The V-type H$^+$-ATPase in Malpighian tubules of *Aedes aegypti*: localization and activity

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Summary

The V-type H$^+$-ATPase is thought to provide the driving force for transepithelial electrolyte and fluid secretion in Malpighian tubules. To confirm the presence of this proton pump in Malpighian tubules of the yellow fever mosquito *Aedes aegypti*, we used several antibodies raised against the V-type H$^+$-ATPase of *Manduca sexta*. Western blot analysis confirmed the presence of the V-type H$^+$-ATPase in Malpighian tubules of *Aedes aegypti*. *In situ* immunostaining identified the V-type H$^+$-ATPase at the apical membrane of the mitochondrion-rich brush border of principal cells. The V-type H$^+$-ATPase was not found in stellate cells. Measurements of ATPase activity revealed that bafilomycin-sensitive and NO$_3^-$-sensitive ATPase activity accounted for 50–60% of total ATPase activity in crude extracts of Malpighian tubules. No significant ouabain- or vanadate-sensitive Na$^+$/K$^+$-ATPase activity was detected. These results support the conclusion reached previously in electrophysiological studies that the mechanisms for transepithelial electrolyte secretion in the *Aedes* Malpighian tubules rely on the V-type H$^+$-ATPase as the principal energizer of epithelial transport. Measures of transepithelial Na$^+$ and K$^+$ secretion and estimates of the H$^+$ flux mediated by the V-type H$^+$-ATPase suggest a 1:1 stoichiometry for Na$^+$/H$^+$ and K$^+$/H$^+$ exchange transport across the apical membrane.

Key words: V-type H$^+$-ATPase, Na$^+$/K$^+$-ATPase, Malpighian tubule, electrolyte secretion, Na$^+$/H$^+$ exchange, polyclonal antibody, immunohistochemistry, western blot.

Introduction

The V-type H$^+$-ATPase was first discovered in endomembranes such as the vacuoles of cells (Nelson, 1992). In recent years, this proton pump has increasingly also been found in plasma membranes of cells (Harvey and Wieczorek, 1997). Here, the V-type H$^+$-ATPase functions as an electrogenic pump, transporting protons from the cytoplasm to the extracellular fluid and generating cell-negative membrane voltages. The membrane voltage can then serve to drive ion transport through ion-specific channels, and the electrochemical proton potential can serve to drive secondary active transport processes such as cation/H$^+$ exchange or anion/H$^+$ cotransport (Harvey et al., 1998). In view of the diversity of transport processes supported by the V-type H$^+$-ATPase, Wieczorek and Harvey have introduced the H$^+$ paradigm of epithelial transport, which takes its place next to the classical Na$^+$/K$^+$ paradigm of Koefoed-Johnsen and Ussing (Wieczorek et al., 1999a).

Bafilomycin A$_1$, a specific inhibitor of the V-type H$^+$-ATPase, completely inhibits transepithelial NaCl and KCl secretion, and with it fluid secretion, in Malpighian tubules of *Aedes aegypti* (Beyenbach et al., 2000). Simultaneously, both apical and basolateral membrane voltages and the transepithelial voltage decrease to zero. These studies strongly suggest the H$^+$ paradigm of epithelial transport for Malpighian tubules.

In the present study, we used an antibody specific to the B subunit of the V-type H$^+$-ATPase to localize the proton pump to the apical brush border membrane of principal cells, but not stellate cells, of Malpighian tubules of the yellow fever mosquito. We also measured enzyme activities of the V-type H$^+$-ATPase and the Na$^+$/K$^+$-ATPase. We found much activity of the former and little of the latter.

Materials and methods

Mosquitoes, and preparation of crude extracts of Malpighian tubules

The *Aedes aegypti* (L.) mosquito colony was maintained as described by Pannabecker et al. (1993). On the day of the experiment, a female mosquito (3–7 days post-eclosion) was cold-anesthetized and decapitated. Malpighian tubules were removed under mosquito Ringer solution from their attachment to the gut and transferred to Ringer solution on ice. Mosquito Ringer solution contained: 150 mmol l^{-1} NaCl, 3.4 mmol l^{-1} KCl, 1.7 mmol l^{-1} CaCl$_2$, 1.8 mmol l^{-1}

NaHCO$_3$, 1.0 mmol l^{-1} MgSO$_4$, 5 mmol l^{-1} glucose, 25 mmol l^{-1} Hepes, pH 7.1. For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot, 250 Malpighian tubules were isolated from 50 female mosquitoes and collected in 1.5 ml of ice-cold Ringer solution. The Ringer solution was then aspirated and replaced with 100 µl of ice-cold lysis buffer, consisting of 20 mmol l^{-1} Tris, 2 mmol l^{-1} EGTA, 2 mmol l^{-1} EDTA, 25 µg aprotinin, 0.5 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF), 25 µg leupeptin and 1% Triton X-100. The tubules were homogenized on ice with a Teflon-coated pestle.

Protein content of Malpighian tubules

Total protein in Malpighian tubules of *Aedes aegypti* was determined with a BioRad *DC* Protein Assay kit (BioRad, Hercules, CA, USA). The method is similar to the well-documented Lowry assay, which is based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent (Lowry et al., 1951). In a typical protein determination, we prepared crude tubule extracts (CTE) as described above using 100 Malpighian tubules from female mosquitoes only. The CTE volume was 100 µl. To 20 µl of this CTE and to 20 µl of bovine serum albumin (BSA) standards, 100 µl of reagent A (alkaline copper tartrate solution; BioRad) and then 800 µl of reagent B (Folin reagent; BioRad) were added. After 15 min, the absorbance was read at 750 nm using a Beckman spectrophotometer (DU-65) against the BSA standards series (ranging from 0.25 mg ml^{-1} to 1.5 mg ml^{-1}).

SDS–PAGE and western blot

SDS–PAGE was performed as described previously (Wieczorek et al., 1990). In brief, 10 µl of sample buffer (5-fold strength) was added to 40 µl of CTE such that final concentrations of sample buffer were 125 mmol l^{-1} Tris-HCl, 5% sucrose, 2% SDS, 0.005% bromophenol blue and 2% β-mercaptoethanol in a final volume of 50 µl at pH 6.8. After boiling for 3 min on a hotplate and then cooling on ice, 7 µl aliquots (approximately 11 µg protein) were loaded on each lane of the gel (BioRad Mini Protean 3 chamber, T 17%/C 0.4%). The electrophoresis was started with a current of 20 mA. After the sample had entered the stacking gel, the current was increased to 45 mA. The proteins were transferred to nitrocellulose membranes by semidry-blotting (60 min, 1 mA cm^{-2}) using a three-buffer system according to Kyhse Andersen (1984), modified by the addition of 20% methanol. SDS–PAGE lanes in the nitrocellulose membrane were cut from the western blot lanes and stained with Ponceau S (Sigma, St Louis, MO, USA).

The western blot membrane (nitrocellulose) was incubated for 60 min in blocking solution consisting of TBSNT (20 mmol l^{-1} Tris-HCl, pH 7.5, 500 mmol l^{-1} NaCl, 0.02% NaN$_3$, 0.05% Tween) fortified with 3% fish gelatine. The membrane was then treated for 60 min with three different primary antibodies diluted in TBSNT plus 1% fish gelatine (1:1000). The antibodies were: (1) Ab 353-2 against the V$_1$ complex of the V-type H$^+$-ATPase (Huss, 2001), (2) Ab 488-1 against the C subunit of the V$_1$ complex of the V-type H$^+$-ATPase (Merzendorfer et al., 2000) and (3) Ab C23 against the B subunit of the V$_1$ complex of the V-type H$^+$-ATPase (Huss, 2001). In each case, the antigen was isolated from *Manduca sexta* in the Wieczorek laboratory. Antibodies (antisera) were prepared in guinea pigs by Charles River (Sulzfeld, Germany).

The western blot membrane was washed with TBSNT in a shaking bath for 3×5 min. The secondary antibody (anti-guinea-pig alkaline phosphatase conjugated, Sigma A-5062) was added after dilution in TBSNT plus 1% fish gelatine (1:30 000). Sixty minutes later, the membrane was washed again with TBSNT for 3×5 min. After rinsing with double-distilled water (ddH$_2$O), the membrane was treated with 10 ml substrate-solution consisting of 50 mmol l^{-1} Tris-HCl, pH 9.5, 100 mmol l^{-1} NaCl, 50 mmol l^{-1} MgCl$_2$, 0.34 mg ml^{-1} nitro blue tetrazolium (NBT; Sigma) and 0.17 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate p-toluidine (BCIP; Sigma). When protein bands became visible after 2–10 min, the membrane was rinsed with ddH$_2$O and dried at room temperature.

Immunohistochemistry

One hundred Malpighian tubules were removed from female mosquitoes as described above and collected in approximately 1 ml of mosquito Ringer solution (Yu and Beyenbach, 2001). The Ringer solution was aspirated and the tubules were transferred for fixation to 5 ml of 10% formaldehyde buffered with 33.3 mmol l^{-1} NaH$_2$PO$_4$ and 45.8 mmol l^{-1} Na$_2$HPO$_4$ at pH 7.2–7.4. After 2.5 h of fixation, the tubules were transferred to a stainless steel embedding mold, dehydrated in a series of ethanol concentrations, ranging from 30% to 100% at 10% increments, and embedded in paraffin wax. Serial sections were cut to a thickness of 4 µm. The sections were deparaffinized in xylene, rehydrated in a series of ethanol (100%, 95%, 70%) and washed in phosphate-buffered saline (PBS: 145 mmol l^{-1} NaCl, 3.2 mmol l^{-1} NaH$_2$PO$_4$, 7.2 mmol l^{-1} Na$_2$HPO$_4$, pH 7.2–7.4). The sections were then treated with 0.5% H$_2$O$_2$ for 10 min to suppress endogenous peroxidase activity.

Slides treated in conventional ways showed little staining with the antibody C23. By contrast, pre-treating slides for 5 min in 0.1 mol l^{-1} citric buffer at pH 6.0 and 80–90°C (microwave) markedly improved the localization of antibody. The method is known as heat-induced antigen retrieval (HIAR) and is frequently used to increase the 'antigenicity' of the antigens in formalin-fixed and paraffin-embedded sections (Shi et al., 2001). Although the mechanism of action of HIAR is not clear, it is believed that the procedure loosens or breaks cross-linkages of antigen and fixative, freeing epitopes for binding to antibody (Shi et al., 2001).

Unspecific binding was blocked with 10% normal rabbit serum (Zymed, San Francisco, CA, USA) for 20 min before the slides were treated with primary antibody at 37°C for 2 h. The primary antibody, Ab C23, was the same polyclonal antibody used in western blot analysis (diluted 1:2000 in PBS).

The secondary antibody, biotinylated rabbit anti-guinea-pig IgG (Zymed) was 50-fold diluted in PBS and applied at room temperature for 20 min. Immunoreactivity was visualized by incubating the sections in streptavidin/peroxidase solution (prediluted; Zymed histostain® kit; Zymed) for 15 min and then in aminoethyl carbazole (AEC) chromogen substrate solution (Zymed) for 2 min. Finally, the sections were counterstained with hematoxylin stain Gill's Formation #2 (Fisher, Fair Lawn, NJ, USA) for 10 s at room temperature.

ATPase activity measurements

Total ATPase activity was measured spectrophotometrically as the oxidation of NADH, which was coupled to ATP hydrolysis as described by Scharschmidt et al. (1979). The activity of the Na^+/K^+-ATPase was measured as the ouabain- or vanadate-sensitive ATPase activity, and the V-type H^+-ATPase activity was measured as the bafilomycin- or nitrate-sensitive ATPase activity, as described by Lin and Randall (1993).

To free ATPase, we lysed Malpighian tubules in hypo-osmotic lysis buffer (20 mmol l^{-1} Tris-HCl, 2 mmol l^{-1} EGTA, pH 7.1). In a typical experiment, 225 Malpighian tubules from 45 female mosquitoes were homogenized on ice with a Teflon-coated pestle in 130 μl of lysis buffer. After ultrasonication for 1 min, the tubule extract was divided into six aliquots of 20 μl each and stored at –20°C.

On the day of the assay, the reaction buffer (125 mmol l^{-1} Tris buffer, 1 mmol l^{-1} EGTA, 120 mmol l^{-1} NaCl, 12.5 mmol l^{-1} KCl, 5 mmol l^{-1} NaN$_3$, 5 mmol l^{-1} MgCl$_2$, 5 mmol l^{-1} ATP, 2.5 mmol l^{-1} phosphoenolpyruvate, with or without ATPase inhibitor) was preincubated with 0.125 mmol l^{-1} NADH (Sigma) and 10 units each of L-lactic dehydrogenase (LDH, type XI; Sigma) and pyruvate kinase (PK; Sigma) for 30 min at room temperature. The ATPase reaction was started by adding 20 μl of tubule extract in a cuvette. The closed cuvette was quickly inverted for mixing and inserted into the spectrophotometer (General purpose UV/VIS DU520; Beckman).

The oxidation of NADH was measured as a function of time at 340 nm, the wavelength of NADH absorption. The linear portion of this function [the slope optical density (OD) per hour] was divided by the NADH extinction coefficient (6.22 OD mmol^{-1}) and normalized to protein concentration to obtain the ATPase activity in tubule extracts (Scharschmidt et al., 1979).

The V-type H^+-ATPase was calculated as the bafilomycin- or NO_3^--suppressible portion of the total ATPase activity, while the Na^+/K^+-ATPase activity was determined as the ouabain- or vanadate-suppressible portion. Bafilomycin was used at a concentration of 0.025 mmol l^{-1}, NO_3^- at 100 mmol l^{-1}, ouabain (Sigma) at 1 mmol l^{-1}, and vanadate at 0.1 mmol l^{-1} (Lin and Randall, 1993).

Statistical treatment of the data

ATPase activity data are presented as means ± S.E.M. The paired Student's *t*-test was used for the significant difference (*P*<0.05) between control and experimental groups.
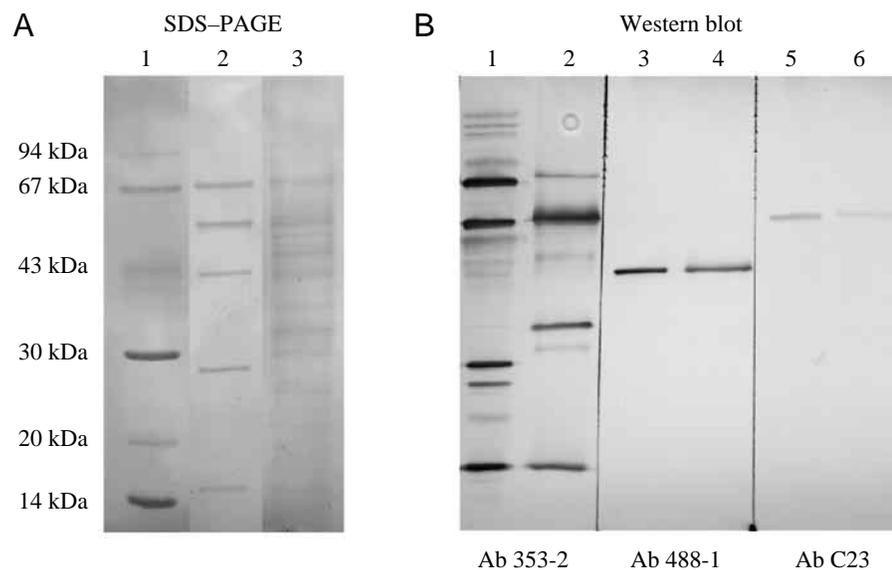
Results

Protein content of Malpighian tubules

The protein concentration in 20 μl of CTE, equivalent to 20 Malpighian tubules, was 0.77±0.04 μg μl^{-1} in five determinations. Hence, the protein content is, on average, 0.77 μg per tubule.

SDS–PAGE and western blot

SDS–PAGE of the extract of *Aedes* Malpighian tubules reveals protein bands that co-localize with proteins of the V-type H^+-ATPase purified from the tobacco hornworm *Manduca sexta*, suggesting that the tubule extract contains proteins of the V-type H^+-ATPase (Fig. 1A). Western blot analysis confirms the presence of the V-type H^+-ATPase in *Aedes* Malpighian tubules (Fig. 1B). The mixture of antibodies (Ab 353-2) raised against proteins of the cytoplasmic V_1 complex identified more than 15 proteins in the purified V-type H^+-ATPase of *Manduca sexta* (lane 1, Fig. 1B). Five protein bands, with molecular masses of 16 kDa, 27 kDa, 43 kDa, 56 kDa and

Fig. 1. Molecular identification of the V-type H^+-ATPase in Malpighian tubules of the yellow fever mosquito. (A) SDS–PAGE: lane 1, standard molecular mass proteins; lane 2, holoenzyme of the V-type H^+-ATPase of *Manduca sexta*; lane 3, crude tubule extract of Malpighian tubules of *Aedes aegypti*. (B) Western blot: antibodies raised against the V-type H^+-ATPase of *Manduca sexta* recognized similar proteins in extracts of Malpighian tubules of *Aedes aegypti*. Lanes 1, 3 and 5 are the *Manduca* holoenzyme, and lanes 2, 4 and 6 are the crude extracts of *Aedes* Malpighian tubules. Ab 353-2, Ab 488-1 and Ab C23 are antibodies against the V_1 complex, C subunit and B subunit of the proton pump, respectively.

67 kDa, stained prominently (lane 1, Fig. 1B). Three of these proteins, with molecular masses of 16 kDa, 56 kDa and 67 kDa, were also recognized by the antibody mixture in the extract of *Aedes* Malpighian tubules (lane 2). The 67 kDa protein is most likely the A subunit of the V_1 complex (Wieczorek et al., 1999b).

Ab 488-1 is a polyclonal antibody raised against the C subunit of the V_1 complex. The antibody clearly recognized subunit C in both the holoenzyme from *Manduca sexta* (lane 3) and the crude extract of *Aedes* Malpighian tubules (lane 4, Fig. 1B). Likewise, the polyclonal antibody Ab C23, raised against the B subunit of the V_1 complex, identified this subunit in the holoenzyme of *Manduca* (lane 5, Fig. 1B) and in the crude extract of *Aedes* Malpighian tubules (lane 6, Fig. 1B).

Immunolabeling performed with preimmuno-serum (control) exhibited no labeling (not shown). Thus, Fig. 1 confirms the presence of the V-type H^+-ATPase in Malpighian tubules of *Aedes aegypti* on the basis of antibodies raised against proteins of the V-type H^+-ATPase purified from *Manduca sexta*.

Immunohistochemistry

Fig. 2 illustrates two sequential microtome sections from the same paraffin block of Malpighian tubules of *Aedes aegypti*. The two sections received the same experimental treatment, including heat-induced antigen retrieval, exposure to secondary antibody and staining with hematoxylin. The only difference is the additional exposure of the section shown in Fig. 2B to Ab C23, the primary antibody specific to the B subunit of the V-type H^+-ATPase. The section shown in Fig. 2A was exposed to the preimmuno-serum.

Tubules appear in a mix of longitudinal and oblique sections (Fig. 2). The tubule lumen occasionally comes into view as the unstained clear space enclosed by the apical brush border of mostly principal cells. The single principal cell of the tubule reveals a large nucleus (18 μm diameter) when the cut has passed through the center. Principal cells are further characterized by a tall dense brush border. Most cells of the tubule are principal cells (86%); the remainder are stellate cells (Satmary and Bradley, 1984).

The obvious difference between the sections shown in Fig. 2A and Fig. 2B is the positive stain of the antibody specific to the V-type H^+-ATPase in Fig. 2B. The antibody C23 recognized the B subunit of the V-type H^+-ATPase most prominently in the brush border of principal cells, which is consistent with a high density of this proton pump at the apical membrane. Light staining of the cytoplasm of principal cells suggests the presence of the V-type H^+-ATPase (or parts thereof containing the B subunit) associated with cytoplasmic structures. However, the antibody did not stain stellate cells, neither plasma membrane nor cytoplasm.

ATPase activities

Fig. 3 illustrates the enzyme activities of the V-type H^+-ATPase and the Na^+/K^+-ATPase in extracts of *Aedes* Malpighian tubules. Total ATPase activity was $3.14\pm0.35\ \mu mol\ h^{-1}\ mg^{-1}$ protein in 10 control determinations. The bafilomycin A_1-sensitive ATPase activity was $1.58\pm0.30\ \mu mol\ h^{-1}\ mg^{-1}$ protein in 10 determinations, which is significantly different from zero ($P=2.5\times10^{-4}$). The KNO_3-sensitive ATPase activity was $1.88\pm0.20\ \mu mol\ h^{-1}\ mg^{-1}$ protein in seven determinations, which is also significantly different from zero ($P=4.8\times10^{-5}$). The two V-type H^+-ATPase activities measured with two different pump inhibitors, bafilomycin A_1 and NO_3^-, were not significantly different, consistent with the complete inhibition of the V-type H^+-ATPase.

Inhibitors of the Na^+/K^+-ATPase had no significant effect on the total ATPase activity. The ouabain-sensitive ATPase activity was $0.26\pm0.12\ \mu mol\ h^{-1}\ mg^{-1}$ protein in 10 determinations, which is not significantly different from zero. Likewise, the nonsensical negative ATPase activity ($-0.61\pm0.70\ \mu mol\ h^{-1}\ mg^{-1}$ protein) measured in the presence
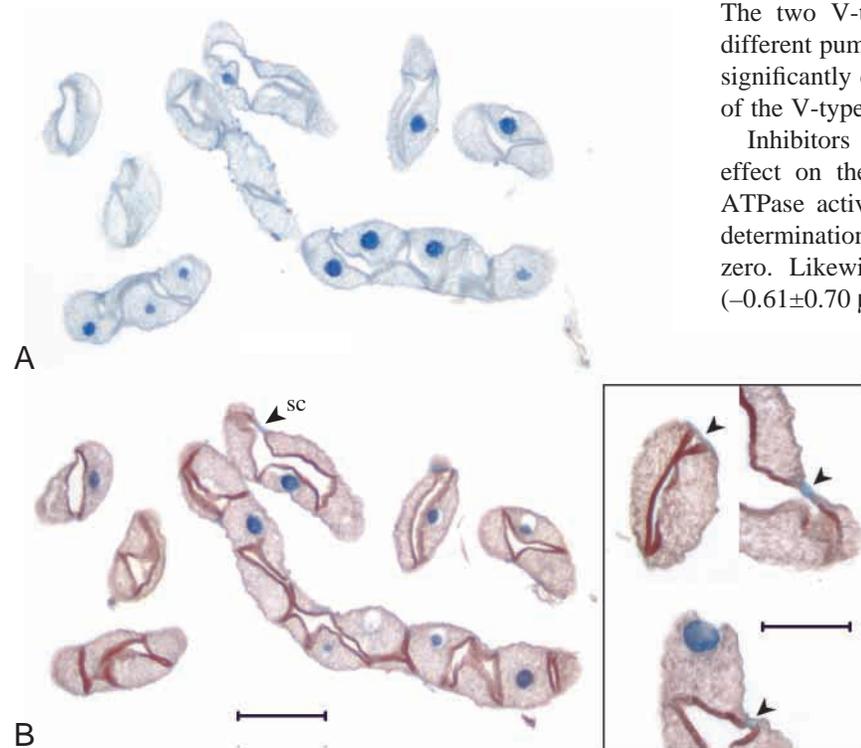

Fig. 2. Immunolocalization of the V-type H^+-ATPase in Malpighian tubules of *Aedes aegypti*. Paraffin sections of mosquito Malpighian tubules were labeled with a polyclonal antibody specific to the 56 kDa B subunit of the V-type H^+-ATPase (see Fig. 1B). (A) Paraffin section labeled with preimmuno-serum as a negative control. (B) Paraffin section labeled with C23 polyclonal antibody; sc, stellate cell. Scale bar, 100 μm. The inset section illustrates stellate cells without staining. Arrowheads point to small stellate cells without a prominent brush border. Scale bar, 200 μm.

of vanadate is not significantly different from zero. Again, the use of two different inhibitors, ouabain and vanadate, yielded similar activities of the Na^+/K^+-ATPase, consistent with complete inhibition.

Discussion

The presence of the V-type H^+-ATPase in Malpighian tubules of Aedes aegypti

The V-type H^+-ATPase is a multi-subunit protein composed of two major functional domains, a cytoplasmic V_1 complex and a membrane-integrated V_0 complex. The V_1 complex is the site for ATP binding and hydrolysis. It is composed of eight different subunits (A–H), including subunit B, which was targeted in the present study using the antibody C23. The B subunit is a 56 kDa polypeptide with high sequence identity among several species (Novak et al., 1992). It is one of the two sites in the V_1 complex that binds ATP; the other is the A subunit (Wieczorek et al., 1999a). The C subunit is a 43 kDa polypeptide to which polyclonal antibody 488-1 binds. The function of the C subunit is not well understood. It is thought to be located outside the V_1 complex and to be part of the cytoplasmic peripheral linkage that couples V_1 and V_0 complexes. The V_0 complex is composed of five different subunits, designated a–d, that collectively function as the proton translocation pathway (Forgac, 1998).

The two polyclonal antibodies, C23 and 488-1, raised against the B and C subunits, respectively, of the proton pump in the midgut of *Manduca sexta*, identified their intended targets in the purified V-type H^+-ATPase and in crude extract of *Aedes* Malpighian tubules (Fig. 1B, lanes 3–6), thereby confirming the presence of the two subunits and hence the presence of the V-type H^+-ATPase in Malpighian tubules of the yellow fever mosquito. Other laboratories have successfully used antibodies raised against various subunits of the V-type H^+-ATPase to identify this proton pump in Malpighian tubules of the ant (*Formica polyctena*), moth (*Heliothis virescens*) and locust (*Locusta migratoria*) (Garayoa et al., 1995; Lezaun et al., 1994; Pietrantonio and Gill, 1995).

Localization of the V-type H^+-ATPase in the brush border membrane of principal cells

The V-type H^+-ATPase was first identified as an enzyme associated with endosomal membranes of lysosomes, clathrin-coated vesicles and vacuoles of yeast and plants (Nelson, 1992; Stevens and Forgac, 1997). In endosomal membranes, the V_1 complex faces the cytoplasm and the V_0 complex points into the endosomal compartment. Thus, protons are moved from the cytoplasm to the endosomal compartment, raising endosomal H^+ concentrations above cytoplasmic concentrations and generating an endosomal membrane potential that is positive inside. Both H^+ and voltage gradients serve a variety of functions. Voltage may drive the entry of Cl^- into the endosome, acidifying the endosomal compartment with HCl,
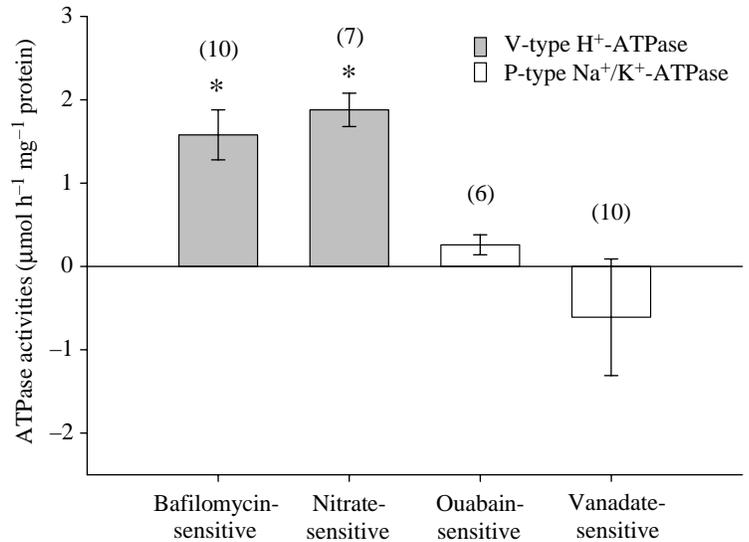

Fig. 3. ATPase activities in crude extracts of Malpighian tubules of *Aedes aegypti*. The V-type H^+-ATPase activity was measured as the bafilomycin- and NO_3^--sensitive ATPase activity. The Na^+/K^+-ATPase activity was measured as the ouabain- and vanadate-sensitive ATPase activity. The following inhibitor concentrations were used: bafilomycin A_1, 0.025 mmol l^{-1}; nitrate, 100 mmol l^{-1}; ouabain, 1 mmol l^{-1}; vanadate, 0.1 mmol l^{-1}. Asterisks represent $P<0.01$. The number of determinations is shown in parentheses.

when the endosomal membrane houses Cl^- channels next to the V-type H^+-ATPase (Marshansky and Vinay, 1996). The presence of malate channels serves electrogenic uptake of malate ions (Pantoja and Smith, 2002).

In epithelial membranes of animal cells, the V-type H^+-ATPase is often located at the apical side, where again voltage and H^+ gradients can serve activities ranging from signal transduction (Camello et al., 2000) to nutrient uptake (Zhuang et al., 1999) and electrolyte transport (Wieczorek et al., 1999a). The present study shows that the V-type H^+-ATPase is densely expressed in the brush border of principal cells of Malpighian tubules of *Aedes aegypti* (Fig. 2). The brush border is also densely populated by mitochondria (Beyenbach, 2001). Virtually every microvillus is home to a mitochondrion (Beyenbach, 2001). The close spatial relationship between ATP synthesis (mitochondria) and ATP utilization (the V-type H^+-ATPase) suggests a close temporal relationship between metabolism and transepithelial transport (Fig. 4A). Indeed, the inhibition of ATP synthesis by dinitrophenol depolarizes the apical membrane voltage from 111 mV to 9 mV within 1 min, consistent with the rapid inhibition of transepithelial transport (Pannabecker et al., 1992). More recent studies confirm that intracellular ATP concentration and electrogenesis by the V-type H^+-ATPase in the apical, microvillar plasma membrane are intimately coupled (Wu and Beyenbach, 2003).

In *Aedes* Malpighian tubules, the V-type H^+-ATPase energizes transport not only across the apical membrane but also across the epithelial shunt and across the basolateral membrane on the other side of the cell (Beyenbach, 2001). As
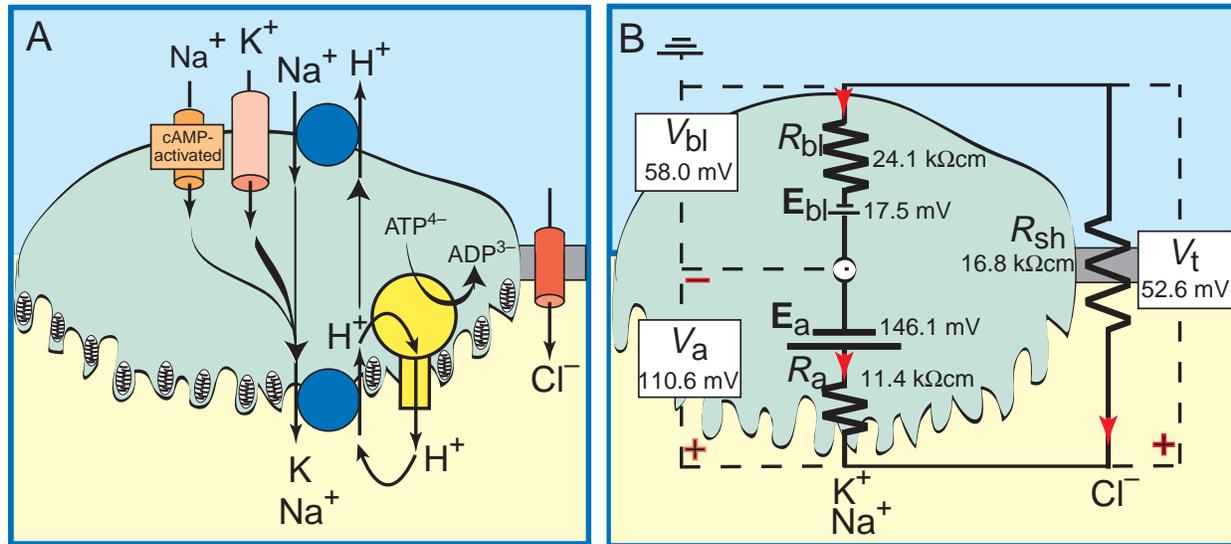
Fig. 4. Models of transepithelial NaCl and KCl secretion across Malpighian tubules of the yellow fever mosquito. (A) Molecular model of transcellular Na$^+$ and K$^+$ secretion and paracellular Cl$^-$ secretion. (B) Electrical equivalent circuit of transepithelial electrolyte secretion. Na$^+$ and K$^+$ are actively transported through principal cells, and Cl$^-$ is passively transported from hemolymph to tubule lumen through the shunt pathway. **E**, electromotive force; *V*, voltage; *R*, resistance; t, transepithelial; bl, basolateral membrane; a, apical membrane; sh, shunt. Reproduced with permission from Beyenbach (2001).

illustrated in Fig. 4, the transport of protons from the microvillar cytoplasm to the extracellular space of the brush border constitutes a pump current that must return to the cytoplasmic side of the V-type H$^+$-ATPase (Beyenbach, 2001). Current passing through the epithelial shunt is carried by Cl$^-$ passing from hemolymph to tubule lumen as the mechanism of transepithelial Cl$^-$ secretion (Masia et al., 2000). Current passing from the hemolymph into principal cells is carried by K$^+$ as the major mechanism of secretory K$^+$ entry into the cell (Beyenbach and Masia, 2002).

Although we expected to find the V-type H$^+$-ATPase at the apical membrane, we cannot be sure about the immunohistochemical evidence for the presence of this proton pump in the cytoplasm of principal cells (Fig. 2). The staining of the cytoplasm may reflect antibody binding to parts of the holoenzyme such as the V$_1$ complex. The reversible dissociation of the V$_1$ complex from the holoenzyme is known as a mechanism for regulating the transport activity of the V-type H$^+$-ATPase (Wieczorek et al., 1999a). But this dissociation should leave the V$_1$ complex in close proximity to the site of its dissociation from the holoenzyme, namely in microvilli of the brush border. It should not yield the diffuse distribution of the B subunit in the cytoplasm. For these reasons, we believe that the cytoplasmic signal reflects various aspects of holoenzyme synthesis, sorting and trafficking, although the association of the V-type H$^+$-ATPase with endosomal membranes of principal cells cannot be excluded.

The V-type H$^+$-ATPase is absent from stellate cells in Malpighian tubules of *Aedes aegypti* (Fig. 2). Stellate cells in Malpighian tubules of *Drosophila melanogaster* do not express

the V-type H$^+$-ATPase according to enhancer trapping studies in Dow's lab (Sözen et al., 1997). Moreover, stellate cells are thought to provide the transepithelial Cl$^-$ shunt pathway in *Drosophila* Malpighian tubules (O'Donnell et al., 1998). Such a transport role also fits our finding of Cl$^-$ channels in apical membrane patches of *Aedes* Malpighian tubules (O'Connor and Beyenbach, 2001). Mediating passive transepithelial Cl$^-$ transport, stellate cells may not need the V-type H$^+$-ATPase.

ATPase activities

Between 50% and 60% of the total ATPase activity in extracts of *Aedes* Malpighian tubules can be attributed to a nitrate- and bafilomycin-sensitive component that reflects the activity of the V-type H$^+$-ATPase. The remaining ATPase activity is probably due to protein kinases, nucleotide cyclases, myosin, DNA helicases and other ATP-consuming processes. Bafilomycin A$_1$ is known to inhibit the free V-type H$^+$-ATPase with an I_{50} of 0.4 nmol mg^{-1} protein (Bowman et al., 1988). Our use of a concentration 6000 times (2500 nmol mg^{-1}) that much should have completely inhibited the V-type H$^+$-ATPase in the tubule extracts used in the present study. Consistent with the complete inhibition by bafilomycin is a similar V-type H$^+$-ATPase activity measured in Malpighian tubules with a maximal dose of nitrate (Fig. 3). Nitrate inhibits the free V-type H$^+$-ATPase with an I_{50} of 50 mmol l^{-1} (Dschida and Bowman, 1995).

Bafilomycin is thought to block the proton channel of the V-type H$^+$-ATPase (Zhang et al., 1994a). By contrast, there are two mechanisms for inhibiting the V-type H$^+$-ATPase by nitrate: (1) *via* oxidation of the cystine residue on the A subunit, with the effect of preventing ATP hydrolysis, and (2)

via dissociation of the V_1 complex from the V_0 complex (Dschida and Bowman, 1995).

Even though the V-type H^+-ATPase activity was measured in a crude extract of Malpighian tubules, the activity most probably reflects the activity of the intact proton pump, i.e. the holoenzyme. The cytosolic V_1 complex is capable of hydrolyzing ATP in a Ca^{2+}-dependent manner, but this hydrolysis is blocked by Mg^{2+} concentrations as low as 0.1 mmol l^{-1} (Gräf et al., 1996). The Mg^{2+} concentration in our ATPase assays was 5 mmol l^{-1}, which should have inhibited any ATP hydrolysis by dissociated V_1 complexes. When the V_1 complex is attached to the V_0 complex and in membrane-bound form, the hydrolysis of ATP is Mg^{2+}- rather than Ca^{2+}-dependent (Gräf et al., 1996). It is believed that the hydrophobic environment of the membrane changes the conformation of the V_1 complex, thereby switching the metal requirement to Mg^{2+} (Gräf et al., 1996). Accordingly, the presence of 5 mmol l^{-1} Mg^{2+} in our ATP assay should have maximized V-type H^+-ATPase activity of the holoenzyme in apical membrane fragments but inhibited ATP hydrolysis by the dissociated V_1 complexes.

In the present study, we measured no detectable ouabain- or vanadate-sensitive Na^+/K^+-ATPase activity in crude extracts of *Aedes* Malpighian tubules (Fig. 3). Likewise, we observed no immediate effects of 1 mmol l^{-1} ouabain on the transepithelial voltage and resistance of isolated *Aedes* Malpighian tubules (Williams and Beyenbach, 1984). By contrast, significant effects of ouabain, such as the partial (approximately 50%) inhibition of transepithelial fluid secretion, were observed after ouabain treatment for more than 30 min (Hegarty et al., 1991). In view of the strong expression of the V-type H^+-ATPase detected by immunostaining (Fig. 2) and by biochemical (Fig. 3) and electrophysiological (Beyenbach, 2001; Wu and Beyenbach, 2003) assays, it appears that the V-type H^+-ATPase first and foremost serves epithelial transport mechanisms, and that the Na^+/K^+-ATPase, if it is present, may perhaps serve cell housekeeping functions. Accordingly, ouabain will have no immediate effect on transepithelial transport but it may compromise transepithelial transport after normal cell housekeeping functions have been impaired. Further experiments using a more sensitive assay for the Na^+/K^+-ATPase activity, or immunostaining methods, may help to reveal the presence of the Na^+/K^+ pump in Malpighian tubules of *Aedes aegypti*.

Kinetics and thermodynamics of transport across the apical membrane

Fig. 4 illustrates the present model of transepithelial NaCl and KCl secretion by Malpighian tubules of the yellow fever mosquito dependent on the central role of the V-type H^+-ATPase located in the apical membrane of principal cells. Since proton-translocating ATPases can transport as many as three protons per ATP molecule hydrolyzed (Tomashek and Brusilow, 2000), the hydrolysis of 1.7 μmol ATP h^{-1} mg^{-1} protein is equivalent to H^+ transport across the apical membrane of the tubule at a rate of 5.1 μmol h^{-1} mg^{-1} protein.

A single Malpighian tubule of the yellow fever mosquito contains approximately 0.77 μg of protein. Hence, a proton transport rate of 65 pmol min^{-1} can be estimated for the whole tubule under control conditions. The estimated proton transport rate comes close to 74 pmol min^{-1}, the rate of transepithelial secretion of Na^+ and K^+ (Beyenbach, 2001). The approximation suggests electroneutral Na^+/H^+ and K^+/H^+ exchange transport across the apical membrane with a stoichiometry of 1:1.

Since electroneutral Na^+/H^+ and K^+/H^+ cannot be driven by voltage, the question arises of whether the pH difference across the apical membrane has the thermodynamic strength to drive Na^+ and K^+ from cell to lumen in exchange for H^+. A proton transport rate of 65 pmol min^{-1} across the apical membrane is expected to decrease the pH in the tubule lumen to values less than 1 in the time of only 1 min, providing a driving force far greater than needed. However, measurements of the pH in the tubule lumen of Malpighian tubules are invariably close to 7, and measures of cytoplasmic pH are not that far off (Bertram and Wessing, 1994; Petzel et al., 1999; Zhang et al., 1994b). What then is the minimum pH difference across the apical membrane that is needed to drive Na^+/H^+ and K^+/H^+ exchange transport in *Aedes* Malpighian tubules? The answer to this question requires knowledge of cytoplasmic cation concentrations in principal cells.

In Malpighian tubules of ants, the intracellular K^+ concentration (67 mmol l^{-1}) is near electrochemical equilibrium with the K^+ concentration in the hemolymph (Leyssens et al., 1994). The equilibrium distribution of K^+ probably also holds true in Malpighian tubules of *Aedes aegypti* in view of a K^+ conductance as large as 64% of the basolateral membrane conductance (Beyenbach and Masia, 2002). Accordingly, in the presence of a peritubular K^+ concentration of 3.4 mmol l^{-1} and a basolateral membrane voltage of –58 mV, the intracellular K^+ concentration can be estimated as 31.5 mmol l^{-1} under control conditions, when the K^+ concentration in secreted (luminal) fluid is 91 mmol l^{-1} (Beyenbach, 2001). Thus, the movement of K^+ from 31.5 mmol l^{-1} in the cell to 91 mmol l^{-1} in the lumen requires a nearly 3-fold H^+ concentration difference, or a pH difference of 0.5 across the apical membrane. If the intracellular pH is 7.2, then a lumen pH of 6.7 would be sufficient to drive K^+/H^+ exchange across the apical membrane with a transport stoichiometry of 1:1.

Since fluid secreted into the tubule lumen is not sufficiently acidic to drive cation/H^+ antiport, the pH of an unstirred layer in the brush border might be low enough, as in amphibian skin (Larsen et al., 1996). In addition, large negative surface charges of the apical membrane due to fixed negative charges of glycosylated proteins, glycolipids and adsorbed proteins may support a surface pH considerably lower than that in the aqueous solution of the tubule lumen (Aronson and Giebisch, 1997). Finally, the glycocalyx of the brush border may limit the diffusion of H^+, generating an acid microenvironment akin to that in the intestinal brush border (Shimada, 1987). These considerations of the kinetics and thermodynamics of transport

suggest, but do not prove, that the transport of K$^+$/H$^+$ and Na$^+$/H$^+$ across the apical membrane of principal cells of the *Aedes* Malpighian tubule can be electroneutral, as previously suggested for Malpighian tubules of the ant *Formica* (Leyssens et al., 1993; Zhang et al., 1994b). Additional experiments are needed to resolve the mechanism, stoichiometry and regulation of cation/H$^+$ exchange across the apical membrane.

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