughout the whole length of the gut (Dow, 1984). Even in the posterior midgut where the pH decreases to values lower than 9 or, more extreme, even at a neutral pH, the main driving force for active $K^+$ transport has to be the voltage. Thus, the electrophoretic properties of the midgut $K^+/2H^+$ antiporter may be an evolutionary adaptation to the extracellular pH. Similarly, all prokaryotic $Na^+/H^+$ antiporters tested thus far appear to be electrophoretic, exchanging more than one $H^+$ for one $Na^+$ (Padan and Schuldiner, 1993); this stoichiometry has been interpreted to be an evolutionary adaptation to the necessity of acidifying the cytoplasm and of $Na^+$ excretion in an alkaline environment where only the voltage, but not a pH gradient, can drive the exchange. It is tempting to speculate that insect and bacterial cation/proton antiporters may be somewhat homologous and that their similar functions may be based on more than simple convergence due to the same selective pressure.

This work was supported by German Research Foundation Grant Wi 698, European Economic Community Grant SC1*-CT90-0480, National Institutes of Health Grant AI22444 and the Ministry of Education, Science and Culture of Japan (International Research Fellowship, 1992-Y-146). We thank Ralph Gräf for critically reading the manuscript.

## References

ANDERSON, E. AND HARVEY, W. R. (1966). Active transport by the *Cecropia* midgut. II. Fine structure of the midgut epithelium. *J. Cell Biol.* **31**, 107–137.

BIANCHINI, L. AND POUYSSÉGUR, J. (1994). Molecular structure and regulation of vertebrate $Na^+/H^+$ exchangers. *J. exp. Biol.* **196**, 337–345.

CIOFFI, M. AND WOLFERSBERGER, M. G. (1983). Isolation of separate apical, lateral and basal plasma membrane from cells of an insect epithelium. A procedure based on tissue organization and ultrastructure. *Tissue & Cell* **15**, 781–803.

CLEGG, J. C. S. (1982). Glycoprotein detection in nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: application to arenavirus and flavivirus proteins. *Analyt. Biochem.* **127**, 389–394.

DE COUET, H. G., BUSQUETS-TURNER, L., GRESHAM, A. AND AHEARN, G. A. (1993). Electrogenic 2 Na/1 H antiport in crustacean epithelium is inhibited by a monoclonal antibody. *Am. J. Physiol.* **264**, R804–R810.

DOW, J. A. T. (1984). Extremely high pH in biological systems: a model for carbonate transport. *Am. J. Physiol.* **246**, R633–R636.

DOW, J. A. T., GOODWIN, S. F. AND KAISER, K. (1992). Analysis of the gene endocing a 16-kDa proteolipid subunit of the vacuolar $H^+$-ATPase from *Manduca sexta* midgut and tubules. *Gene* **122**, 355–360.

DOW, J. A. T., GUPTA, B. L., HALL, T. A. AND HARVEY, W. R. (1984). X-ray microanalysis of elements in frozen-hydrated sections of an electrogenic $K^+$ transport system: The posterior midgut of tobacco hornworm (*Manduca sexta*) *in vivo* and *in vitro*. *J. Membr. Biol.* **77**, 223–241.

DOW, J. A. T. AND PEACOCK, J. M. (1989). Microelectrode evidence for the electrical isolation of goblet cell cavities in *Manduca sexta* middle midgut. *J. exp. Biol.* **143**, 101–114.

GRÄF, R., HARVEY, W. R. AND WIECZOREK, H. (1994). Cloning, sequencing and expression of cDNA encoding an insect V-ATPase subunit E. *Biochim. biophys. Acta* **1190**, 193–196.

GRÄF, R., NOVAK, F. J. S., HARVEY, W. R. AND WIECZOREK, H. (1992). Cloning and sequencing of cDNA encoding the putative insect plasma membrane V-ATPase subunit A. *FEBS Lett.* **300**, 119–122.

GRINSTEIN, S. AND WIECZOREK, H. (1994). Cation antiport in animal plasma membranes. *J. exp. Biol.* **196**, 307–318.

HAROLD, F. M. (1986). *The Vital Force. A Study of Bioenergetics.* New York: Freeman. 577pp.

HARVEY, W. R. (1992). Physiology of V-ATPases. *J. exp. Biol.* **172**, 1–17.

HARVEY, W. R., CIOFFI, M., DOW, J. A. T. AND WOLFERSBERGER, M. G. (1983*a*). Potassium ion transport ATPase in insect epithelia. *J. exp. Biol.* **106**, 91–117.

HARVEY, W. R., CIOFFI, M. AND WOLFERSBERGER, M. G. (1981). Portasomes as coupling factors in active ion transport and oxidative phosphorylation. *Am. Zool.* **21**, 775–791.

HARVEY, W. R., CIOFFI, M. AND WOLFERSBERGER, M. G. (1983*b*). Chemiosmotic potassium ion pump of insect epithelia. *Am. J. Physiol.* **244**, R163–R175.

HARVEY, W. R., HASKELL, J. A. AND ZERAHN, K. (1967). Active transport of potassium and oxygen consumption in the isolated midgut of *Hyalophora cecropia*. *J. exp. Biol.* **46**, 235–248.

HARVEY, W. R. AND NEDERGAARD, S. (1964). Sodium-independent active transport of potassium in the isolated midgut of *Cecropia* silkworm. *Proc. natn. Acad. Sci. U.S.A.* **51**, 757–765.

HAWKES, R. (1982). Identification of concanavalin A-binding proteins after sodium dodecylsulfate–gel electrophoresis and protein blotting. *Analyt. Biochem.* **123**, 143–146.

JOHNSON, R. G., BEERS, M. F. AND SCARPA, A. (1982). $H^+$ ATPase of chromaffin granules: kinetics, regulation and stoichiometry. *J. biol. Chem.* **257**, 10701–10707.

JUNGREIS, A. M. AND VAUGHAN, G. L. (1977). Insensitivity of lepidopteran tissues to ouabain: Absence of ouabain binding and $Na^+$–$K^+$ ATPases in larval and adult midgut. *J. Insect Physiol.* **23**, 503–509.

KIMURA, C., AHEARN, G. A., BUSQUETS-TURNER, L., HALEY, S. R., NAGAO, C. AND DE COUET, H. G. (1994). Immunolocalization of an antigen associated with the invertebrate electrogenic $2Na^+/1H^+$ antiporter. *J. exp. Biol.* **189**, 85–104.

MANDEL, L. J., RIDDLE, T. G. AND STOREY, J. M. (1980). Role of ATP in respiratory control and active transport in tobacco hornworm midgut. *Am. J. Physiol.* **238**, C10–C14.

MOFFETT, D. F. AND KOCH, A. R. (1988). Electrophysiology of $K^+$ transport by midgut epithelium of lepidopteran insect larvae. II. The transapical electrochemical gradients. *J. exp. Biol.* **135**, 39–49.

NOVAK, F. J. S., GRÄF, R., WARING, R., WOLFERSBERGER, M. G., WIECZOREK, H. AND HARVEY, W. R. (1992). Primary structure of V-ATPase subunit B from *Manduca sexta* midgut. *Biochim. biophys. Acta* **1132**, 67–71.

PADAN, E. AND SCHULDINER, S. (1993). $Na^+/H^+$ antiporters, molecular devices that couple the $Na^+$ and $H^+$ circulation in cells. *J. Bioenerg. Biomembr.* **25**, 647–669.

SARDET, C., FRANCHI, A. AND POUYSSEGUR, J. (1989). Molecular cloning, primary structure and expression of the human growth factor-activable $Na^+/H^+$ antiporter. *Cell* **56**, 271–280.

SCHMIDT, A. L. AND BRISKIN, D. P. (1993). Energy transduction in tonoplast vesicles from red beet (*Beta vulgaris*) storage tissue – $H^+$/substrate stoichiometries for the $H^+$-ATPase and $H^+$-PPase. *Archs Biochem. Biophys.* **301**, 165–173.

SCHWEIKL, H., KLEIN, U., SCHINDLBECK, M. AND WIECZOREK, H. (1989). A vacuolar-type ATPase, partially purified from potassium transporting plasma membranes of tobacco hornworm midgut. *J. biol. Chem.* **264**, 11136–11142.

SIMON, B. J. AND BURCKHARDT, G. (1990). Characterization of inside-out oriented $H^+$-ATPases in cholate-pretreated renal brush-border membrane vesicles. *J. Membr. Biol.* **117**, 141–151.

SKOU, J. C. (1957). The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. biophys. Acta* **23**, 394–401.

TSE, M., LEVINE, S., YUN, C., BRANT, S., COUNILLON, L. T., POUYSSEGUR, J. AND DONOWITZ, M. (1993). Structure/function studies of the epithelial isoforms of the mammalian $Na^+/H^+$ exchanger gene family. *J. Membr. Biol.* **135**, 93–108.

TURNER, R. J. AND MORAN, A. (1982). Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. *J. Membr. Biol.* **67**, 73–80.

WIECZOREK, H. (1992). The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport: molecular analysis of electrogenic potassium transport in the tobacco hornworm midgut. *J. exp. Biol.* **172**, 335–343.

WIECZOREK, H., CIOFFI, M., KLEIN, U., HARVEY, W. R., SCHWEIKL, H. AND WOLFERSBERGER, M. G. (1990). Isolation of goblet cell apical membrane from tobacco hornworm midgut and purification of its vacuolar-type ATPase. *Meth. Enzymol.* **192**, 608–616.

WIECZOREK, H., PUTZENLECHNER, M., ZEISKE, W. AND KLEIN, U. (1991). A vacuolar-type proton pump energizes $K^+/H^+$-antiport in an animal plasma membrane. *J. biol. Chem.* **266**, 15340–15347.

WIECZOREK, H., WEERTH, S., SCHINDLBECK, M. AND KLEIN, U. (1989). A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. biol. Chem.* **264**, 11143–11148.

WIECZOREK, H., WOLFERSBERGER, M. G., CIOFFI, M. AND HARVEY, W. R. (1986). Cation stimulated ATPase activity in purified plasma membranes from tobacco hornworm midgut. *Biochim. biophys. Acta* **857**, 271–281.

WOLFERSBERGER, M. G. (1979). A potassium-modulated plasma membrane adenosine triphosphatase from the midgut of *Manduca sexta* larvae. *Fedn Proc. Fedn Am. Socs exp. Biol.* **38**, 242.

WOLFERSBERGER, M. G., HARVEY, W. R. AND CIOFFI, M. (1982). Transepithelial potassium transport in insect midgut by an electrogenic alkali metal ion pump. *Curr. Topics Membr. Transport* **16**, 109–133.

WOOD, J. L., FARRAND, P. S. AND HARVEY, W. R. (1969). Active transport of potassium by the *Cecropia* midgut. VI. Microelectrode potential profile. *J. exp. Biol.* **50**, 169–178.