The Journal of Experimental Biology 212, 1611-1619
Published by The Company of Biologists 2009
doi:10.1242/jeb.030007

Review

Vacuolar-type proton pumps in insect epithelia

Helmut Wieczorek[1,*], Klaus W. Beyenbach[2], Markus Huss[1] and Olga Vitavska[1]

[1]Department of Biology/Chemistry, University of Osnabrück, 49069 Osnabrück, Germany and [2]Department of Biomedical Sciences, Cornell University, Ithaca, NY 14853, USA

*Author for correspondence (e-mail: wieczorek@biologie.uni-osnabrueck.de)

Accepted 18 March 2009

Summary

Active transepithelial cation transport in insects was initially discovered in Malpighian tubules, and was subsequently also found in other epithelia such as salivary glands, labial glands, midgut and sensory sensilla. Today it appears to be established that the cation pump is a two-component system of a H^+-transporting V-ATPase and a cation/nH^+ antiporter. After tracing the discovery of the V-ATPase as the energizer of K^+/nH^+ antiport in the larval midgut of the tobacco hornworm *Manduca sexta* we show that research on the tobacco hornworm V-ATPase delivered important findings that emerged to be of general significance for our knowledge of V-ATPases, which are ubiquitous and highly conserved proton pumps. We then discuss the V-ATPase in Malpighian tubules of the fruitfly *Drosophila melanogaster* where the potential of post-genomic biology has been impressively illustrated. Finally we review an integrated physiological approach in Malpighian tubules of the yellow fever mosquito *Aedes aegypti* which shows that the V-ATPase delivers the energy for both transcellular and paracellular ion transport.

Key words: H^+-translocating vacuolar-type ATPase, V-ATPase, insect epithelia, *Manduca sexta*, tobacco hornworm, *Drosophila melanogaster*, *Aedes aegypti*.

The potassium ion pump of insect epithelia: from K^+-ATPase to H^+-ATPase

Insects exhibit a unique alkali metal ion pump which actively transports, depending on the species and/or the physiological circumstances, K^+ and/or Na^+ across epithelia. The pump was initially detected in Malpighian tubules from various insect species (Ramsay, 1953) where it was suggested to actively transport K^+ from the hemolymph into the tubule lumen. Subsequently, the alkali metal ion pump was (1) established in other ion-transporting epithelia such as salivary glands, labial glands, midgut and sensory sensilla, (2) shown to be located in the apical membranes of epithelial cells, and (3) demonstrated to be electrogenic and energized by the hydrolysis of ATP (for a review, see Harvey, 1982).

One of the longstanding model systems for the investigation of active transepithelial K^+ transport is the larval midgut of the tobacco hornworm *Manduca sexta*. Over the years, Bill Harvey and his coworkers as well as David Moffett and Alan Koch have provided many important and useful insights into the function of the midgut K^+ pump. Moira Cioffi and Mike Wolfersberger from the Harvey laboratory were the first to explore the biochemical identity of the pump. They identified a K^+-stimulated ATPase activity in partially purified plasma membranes and then in highly purified apical membranes of goblet cells (Wolfersberger et al., 1982; Harvey et al., 1983). One of us (H.W.) joined the Harvey laboratory for a collaboration that led to the unequivocal identification and characterization of the K^+-stimulated ATPase activity (Wieczorek et al., 1986), thanks to the availability of (1) highly purified goblet cell apical membranes (Cioffi and Wolfersberger, 1983) and (2) a very sensitive method for measuring inorganic phosphate as the product of ATP hydrolysis (Wieczorek, 1982).

Back in Germany, H.W. together with Helmut Schweikl detergent-solubilized and purified the ATPase from apical membranes of goblet cells and found it to be a vacuolar-type ATPase on the basis of the molecular mass of the holoenzyme, its subunit composition, its sensitivity to *N*-ethlymaleimide and its insensitivity to azide and to vanadate (Schweikl et al., 1989). The result was rather baffling because, at that time, V-ATPases were known to be proton pumps of intracellular organelles. The only V-ATPase which had been found in the plasma membrane of cells was that of the vertebrate urinary bladder (Al Awqati, 1978).

In experiments using membrane vesicles, the tobacco hornworm V-ATPase was shown to transport H^+, and not alkali metal ion (Wieczorek et al., 1989). What transported K^+ in these membrane vesicles turned out to be a K^+/H^+ antiporter that these membrane vesicles also housed (Wieczorek et al., 1989; Wieczorek et al., 1991). Thus the molecular correlate of the midgut alkali metal pump emerged as an electrogenic H^+-transporting V-ATPase that energizes K^+/H^+ antiport in the same membrane. Subsequent studies elucidated the electrophoretic stoichiometry of $K^+/2H^+$ (Azuma et al., 1995). The discovery of $K^+/2H^+$ antiport explained why in the earlier studies a K^+-stimulated ATPase activity had been measured. When protons are transported by the V-ATPase into vesicles, a voltage as well as a pH difference develop across the membrane that counteract further H^+ transport, thereby decreasing V-ATPase activity. However, in the presence of $K^+/2H^+$ antiport, H^+ can leave the vesicle, thereby diminishing the voltage and pH difference and maintaining pump activity.

The new paradigm, a H^+-transporting V-ATPase forming a partnership with a K^+/nH^+ antiporter, was quickly accepted by the scientific community and transferred to other ion-transporting epithelia such as Malpighian tubules and salivary glands. Today,

the V-ATPase has been demonstrated in numerous ion-transporting insect epithelia (Harvey and Wieczorek, 1997; Harvey et al., 1998). The molecular evidence for the antiporter, however, has been elusive. Recently genes that may encode the antiporter have been identified (Day et al., 2008; Rheault et al., 2007).

While the V-ATPases were isolated from cell organelles, they also occur in many animal plasma membranes where they energize diverse membrane transport systems (for a review, see Wieczorek et al., 1999). The V-ATPase from goblet cell apical membranes of the tobacco hornworm midgut was the first vacuolar-type ATPase shown to energize secondary active transport in a eukaryotic plasma membrane (Fig. 1). In this review we will first focus on the V-ATPase in the midgut of the tobacco hornworm. We then will review the V-ATPase in *Drosophila* Malpighian tubules where the power of genomic and post-genomic approaches has been convincingly demonstrated. Finally we will turn to an integrated physiological approach in Malpighian tubules of mosquitoes that reveals the V-ATPase as the energizer of both transcellular and paracellular transport pathways.

The tobacco hornworm plasma membrane V-ATPase and its impact for V-ATPases in general

V-ATPases are ubiquitous and highly conserved proton pumps that acidify specific organelles such as endosomes, lysosomes or secretory vesicles in every eukaryotic cell, but also are found in the plasma membrane of many animal cell types where they are involved either in pH homeostasis or in membrane energization (Nishi and Forgac, 2002; Wieczorek et al., 1999; Beyenbach and Wieczorek, 2006). Like the evolutionarily related F-ATPases of mitochondria, chloroplasts and bacteria, V-ATPases are heteromultimeric proteins comprising two functional parts, a peripheral catalytic V_1 complex and a membrane-bound, proton-conducting V_O complex (Fig. 2A). In the midgut of the tobacco hornworm, the V_1 complex of the plasma membrane V-ATPase contains the eight different subunits A to H, whereas the V_O complex consists of the four different subunits a, c, d and e (Merzendorfer et al., 2000). Thus the insect V-ATPase is, in most of its facets, a typical representative of V-ATPases in general. Even though the yeast V-ATPase has been the main source for insights into the structure, function and regulation of this important family of ion pumps, research on the V-ATPase from tobacco hornworm midgut delivered significant findings of general interest. In the following we will concentrate on these aspects.

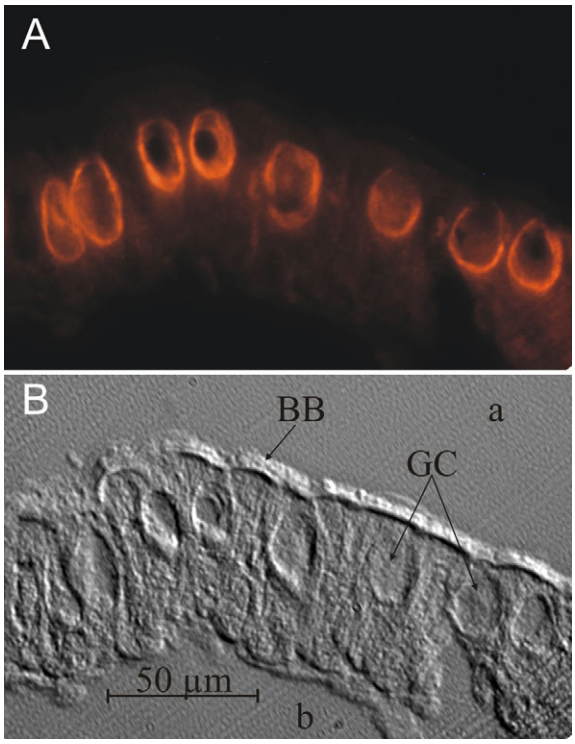

Fig. 1. Cryosection of the tobacco hornworm midgut epithelium. (A) Immunolocalization of the V_1 subunit A by a monoclonal antibody. (B) Same section as in A, but visualized by Hoffman modulation contrast microscopy. a, apical side; b, basal side; BB, brush border the columnar cells; GC goblet cavity of goblet cells. The V-ATPase is present only in the apical membrane of goblet cells.

Subunits F, G and e

The V_1 subunit F was first cloned from tobacco hornworm midgut (Gräf et al., 1994). It was shown to be a constitutive V-ATPase subunit because monospecific antibodies inhibited both ATPase activity and ATP-dependent proton transport. The association of subunit F with the V_1 complex was confirmed by stripping it from the membrane by treatment with the chaotropic agent KI. The 14 kDa subunit F appears to be not as strictly associated with the V_1 complex as the other V_1 subunits because subunit F is absent from the V_1 complex after KI stripping (Gräf et al., 1996). Furthermore, subunit F appears to have a coupling role between the V_1 and V_O parts of the enzyme because neither the assembly of V_1 with V_O nor the assembly of the V_O complex itself takes place in the absence of subunit F (Graham et al., 1994). Most recent cryo-electron microscopy data from the tobacco hornworm holoenzyme also locate subunit F at a position consistent with V_1V_O linkage (Muench et al., 2009).

The V_1 subunit G was initially cloned from yeast and interpreted as a member of the V_O complex, based on cold inactivation results, the properties of the null mutant, and its similarity to the bacterial F_O subunit b (Supeková et al., 1995). However, treatment of midgut goblet cell apical membranes and of Malpighian tubule brush border membranes from tobacco hornworm with chaotropic iodide as well as cold inactivation led to the conclusion that subunit G is a peripheral V-ATPase subunit (Lepier et al., 1996; Gräf et al., 1996). The finding that subunit G stimulates the ATPase activity of the reassembled V_1 complex supported the designation as a V_1 subunit (Xie, 1996). Structural and further biochemical studies also unequivocally allocate subunit G to the V_1 complex (Ohira et al., 2006; Zhang et al., 2008; Muench et al., 2009).

Subunit e was first cloned as M9.2, an extremely hydrophobic 9.2 kDa protein present in bovine chromaffin granule membranes (Ludwig et al., 1998). Because it showed sequence and structural similarity to Vma21p, a yeast protein required for the assembly of the V-ATPase, it was unclear whether it was a component of the mature enzyme. Studies with the tobacco hornworm V-ATPase revealed that this subunit indeed is a member of the V_O complex, and that it is a constitutive subunit of the mature holoenzyme (Merzendorfer et al., 1999). Meanwhile subunit e was also described as a member of the yeast V-ATPase and shown to be essential for enzyme activity (Sambade and Kane, 2004). Unlike the yeast and bovine subunit e, the insect subunit e from midgut and Malpighian tubules of the tobacco hornworm is highly glycosylated, with half of the 20 kDa molecular mass consisting of sugar residues (Merzendorfer et al., 1999). Whether this is typical for plasma membrane V-ATPases in general remains an open question.

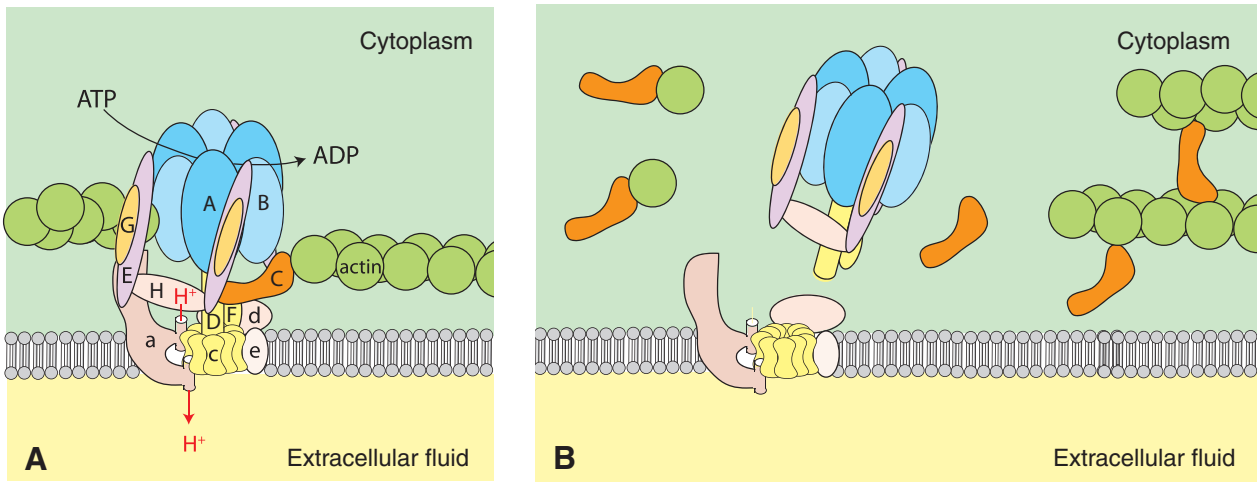

Fig. 2. Model of a eukaryotic V-ATPase. (A) The peripheral V_1 complex consists of eight different subunits identified with capital letters A–H. The integral membrane V_O complex consists of at least four different subunits identified with lowercase letters (a, c, d, e). In yeast subunit c occurs together with its isoforms c′ and c″. Actin filaments bind to subunits B (Holliday et al., 2000) and C (Vitavska et al., 2003). (B) Disassembly of the V-ATPase into its V_1 and V_O complexes. Subunit C, which dissociates from the V_1 complex, binds not only to F actin but also to monomeric G-actin (Vitavska et al., 2005).

Structure of the V_1 complex and of the V_1V_O holoenzyme

In his excellent textbook '*The Vital Force: A Study of Bioenergetics*' Frank Harold provides a figure showing the schematic and hypothetical structure of the F-ATPase with the following caveat (Harold, 1986): 'After several authors, none of whom should be held responsible'. That was the situation for V-ATPases as well until several papers appeared illuminating the picture further in the late nineties (Boekema et al., 1997; Boekema et al., 1998; Wilkens et al., 1999). The first examination of the quaternary structure of the isolated V_1 complex, performed by small-angle X-ray scattering of the tobacco hornworm V_1, revealed homologies to but also differences from the bacterial F_1 complex (Svergun et al., 1998). Electron microscopy of the negatively stained tobacco hornworm V_1 complex supported the hexagonal arrangement of the A and B subunits together with a seventh mass either centrally or asymmetrically to the hexamer (Radermacher et al., 1999). The analysis of the three-dimensional structure of the V_1 complex at a resolution of 18 Å revealed further internal features of this protein (Radermacher et al., 2001). Moreover, the most detailed image of the whole V-ATPase was obtained by cryo-electron microscopy of the tobacco hornworm enzyme at a resolution of 17 Å (Muench et al., 2009) that is approximately 2-fold higher than previous cryo-EM models of either the V-ATPase or the F-ATPase holoenzyme. This unprecedented resolution of the holoenzyme uncovered a third stalk, indicating that there are three (and not just two) peripheral connections (stators) between the V_1 and the V_O complexes. Each stalk most probably consists of the V_1 subunits E and G (see Fig. 2).

Inhibitors: old and new players

The plecomacrolides bafilomycin and concanamycin were the first specific and highly potent inhibitors of V-ATPases to be detected, with IC_{50} values at nanomolar concentrations (Bowman et al., 1988; Dröse et al., 1993). In *Manduca* the IC_{50} values were shown to be approximately 10 nmol l^{-1} (Huss et al., 2002). Despite intensive investigation of the structure–activity relationship the location of the binding site in the enzyme remained elusive for a long time. Studies with the chromaffin granule V-ATPase suggested that the membrane-bound V_O complex lodged the binding site (Hanada et al., 1990), and later studies with the bovine clathrin-coated vesicle V-ATPase reinforced this assumption, pointing to the V_O subunit a as the target (Crider et al., 1994; Zhang et al., 1994). Conversely, indirect evidence suggested the V_O subunit c as a candidate (Rautiala et al., 1993). Finally, mutational analysis of the V-ATPase from *Neurospora crassa* (Bowman and Bowman, 2002) as well as radiolabeling studies of the insect V-ATPase with a semi-synthetic derivative of concanamycin (Huss et al., 2002) proved that subunit c indeed contains the biggest part of the plecomacrolide binding site; a minor contribution of the V_O subunit a could be demonstrated by site-directed mutagenesis (Wang et al., 2005). The availability of a high resolution structure of the bacterial Na^+–V_O ring from *Enterococcus hirae*, in combination with the present data from mutational analysis, revealed the location of the binding pocket in the luminal half of subunit c (Bowman et al., 2006; Murata et al., 2005).

More recently it was shown, using the tobacco hornworm midgut V-ATPase as a target, that the myxobacterial antibiotics archazolid and apicularen are further highly efficient and specific novel inhibitors of V-ATPases, with IC_{50} values of approximately 20 nmol l^{-1} (Huss et al., 2005). Archazolid, in spite of its different structure compared with the plecomacrolides, probably has a similar mode of inhibition and binding site in the V-ATPase as it prevents radiolabeling of subunit c with the concanamycin derivative. Compared with this, the benzolacton enamides apicularen and salicylihalamide appear to exhibit a manner of inhibition which is different from that of the plecomacrolides as, on the one hand, they did not compete with concanamycin for its binding site and, on the other hand, they are the first V-ATPase inhibitors which discriminate between various species as they do not inhibit fungal V-ATPases. For a more comprehensive outline of V-ATPase inhibitors we refer to the recent review by Huss and Wieczorek (Huss and Wieczorek, 2009).

Reversible disassembly of the V_1 complex from the membrane

The reversible disassembly of the V_1 complex as an *in vivo* control mechanism of V-ATPase activity was first demonstrated in the midgut of moulting tobacco hornworms. In the midgut, more than

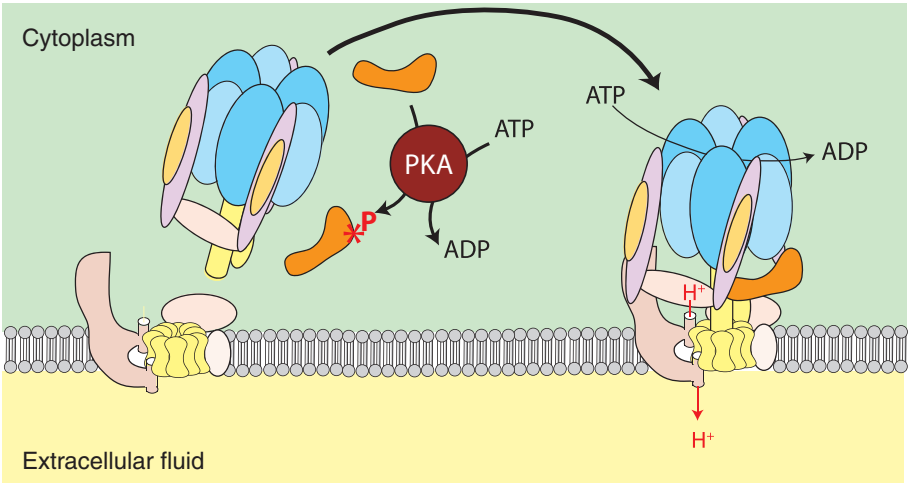

Fig. 3. Phosphorylation of the V_1 subunit C by protein kinase A (PKA) and its assumed involvement in the process of reversible V_1V_O disassembly.

10% of the animal's total ATP production is utilized by the H^+-translocating V-ATPase to generate a voltage of more than 200 mV across the apical membrane of goblet cells which drives secondary $K^+/2H^+$ antiport and thus net active K^+ secretion (Wieczorek and Harvey, 1995). The resulting K^+ electrochemical potential energizes secondary transport processes across the midgut epithelium such as the uptake of amino acids by K^+/amino acid symport. When moulting or starving, the caterpillars do not feed and thus there is no need for secondary transport. One would therefore expect for reasons of economy that active transepithelial K^+ transport is diminished if not altogether stopped. Indeed, this was found to happen during moult due to dissociation of the peripheral V_1 subunits from the membrane V_O complex (Sumner et al., 1995). The V_1 subunits appeared to dissociate as a whole (Fig. 2B), as the cytosolic concentration of the V_1 complex was doubled during moult (Gräf et al., 1996). Subunit C seemed to be released separately to the cytoplasm (Fig. 2B), because the purified V_1 complex lacked most of it (Merzendorfer et al., 2000). The reverse process, namely the reassembly of the V_1V_O holoenzyme, evidently does not require biosynthesis of new V_1 subunits, because it was not influenced by inhibition of translation in moulting larvae with cycloheximide (Jäger and Klein, 1996). As expected, the V_1 complex dissociates reversibly from the V_O complex not only during moult but also during starvation, indicating that this type of V-ATPase regulation is a response to a drop in energy supply (Gräf et al., 1996). *In vitro* experiments with the isolated V_1V_O holoenzyme clearly showed that the dissociation is dependent on the ATP/ADP ratio, which represents the energy load of the cell (Huss and Wieczorek, 2007).

Shortly after detecting the reversible disassembly of the V_1V_O complex in the tobacco hornworm, it was also observed in the yeast *Saccharomyces cerevisiae* (Kane, 1995). In cells grown in the presence of glucose, a rich carbon source, disassembly was induced by brief glucose deprivation. Upon restoration of glucose, rapid reassembly was observed, without the need for new subunit biosynthesis. Reversible disassembly was afterwards also detected in mammalian cells (Trombetta et al., 2003; Sautin et al., 2005). The observation of the reversible assembly of V_1 and V_O complexes in three evolutionarily distant organisms (insects, vertebrates and yeast) indicates an ancient mechanism for regulating V-ATPase that has been conserved.

The regulation of V-ATPase activity by reversible disassembly has been best characterized in yeast. Apparently disassembly and reassembly are separately controlled processes, because disassembly but not reassembly requires intact microtubules (Xu and Forgac, 2001), and because reassembly but not disassembly is aided by the heterotrimeric protein complex RAVE (Seol et al., 2001; Smardon et al., 2002). The glycolytic enzyme aldolase which binds V-ATPase subunits may play a role in stabilizing the assembled holoenzyme because overexpressing aldolase blocks V-ATPase disassembly upon removal of glucose (Lu et al., 2007). Recently it was shown that yeast cells with constitutively active protein kinase A (PKA) do not respond to glucose depletion by disassembly of the V-ATPase (Bond and Forgac, 2008). This means that either activated PKA causes the V-ATPase to (re)assemble or it prevents its disassembly.

In salivary glands of the blowfly *Calliphora vicina* serotonin induces a cAMP-mediated reversible assembly of the V-ATPase resulting in increased V-ATPase-driven proton transport (Dames et al., 2006). It could be shown that PKA is the target of cAMP, implying that there must be at least one protein in the signaling pathway leading to V-ATPase (re)assembly that is phosphorylated by PKA (Rein et al., 2008). One of the candidates may be the V_1 subunit C which is, in the tobacco hornworm V-ATPase, the only subunit that can be phosphorylated by PKA (Voss et al., 2007) (see also Fig. 3). Subunit C can be phosphorylated as a single recombinant protein as well as when embedded in the V_1 complex. In contrast it cannot be phosphorylated as part of the V_1V_O holoenzyme. Both the phosphorylated and the unphosphorylated forms of subunit C are able to reassociate with the V_1 complex from which subunit C had been removed before. In salivary glands of the blowfly a membrane-permeable cAMP analog as well as serotonin cause phosphorylation of subunit C but this is abolished by the PKA inhibitor H-89. These data are in line with the assumption that subunit C is a target for PKA-mediated phosphorylation and that this phosphorylation may be a regulatory switch for the (re)assembly of the V-ATPase. Indeed, subunit C is unique among V-ATPase subunits as it is released into the cytosol upon dissociation of the holoenzyme into its V_1 and V_O complexes (Kane, 2000; Merzendorfer et al., 2000). Subunit C is an elongated molecule (Drory et al., 2004) that is proposed to form part of a stator linking the V_1 with the V_O complex (Muench et al., 2009). Moreover, subunit C binds to actin filaments and this interaction may be involved in stabilizing the proton pump in its assembled state (see below) (Vitavska et al., 2003; Vitavska et al., 2005). These properties make subunit C suited to the control of V-ATPase (re)assembly state and to the mediation of the relevant cellular signals.

Binding of the V_1 subunit C to G-actin and F-actin

The interaction of the V-ATPase with the actin cytoskeleton was first demonstrated in mouse osteoclasts (Lee et al., 1999). Immunocytochemical and biochemical studies with a mouse bone marrow culture as well as with the bovine kidney V-ATPase suggested that the V-ATPase not only co-localizes with the actin cytoskeleton *in vivo* but also binds directly to actin filaments *via* its subunit B *in vitro* (Holliday et al., 2000).

In the tobacco hornworm, the co-localization of the V-ATPase with actin filaments was demonstrated at the apical membrane of midgut goblet cells as well as at the apical brush border membranes of epithelial cells from Malpighian tubules and salivary glands (Vitavska et al., 2003). In midgut goblet cells, no co-localization was observed under conditions where the V_1 complex detaches from the apical membrane. In co-pelleting assays, both the V_1 complex and the V_1V_O holoenzyme bound to actin filaments in a concentration-dependent manner. Overlay blots revealed that not only subunit B but also subunit C binds to F-actin. Experiments with the recombinant subunit C also confirmed its interaction with F-actin under native conditions, which may explain why the holoenzyme, fully equipped with subunit C, exhibits a higher affinity for F-actin than the V_1 complex, which contains subunit C only in substoichiometric amounts. When the V_1 complex was reconstituted with the recombinant subunit C, enhanced binding to F-actin was observed. Incubation of F-actin with subunit C led to cross-linking of actin filaments, forming bundles of varying thickness (Vitavska et al., 2005). Subunit C was also shown to bind to monomeric G-actin with high affinity in the nanomolar range, and no difference could be found between ATP–G-actin or ADP–G-actin.

What may be the biological function of actin binding by subunit C? The goblet cell apical membrane of the tobacco hornworm midgut is densely packed with several thousand copies of V-ATPase. Therefore one may assume an actin-anchoring function, especially as spectrin, which usually links membrane proteins to the actin cytoskeleton, is absent in the apical sphere of goblet cells (Bonfanti et al., 1992). The high concentration of up to $10\,\mu mol\,l^{-1}$ free subunit C in the midgut cytoplasm allows us to extend this view. As subunit C binds non-preferentially to actin filaments and not only to their plus or minus ends, it could be an ideal candidate for the organization of actin dynamics in the apical cytoskeletal web.

The activity of the V-ATPase in Malpighian tubules of *Aedes aegypti* appears to correlate with the state and distribution of the actin cytoskeleton (Karas et al., 2005). Treatment of tubules with cAMP or blood feeding of adult mosquitos led not only to an increase of fluid secretion, indicating V-ATPase activation, but also to the redistribution and enrichment of actin at the apical membranes of principal cells (Karas et al., 2005). Accordingly, the protein associations described above for the tobacco hornworm may be vital for the secretory response to the blood meal in mosquito Malpighian tubules.

Drosophila Malpighian tubules: the genomic model for insect V-ATPase research

The importance of the fruitfly *Drosophila* as a model organism for developmental and genetic research is amply documented by several Nobel prizes awarded to researchers working with *Drosophila*, beginning with the award to Thomas Hunt Morgan in 1933. Less well known are the essential contributions of the little fly to the field of V-ATPase research. Soon after the discovery that a V-ATPase energizes the midgut epithelium of the tobacco hornworm (Schweikl et al., 1989; Wieczorek et al., 1989) it was shown in *Drosophila hydei* that a V-ATPase also plays a central role in fluid secretion in the insect kidney, the Malpighian tubules (Bertram et al., 1991). There and in subsequent studies it was confirmed that the cooperation of a V-ATPase and a cation/H^+ exchanger is essential for the regulation of intracellular pH, the secretion of K^+ and Na^+ and the formation of fluid by the Malpighian tubules (Bertram et al., 1991; Bertram and Wessing, 1994; Wessing et al., 1993). Since then, candidates of the K^+/H^+ antiporter have been suggested in *Drosophila* and mosquitoes (Day et al., 2008; Rheault et al., 2007).

Not entirely unexpected, the first animal with a knockout of a V-ATPase was *Drosophila melanogaster* after Davies and her colleagues were able to assign the *vha55* gene encoding subunit B of the V_1 complex to *SzA*, a lethal locus already described in 1979 (Davies et al., 1996; Gausz et al., 1979). Using the *LacZ* reporter gene of a P-element insertion in *vha55*, they could show an elevated expression of subunit B exactly in tissues where a high number of V-ATPases in the plasma membrane is expected, namely in oviducts, antennal palps, rectum and Malpighian tubules. The expression of *vha55* in the Malpighian tubules matched the nuclei of principal cells whereas stellate cells showed no significant expression (Davies et al., 1996; Dow et al., 1997). The observation that other V-ATPase subunits in the Malpighian tubules are also solely allocated in principal cells (Sözen et al., 1997) was in line with earlier results of intracellular measurements from *Ae. aegypti* indicating cation transport in the tubule epithelium as a unique property of principal cells (Beyenbach and Petzel, 1987). An overall picture of ion transport processes in the Malpighian tubules will be drawn in the paragraph on *Ae. aegyti* (see below).

The use of 700 P{GAL4} enhancer trap lines has revealed a high complexity and diversification of genetic and functional regions along the length of Malpighian tubules in *Drosophila* (Sözen et al., 1997) and opened new vistas for investigating cellular functional diversity. A big advantage of *Drosophila* is its sequenced genome and hence the availability of microarrays which allows a fast access to gene transcripts and the corresponding proteins in the context of defined physiological conditions. In a microarray approach that compared the transcriptome of Malpighian tubules with that of the whole adult fly, a surprisingly new set of players besides the common ones needed for fluid secretion (see Fig. 5) appeared on the scene, allowing us to draw an overall picture of what is necessary to fulfil the manifold tasks of Malpighian tubules as the insect kidney (Wang et al., 2004). Supported by these data a genome-wide survey of the V-ATPase genes was performed using *in situ* hybridization and reporter gene expression (Allan et al., 2005). The results revealed that 33 genes encode V-ATPase subunits, and that 13 of these 33 genes code for the plasma membrane V-ATPase. Furthermore, the clear translucent tubule phenotype in *Drosophila* embryos, a general feature of lethal plasma membrane V-ATPase alleles, was identified. This phenotype has been suggested to be the consequence of the failure to sufficiently acidify the lumen of the Malpighian tubules; because above a certain pH excreted urate cannot be precipitated to uric acid crystals, the tubule lumen remains translucent (Allan et al., 2005; Dow, 1999; Gausz et al., 1979). This finding is of particular medical interest as there are also human renal disorders due to mutations in kidney-specific isoforms of V-ATPase subunits; therefore the Malpighian tubules of *Drosophila* may serve as a model for human diseases (Dow, 2009; Karet et al., 1999; Smith et al., 2000).

Driving transcellular and paracellular transport in *Aedes* Malpighian tubules

In Malpighian tubules of the yellow fever mosquito, *Ae. aegypti*, the V-ATPase drives the transepithelial secretion of NaCl, KCl and possibly other solutes. Located at the apical membrane of principal cells (Fig. 4) it energizes not only that membrane but also the basolateral membrane and the paracellular pathway (Beyenbach, 2001).

The V-ATPase translocates protons across the apical membrane of Malpighian tubules, thereby producing a H^+ electrochemical potential across this membrane. In considering how this proton-motive force can drive, for example, the transepithelial secretion of cations and anions, it is useful to examine separately chemical and electrical potentials.

A V-ATPase located at the apical membrane is expected to acidify the tubule lumen. However, the measured concentration of H^+ in the lumen (pH 7.2) is lower than that in the cell (pH 7.0) (Petzel et al., 1999). Clearly, the chemical H^+ potential difference has the wrong orientation to drive K^+ or Na^+ into the tubule lumen *via* cation/H^+ exchange (Fig. 5).

The situation changes markedly when the cation/H^+ exchanger returns 2 H^+ ions to the cell for each cation (Na^+ or K^+) moved from cell to lumen (Fig. 5) as has been demonstrated for the respective exchanger in goblet cell apical membranes from the tobacco hornworm (Azuma et al., 1995). Operating with a stoichiometry of n=2, the exchange transporter can take advantage of the large membrane voltage (123 mV) existing across the apical membrane. Eqn 1 describes the effect of stoichiometry (n) and voltage on the K^+ concentration difference across the apical membrane:

$$\frac{[K^+]_{lu}}{[K^+]_{cell}} = \left(\frac{[H^+]_{lu}}{[H^+]_{cell}}\right)^n \frac{FV}{e^{RT(n-1)}}, \quad (1)$$

where F is the Faraday constant, V is the apical membrane voltage (123.5 mV), n is the stoichiometry of exchange transport, R is the gas constant and T is the temperature (K). For electroneutral K^+/H^+exchange with n=1, the lumen/cell $[K^+]$ ratio is only 0.63, yielding a luminal $[K^+]$ no greater than 48 mmol l^{-1} when the cell $[K^+]$ is 75 mmol l^{-1} (Petzel et al., 1999). In contrast, for n=2 the lumen/cell $[K^+]$ ratio jumps to 40, or a luminal $[K^+]$ of 3 mol l^{-1}! The calculations illustrate the profound effects the electrical potential generated by the V-ATPase can have on electrogenic transporters.

Next to the high apical membrane voltage generated by the V-ATPase, there is another consequence to the activity of the proton pump. Because the V-ATPase translocates H^+ across the apical membrane without exchange of a cation (or cotransport of an anion), the transport of H^+ constitutes a current across the apical membrane that must return to the cytoplasmic face of the V-ATPase (Fig. 5). If the pump current were not allowed to return, the voltage across the apical membrane would quickly rise to the electron-motive force of the V-ATPase. In this case, the ATPase would stop pumping, because the energy of ATP hydrolysis would reach the maximum H^+ electrochemical potential across the apical membrane. Thus, normal conditions of transepithelial secretion of NaCl and KCl require that apical membrane current (carried by H^+) returns to the cytoplasmic face of the V-ATPase. Some of this current may be carried by H^+ itself *via* the hypothetical cation exchanger/nH^+ discussed above (Fig. 5). Current may also return through stellate cells, where current across the apical membrane is carried by Cl^- passing from cell to lumen *via* Cl^- channels that have been identified (O'Connor and Beyenbach, 2001). The current would then presumably pass into principal cells carried by HCO_3^- diffusing from principal cells to stellate cells *via* gap junctions (Fig. 5) that recently have been identified in *Aedes* Malpighian tubules (Weng et al., 2008). A second route for the return of current is through the paracellular septate junction and then through ion channels in the basolateral membrane of principal cells. Here, current passing through the septate junction from tubule lumen to hemolymph is carried by Cl^- passing in the opposite direction (Fig. 5). From the hemolymph current reaches the cytoplasmic face of the V-ATPase *via* K^+ channels that dominate the basolateral membrane conductance of principal cells (Fig. 5) (Beyenbach and Masia, 2002). Ion channels in the basolateral membrane of stellate cells have not yet been identified.

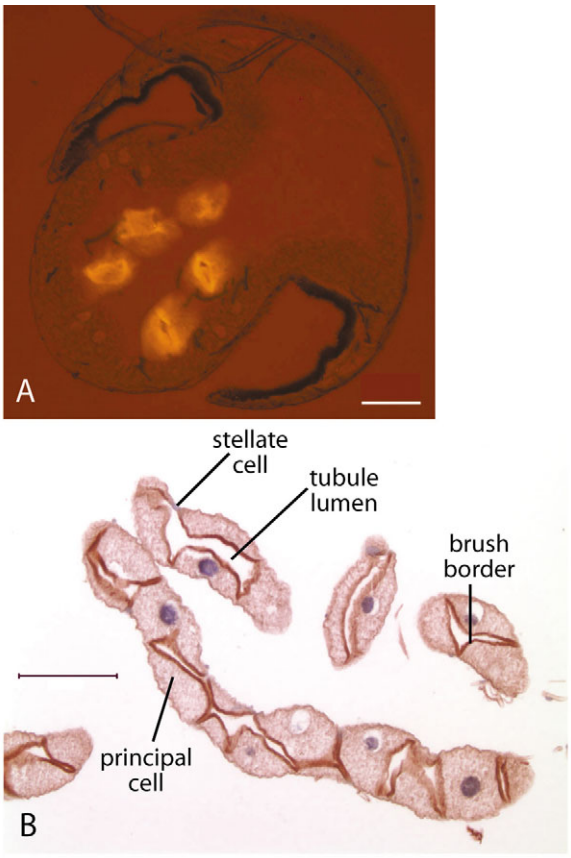

Fig. 4. The V-ATPase in Malpighian tubules of the mosquito *Aedes aegypti*. (A) Cross-section of the abdomen. Immunofluorescence labeling of subunit C of the V-type H^+-ATPase at the luminal brush border of Malpighian tubules (courtesy of H. Merzendorfer). (B) Sections of Malpighian tubules. Immunoperoxidase labeling of the B-subunit of the V-type H^+-ATPase at the brush border of principal cells. The V-ATPase is not present in stellate cells (adapted from Weng et al., 2003). Scale bars in A and B are 100 μm.

The effects of two diuretic hormones on tubule function illustrate the importance of the intraepithelial currents generated by the V-ATPase. One hormone is the mosquito natriuretic peptide (MNP) which the laboratory of Coast identified as Anoga-DH_{31} (Coast et al., 2005). Anoga-DH_{31} selectively increases the rates of transepithelial secretion of NaCl and water, but not of KCl. The Na^+ selectivity stems from the activation of Na^+ channels in the basolateral membrane of principal cells *via* cAMP, thereby allowing the return current to be carried by Na^+ over K^+ (Beyenbach, 2003a; Sawyer and Beyenbach, 1985). In contrast,

peptides of the kinin family of diuretic hormones trigger a sharp decrease in the paracellular resistance to produce a near transepithelial short circuit (Beyenbach, 2003b; Pannabecker et al., 1993). The drop in paracellular resistance enhances the paracellular secretion of Cl^- with the effect of increasing the transepithelial secretion of both NaCl and KCl and consequently water (Yu and Beyenbach, 2001; Yu and Beyenbach, 2004).

The above analysis reveals that the primary energizer of epithelial currents and transepithelial electrolyte secretion is the electrical potential generated by the V-ATPase, at least in Malpighian tubules of the yellow fever mosquito.

Perspectives

The Krogh principle is central to Comparative Physiology (Krogh, 1929): '*For a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied*'. The converse is also true, where an unusual model system leads to fruitful avenues of investigation. The scientific history of the insect V-ATPase is a case in point, where interest in intestinal transport mechanisms in the world champion of growth, the larval *Manduca sexta*, led to the discovery of a V-ATPase embedded in a plasma membrane and not a vacuolar or subcellular membrane. Moreover, it was found that the V-ATPase and not the Na^+/K^+-ATPase energized secondary active transport not only in the plasma membrane where the proton pump is located but also at other sites in the epithelium. The natural abundance of the V-ATPase in the larval *Manduca* midgut facilitated its purification and opened the possibility of studying its structure, function and regulation.

Studies in *Manduca* have revealed a novel mechanism for regulating transport activity: the remarkable dissociation/re-association of V_O and V_1 complexes in ways that couple the availability of nutrients to the assembly and activation of the proton pump. The disassembly of the V-ATPase may reflect the endocrinology of moulting in *Manduca sexta*, and it may reflect the absence of glucose in yeast. Signaling pathways to and from the proton pump are largely unknown as the first examples of phosphorylation of some subunits are now being identified. But phosphorylation is just one of many mechanisms for post-translational modification of proteins. Accordingly, other mechanisms for regulating pump activity are likely to emerge. These mechanisms may also focus on the assembled, intact proton pump, i.e. on rates of ATP hydrolysis, on changes in the ATP/H^+ transport-coupling ratio, or on the mobility of the rotor within the plasma membrane. Interactions of the V-ATPase with scaffolding and cytoskeletal proteins (that often mediate signal transduction) are likely to influence the transport activity of the holoenzyme, but these interactions have not yet been explored. Finally, the V-ATPase appears to be an ideal model for investigating the effects of voltage on an electrogenic pump.

It is puzzling why the presently known pharmaceutical inhibitors of the V-ATPase such as bafilomycin, concanamycin, archazolid and apicularen all target the V_O complex, while specific inhibitors of the V_1 complex have not yet been found. Do macrolides identify particularly vulnerable part(s) of the proton pump? If so, could endogenous agents that mimic plecomacrolides account for diseases associated with defects of the V-ATPase? Clearly, the discovery of additional inhibitors and activators of the V-ATPase will figure importantly in completing the molecular and mechanistic models of this proton pump.

The power of genetics and its post-genomic biology is unquestioned. Genetic and molecular approaches illuminate molecular processes in great detail. Thus, as we near the first decade of the 21st century, we have a decent structural model of the V-ATPase, and we know some of its functional properties. The structural complexity signals functional complexity beyond the roles of proteins functioning as mechanical devices of stator and rotor. While molecular and genetic studies will continue to reach the deepest bases for understanding physiology, *in vivo* experiments – as difficult as they are and as uncertain as they can be – will continue to differentiate between genetic/molecular potential and physiological reality in the living cell.

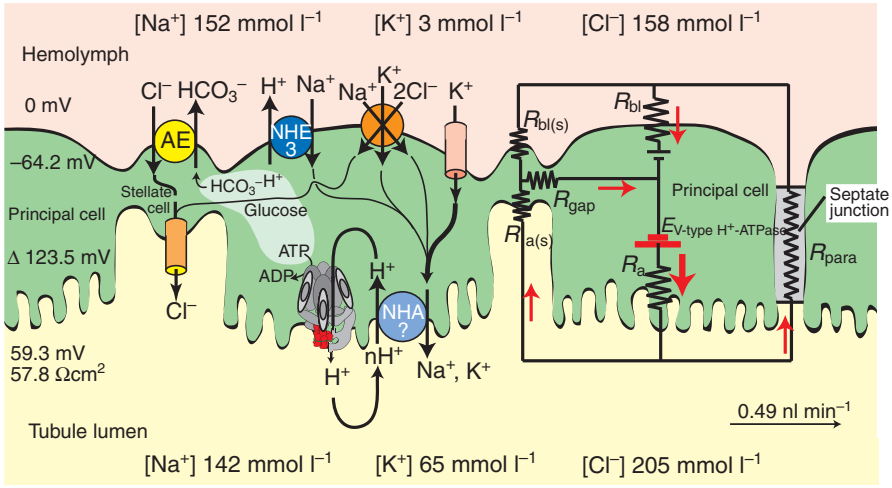

Fig. 5. Molecular and electrical models for powering the transepithelial secretion of NaCl, KCl and water in Malpighian tubules of *Ae. aegypti* under control conditions. Principal cells mediate the transepithelial active transport of Na^+ and K^+ driven by the V-type H^+ ATPase collaborating with a putative electrogenic H^+/cation exchanger (NHA?) located at the apical membrane. Stellate cells and the septate junction mediate the passive transepithelial secretion of Cl^-. Active and passive transepithelial transport routes are electrically coupled *via* septate and gap junctions. Red arrows indicate the flow of positive charge, where current can be carried by negative charge (anion) flowing in the opposite direction. *R*, resistance; a and a(s) apical membrane of principal and stellate cell, respectively; bl and bl(s), basolateral membrane of principal and stellate cell, respectively; gap, gap junction; para, paracellular pathway; AE, electroneutral anion exchanger; NHE3, electroneutral Na^+/H^+ exchanger type 3. The paracellular pathway between principal and stellate cells is not depicted. Data from previous publications (Beyenbach, 2001; Beyenbach and Masia, 2002; O'Connor and Beyenbach, 2001; Piermarini et al., 2008).

Supported by grants awarded to H.W. and M.H. from the Deutsche Forschungsgemeinschaft (SFB 431) and the VolkswagenStiftung (Funding Initiative 'Interplay between Molecular Conformations and Biological Function') and by NSF grant IOB 0542797 and NIH grant R21 AI072102 awarded to K.W.B. Deposited in PMC for release after 12 months.

References

Al Awqati, Q. (1978). H^+ transport in urinary epithelia. *Am. J. Physiol.* **235**, F77-F88.
Allan, A. K., Du, J., Davies, S. A. and Dow, J. A. (2005). Genome-wide survey of V-ATPase genes in *Drosophila* reveals a conserved renal phenotype for lethal alleles. *Physiol. Genomics* **22**, 128-138.
Azuma, M., Harvey, W. R. and Wieczorek, H. (1995). Stoichiometry of K^+/H^+ antiport helps to explain extracellular pH 11 in a model epithelium. *FEBS Lett.* **361**, 153-156.
Bertram, G. and Wessing, A. (1994). Intracellular pH regulation by the plasma membrane V-ATPase in Malpighian tubules of *Drosophila* larvae. *J. Comp. Physiol. B* **164**, 238-246.
Bertram, G., Schleithoff, L., Zimmermann, P. and Wessing, A. (1991). Bafilomycin A_1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei*: is a vacuolar-type ATPase involved in ion and fluid secretion? *J. Insect Physiol.* **37**, 201-209.
Beyenbach, K. W. (2001). Energizing epithelial transport with the vacuolar H^+-ATPase. *News Physiol. Sci.* **16**, 145-151.
Beyenbach, K. W. (2003a). Transport mechanisms of diuresis in Malpighian tubules of insects. *J. Exp. Biol.* **206**, 3845-3856.
Beyenbach, K. W. (2003b). Regulation of tight junction permeability with switch-like speed. *Curr. Opin. Nephrol. Hypertens.* **12**, 543-550.
Beyenbach, K. W. and Masia, R. (2002). Membrane conductances of principal cells in Malpighian tubules of *Aedes aegypti*. *J. Insect Physiol.* **48**, 375-386.
Beyenbach, K. W. and Petzel, D. H. (1987). Diuresis in mosquitoes: role of a natriuretic factor. *News Physiol. Sci.* **2**, 171-175.
Beyenbach, K. W. and Wieczorek, H. (2006). The V-type H^+ ATPase: molecular structure and function, physiological roles and regulation. *J. Exp. Biol.* **209**, 577-589.
Boekema, E. J., Ubbinkkok, T., Lolkema, J. S., Brisson, A. and Konings, W. N. (1997). Visualization of a peripheral stalk in V-type ATPase-evidence for the stator structure essential to rotational catalysis. *Proc. Natl. Acad. Sci. USA* **94**, 14291-14293.
Boekema, E. J., Ubbinkkok, T., Lolkema, J. S., Brisson, A. and Konings, W. N. (1998). Structure of V-type ATPase from *Clostridium fervidus* by electron microscopy. *Photosyn. Res.* **57**, 267-273.
Bond, S. and Forgac, M. (2008). The Ras/cAMP/protein kinase A pathway regulates glucose-dependent assembly of the vacuolar (H^+)-ATPase in yeast. *J. Biol. Chem.* **283**, 36513-36521.
Bonfanti, P., Colombo, A., Heintzelman, M. B., Mooseker, M. S. and Camatini, M. (1992). The molecular architecture of an insect midgut brush border cytoskeleton. *Eur. J. Cell Biol.* **57**, 298-307.
Bowman, B. J. and Bowman, E. J. (2002). Mutations in subunit c of the vacuolar ATPase confer resistance to bafilomycin and identify a conserved antibiotic binding site. *J. Biol. Chem.* **277**, 3965-3972.
Bowman, B. J., McCall, M. E., Baertsch, R. and Bowman, E. J. (2006). A model for the proteolipid ring and bafilomycin/concanamycin-binding site in the vacuolar ATPase of *Neurospora crassa*. *J. Biol. Chem.* **281**, 31885-31893.
Bowman, E. J., Siebers, A. and Altendorf, K. (1988). Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Nat. Acad. Sci. USA* **85**, 7972-7976.
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.
Coast, G. M., Garside, C. S., Webster, S. G., Schegg, K. M. and Schooley, D. A. (2005). Mosquito natriuretic peptide identified as a calcitonin-like diuretic hormone in *Anopheles gambiae* (Giles). *J. Exp. Biol.* **208**, 3281-3291.
Crider, B. P., Xie, X. S. and Stone, D. K. (1994). Bafilomycin inhibits proton flow through the H^+ channel of vacuolar proton pumps. *J. Biol. Chem.* **269**, 17379-17381.
Dames, P., Zimmermann, B., Schmidt, R., Rein, J., Voss, M., Schewe, B., Walz, B. and Baumann, O. (2006). cAMP regulates plasma membrane vacuolar-type H^+-ATPase assembly and activity in blowfly salivary glands. *Proc. Natl. Acad. Sci. USA* **103**, 3926-3931.
Davies, S. A., Goodwin, S. F., Kelly, D. C., Wang, Z., Sözen, M. A., Kaiser, K. and Dow, J. A. (1996). Analysis and inactivation of *vha55*, the gene encoding the vacuolar ATPase B-subunit in *Drosophila melanogaster* reveals a larval lethal phenotype. *J. Biol. Chem.* **271**, 30677-30684.
Day, J. P., Wan, S., Allan, A. K., Kean, L., Davies, S. A., Gray, J. V. and Dow, J. A. (2008). Identification of two partners from the bacterial Kef exchanger family for the apical plasma membrane V-ATPase of metazoa. *J. Cell Sci.* **121**, 2612-2619.
Dow, J. A. (1999). The multifunctional *Drosophila melanogaster* V-ATPase is encoded by a multigene family. *J. Bioenerg. Biomembr.* **31**, 75-83.
Dow, J. A. (2009). Insights into the Malpighian tubule from functional genomics. *J. Exp. Biol.* **212**, 435-445.
Dow, J. A., Davies, S. A., Guo, Y., Graham, S., Finbow, M. E. and Kaiser, K. (1997). Molecular genetic analysis of V-ATPase function in *Drosophila melanogaster*. *J. Exp. Biol.* **200**, 237-245.
Drory, O., Frolow, F. and Nelson, N. (2004). Crystal structure of yeast V-ATPase subunit C reveals its stator function. *EMBO Rep.* **5**, 1148-1152.
Dröse, S., Bindseil, K. U., Bowman, E. J., Siebers, A., Zeeck, A. and Altendorf, K. (1993). Inhibitory effect of modified bafilomycins and concanamycins on P- and V-type adenosinetriphosphatases. *Biochemistry* **32**, 3902-3906.
Gausz, J., Bencze, G., Gyurkovics, H., Ashburner, M., Ish-Horowicz, D. and Holden, J. J. (1979). Genetic characterization of the 87c region of the third chromosome of *Drosophila melanogaster*. *Genetics* **93**, 917-934.
Gräf, R., Lepier, A., Harvey, W. R. and Wieczorek, H. (1994). A novel 14-kDa V-ATPase subunit in the tobacco hornworm midgut. *J. Biol. Chem.* **269**, 3767-3774.
Gräf, R., Harvey, W. R. and Wieczorek, H. (1996). Purification and properties of a cytosolic V_1-ATPase. *J. Biol. Chem.* **271**, 20908-20913.
Graham, L. A., Hill, K. J. and Stevens, T. H. (1994). *VMA7* encodes a novel 14-kDa subunit of the *Saccharomyces cerevisiae* vacuolar H^+-ATPase complex. *J. Biol. Chem.* **269**, 25974-25977.
Hanada, H., Moriyama, Y., Maeda, M. and Futai, M. (1990). Kinetic studies of chromaffin granule H^+-ATPase and effects of bafilomycin A_1. *Biochem. Biophys. Res. Commun.* **170**, 873-878.
Harold, F. M. (1986). *The Vital Force: A Study of Bioenergetics*. New York: W. H. Freeman.
Harvey, W. R. (1982). Membrane physiology of insects. In *Membrane Physiology of Invertebrates* (ed. R. B. Podesta), pp. 495-565. New York: Marcel Dekker.
Harvey, W. R. and Wieczorek, H. (1997). Chemiosmotic energization of animal plasma membranes by H^+ V-ATPases. *J. Exp. Biol.* **200**, 203-216.
Harvey, W. R., Cioffi, M. and Wolfersberger, M. G. (1983). Chemiosmotic potassium ion pump of insect epithelia. *Am. J. Physiol.* **244**, R163-R175.
Harvey, W. R., Maddrell, S. H. P., Telfer, W. H. and Wieczorek, H. (1998). H^+ V-ATPases energize animal plasma membranes for secretion and absorption of ions and fluids. *Am. Zool.* **38**, 426-441.
Holliday, L. S., Lu, M., Lee, B. S., Nelson, R. D., Solivan, S., Zhang, L. and Gluck, S. L. (2000). The amino-terminal domain of the B subunit of vacuolar H^+-ATPase contains a filamentous actin binding site. *J. Biol. Chem.* **275**, 32331-32337.
Huss, M. and Wieczorek, H. (2007). Influence of ATP and ADP on dissociation of the V-ATPase into its V_1 and V_O complexes. *FEBS Lett.* **581**, 5566-5572.
Huss, M. and Wieczorek, H. (2009). Inhibitors of V-ATPases: old and new players. *J. Exp. Biol.* **212**, 341-346.
Huss, M., Ingenhorst, G., König, S., Gassel, M., Dröse, S., Zeeck, A., Altendorf, K. and Wieczorek, H. (2002). Concanamycin A, the specific inhibitor of V-ATPases, binds to the V_O subunit c. *J. Biol. Chem.* **277**, 40544-40548.
Huss, M., Sasse, F., Kunze, B., Jansen, R., Steinmetz, H., Ingenhorst, G., Zeeck, A. and Wieczorek, H. (2005). Archazolid and apicularen: Novel specific V-ATPase inhibitors. *BMC Biochem.* **6**, 13.
Jäger, D. and Klein, U. (1996). Biogenesis and regulation of V-ATPase in the midgut of the tobacco hornworm. *Proc. Germ. Zool. Soc.* **89**, 327.
Kane, P. M. (1995). Disassembly and reassembly of the yeast vacuolar H^+-ATPase *in vivo*. *J. Biol. Chem.* **270**, 17025-17032.
Kane, P. M. (2000). Regulation of V-ATPases by reversible disassembly. *FEBS Lett.* **469**, 137-141.
Karas, K., Brauer, P. and Petzel, D. (2005). Actin redistribution in mosquito Malpighian tubules after a blood meal and cyclic AMP stimulation. *J. Insect Physiol.* **51**, 1041-1054.
Karet, F. E., Finberg, K. E., Nelson, R. D., Nayir, A., Mocan, H., Sanjad, S. A., Rodriguez-Soriano, J., Santos, F., Cremers, C., Di Pietro, A. et al. (1999). Mutations in the gene encoding B1 subunit of H^+-ATPase cause renal tubular acidosis with sensorineural deafness. *Nat. Genet.* **21**, 84-90.
Krogh, A. (1929). Progress of physiology. *Am J. Physiol.* **90**, 243-251.
Lee, B. S., Gluck, S. L. and Holliday, L. S. (1999). Interaction between vacuolar H^+-ATPase and microfilaments during osteoclast activation. *J. Biol. Chem.* **274**, 29164-29171.
Lepier, A., Gräf, R., Azuma, M., Merzendorfer, H., Harvey, W. R. and Wieczorek, H. (1996). The peripheral complex of the tobacco hornworm V-ATPase contains a novel 13-kDa subunit G. *J. Biol. Chem.* **271**, 8502-8508.
Lu, M., Ammar, D., Ives, H., Albrecht, F. and Gluck, S. L. (2007). Physical interaction between aldolase and vacuolar H^+-ATPase is essential for the assembly and activity of the proton pump. *J. Biol. Chem.* **282**, 24495-24503.
Ludwig, J., Kerscher, S., Brandt, U., Pfeiffer, K., Getlawi, F., Apps, D. K. and Schägger, H. (1998). Identification and characterization of a novel 9.2-Kda membrane sector-associated protein of vacuolar proton-ATPase from chromaffin granules. *J. Biol. Chem.* **273**, 10939-10947.
Merzendorfer, H., Huss, M., Schmid, R., Harvey, W. R. and Wieczorek, H. (1999). A novel insect V-ATPase subunit M9.7 is glycosylated extensively. *J. Biol. Chem.* **274**, 17372-17378.
Merzendorfer, H., Reineke, S., Zhao, X. F., Jacobmeier, B., Harvey, W. R. and Wieczorek, H. (2000). The multigene family of the tobacco hornworm V-ATPase: novel subunits a, C, D, H, and putative isoforms. *Biochim. Biophys. Acta* **25**, 369-379.
Muench, S. P., Huss, M., Phillips, C., Wieczorek, H., Trinick, J. and Harrison, M. A. (2009). The structure of the vacuolar ATPase motor reveals its mechanical and regulatory complexity. *J. Mol. Biol.* **386**, 989-999.
Murata, T., Yamato, I., Kakinuma, Y., Leslie, A. G. and Walker, J. E. (2005). Structure of the rotor of the V-Type Na^+-ATPase from *Enterococcus hira*e. *Science* **308**, 654-659.
Nishi, T. and Forgac, M. (2002). The vacuolar (H^+)-ATPases-nature's most versatile proton pumps. *Nat. Rev. Mol. Cell. Biol.* **3**, 94-103.
O'Connor, K. R. and Beyenbach, K. W. (2001). Chloride channels in apical membrane patches of stellate cells of Malpighian tubules of *Aedes aegypti*. *J. Exp. Biol.* **204**, 367-378.
Ohira, M., Smardon, A. M., Charsky, C. M. H., Liu, J., Tarsio, M. and Kane, P. M. (2006). The E and G subunits of the yeast V-ATPase interact tightly and are both present at more than one copy per V_1 complex. *J. Biol. Chem.* **281**, 22752-22760.
Pannabecker, T. L., Hayes, T. K. and Beyenbach, K. W. (1993). Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membr. Biol.* **132**, 63-76.
Petzel, D. H., Pirotte, P. T. and Van Kerkhove, E. (1999). Intracellular and luminal pH measurements of Malpighian tubules of the mosquito *Aedes aegypti*: The effects of cAMP. *J. Insect Physiol.* **45**, 973-982.
Piermarini, P. M., Grogan, L., Wang, L. and Beyenbach, K. W. (2009). The other half of the insect SLC4 family: a Cl/HCO_3 anion exchanger from mosquito renal tubules. *FASEB J.* **23**, 800.4.

Radermacher, M., Ruiz, T., Harvey, W. R., Wieczorek, H. and Grüber, G. (1999). Molecular architecture of *Manduca sexta* midgut V_1 ATPase visualized by electron microscopy. *FEBS Lett.* **453**, 383-386.

Radermacher, M., Ruiz, T., Wieczorek, H. and Grüber, G. (2001). The structure of the V_1-ATPase determined by three-dimensional electron microscopy of single particles. *J. Struct. Biol.* **135**, 26-37.

Ramsay, J. A. (1953). Active transport of potassium by the Malpighian tubules of insects. *J. Exp. Biol.* **30**, 358-369.

Rautiala, T. J., Koskinen, A. M. and Vaananen, H. K. (1993). Purification of vacuolar ATPase with bafilomycin C1 affinity chromatography. *Biochem. Biophys. Res. Commun.* **194**, 50-56.

Rein, J., Voss, M., Blenau, W., Walz, B. and Baumann, O. (2008). Hormone-induced assembly and activation of V-ATPase in blowfly salivary glands is mediated by protein kinase A. *Am. J. Physiol.* **294**, C56-C65.

Rheault, M. R., Okech, B. A., Keen, S. B., Miller, M. M., Meleshkevitch, E. A., Linser, P. J., Boudko, D. Y. and Harvey, W. R. (2007). Molecular cloning, phylogeny and localization of AgNHA1: the first Na^+/H^+ antiporter (NHA) from a metazoan, *Anopheles gambiae*. *J. Exp. Biol.* **210**, 3848-3861.

Sambade, M. and Kane, P. M. (2004). The yeast vacuolar proton-translocating ATPase contains a subunit homologous to the *Manduca sexta* and bovine e subunits that is essential for function. *J. Biol. Chem.* **279**, 17361-17365.

Sautin, Y. Y., Lu, M., Gaugler, A., Zhang, L. and Gluck, S. L. (2005). Phosphatidylinositol 3-kinase-mediated effects of glucose on vacuolar H^+-ATPase assembly, translocation, and acidification of intracellular compartments in renal epithelial cells. *Mol. Cell. Biol.* **25**, 575-589.

Sawyer, D. B. and Beyenbach, K. W. (1985). Dibutyryl-cAMP increases basolateral sodium conductance of mosquito Malpighian tubules. *Am. J. Physiol.* **248**, R339-R345.

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.

Seol, J. H., Shevchenko, A. and Deshaies, R. J. (2001). Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. *Nat. Cell Biol.* **2**, 384-391.

Smardon, A. M., Tarsio, M. and Kane, P. M. (2002). The RAVE complex is essential for stable assembly of the yeast V-ATPase. *J. Biol. Chem.* **277**, 13831-13839.

Smith, A. N., Skaug, J., Choate, K. A., Nayir, A., Bakkaloglu, A., Ozen, S., Hulton, S. A., Sanjad, S. A., Al-Sabban, E. A., Lifton, R. P. et al. (2000). Mutations in *ATP6N1B*, encoding a new kidney vacuolar proton pump 116-kD subunit, cause recessive distal renal tubular acidosis with preserved hearing. *Nat. Genet.* **26**, 71-75.

Sözen, M. A., Armstrong, J. D., Yang, M., Kaiser, K. and Dow, J. A. (1997). Functional domains are specified to single-cell resolution in a *Drosophila* epithelium. *Proc. Natl. Acad. Sci. USA* **94**, 5207-5212.

Sumner, J. P., Dow, J. A., Earley, F. G., Klein, U., Jäger, D. and Wieczorek, H. (1995). Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. *J. Biol. Chem.* **270**, 5649-5653.

Supeková, L., Supek, F. and Nelson, N. (1995). The *Saccharomyces cerevisiae VMA10* is an intron-containing gene encoding a novel 13-kDa subunit of vacuolar H^+-ATPase. *J. Biol. Chem.* **270**, 13726-13732.

Svergun, D. I., Konrad, S., Huss, M., Koch, M. H. J., Wieczorek, H., Altendorf, K., Volkov, V. V. and Grüber, G. (1998). Quaternary structure of V_1 and F_1 ATPase: Significance of structural homologies and diversities. *Biochemistry* **37**, 17659-17663.

Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M. and Mellman, I. (2003). Activation of lysosomal function during dendritic cell maturation. *Science* **299**, 1400-1403.

Vitavska, O., Wieczorek, H. and Merzendorfer, H. (2003). A novel role for subunit C in mediating binding of the H^+-V-ATPase to the actin cytoskeleton. *J. Biol. Chem.* **278**, 18499-18505.

Vitavska, O., Merzendorfer, H. and Wieczorek, H. (2005). The V-ATPase subunit C binds to polymeric F-actin as well as to monomeric G-actin and induces cross-linking of actin filaments. *J. Biol. Chem.* **280**, 1070-1076.

Voss, M., Vitavska, O., Walz, B., Wieczorek, H. and Baumann, O. (2007). Stimulus-induced phosphorylation of plasma membrane V-ATPase by protein kinase A. *J. Biol. Chem.* **282**, 33735-33742.

Wang, J., Kean, L., Yang, J., Allan, A. K., Davies, S. A., Herzyk, P. and Dow, J. A. (2004). Function-informed transcriptome analysis of *Drosophila* renal tubule. *Genome Biol.* **5**, R69.

Wang, Y., Inoue, T. and Forgac, M. (2005). Subunit a of the yeast V-ATPase participates in binding of bafilomycin. *J. Biol. Chem.* **280**, 40481-40488.

Weng, X. H., Huss, M., Wieczorek, H. and Beyenbach, K. W. (2003). The V-type H(+)-ATPase in Malpighian tubules of *Aedes aegypti*: localization and activity. *J. Exp. Biol.* **206**, 2211-2219.

Weng, X. H., Piermarini, P. M., Yamahiro, A., Yu, M. J., Aneshansley, D. J. and Beyenbach, K. W. (2008). Gap junctions in Malpighian tubules of *Aedes aegypti*. *J. Exp. Biol.* **211**, 409-422.

Wessing, A., Bertram, G. and Zierold, K. (1993). Effects of bafilomycin A1 and amiloride on the apical potassium and proton gradients in Drosophila Malpighian tubules studied by X-ray microanalysis and microelectrode measurements. *J. Comp. Physiol. B* **163**, 452-462.

Wieczorek, H. (1982). A biochemical approach to the electrogenic potassium pump of insect sensilla: potassium sensitive ATPases in the labellum of the fly. *J. Comp. Physiol.* **148**, 303-311.

Wieczorek, H. and Harvey, W. R. (1995). Energization of animal plasma membranes by the proton-motive force. *Physiol. Zool.* **68**, 15-23.

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.

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., 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., Brown, D., Grinstein, S., Ehrenfeld, J. and Harvey, W. R. (1999). Animal plasma membrane energization by proton-motive V-ATPases. *Bioessays* **21**, 637-648.

Wilkens, S., Vasilyeva, E. and Forgac, M. (1999). Structure of the vacuolar ATPase by electron microscopy. *J. Biol. Chem.* **274**, 31804-31810.

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. Transp.* **16**, 109-133.

Xie, X. S. (1996). Reconstitution of ATPase activity from individual subunits of the clathrin-coated vesicle proton pump: the requirement and effect of three small subunits. *J. Biol. Chem.* **271**, 30980-30985.

Xu, T. and Forgac, M. (2001). Microtubules are involved in glucose-dependent dissociation of the yeast vacuolar. *J. Biol. Chem.* **276**, 24855-24861.

Yu, M. and Beyenbach, K. W. (2001). Leucokinin and the modulation of the shunt pathway in Malpighian tubules. *J. Insect Physiol.* **47**, 263-276.

Yu, M. J. and Beyenbach, K. W. (2004). Effects of leucokinin-VIII on *Aedes* Malpighian tubule segments lacking stellate cells. *J. Exp. Biol.* **207**, 519-526.

Zhang, J. M., Feng, Y. and Forgac, M. (1994). Proton conduction and bafilomycin binding by the V-0 domain of the coated vesicle V-ATPase. *J. Biol. Chem.* **269**, 23518-23523.

Zhang, Z., Zheng, Y., Mazon, H., Milgrom, E., Kitagawa, N., Kish-Trier, E., Heck, A. J., Kane, P. M. and Wilkens, S. (2008). Structure of the yeast vacuolar ATPase. *J. Biol. Chem.* **283**, 35983-35995.