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Review

The V-type H^+ ATPase: molecular structure and function, physiological roles and regulation

Klaus W. Beyenbach[1,*] and Helmut Wieczorek[2]

[1]*Department of Biomedical Sciences, VRT 8004, Cornell University, Ithaca, NY 14853, USA and* [2]*Department of Biology/Chemistry, University of Osnabrück, 49069 Osnabrück, Germany*

*Author for correspondence (e-mail: KWB1@CORNELL.EDU)

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Summary

It was nearly 30 years before the V-type H^+ ATPase was admitted to the small circle of *bona fide* transport ATPases alongside F-type and P-type ATPases. The V-type H^+ ATPase is an ATP-driven enzyme that transforms the energy of ATP hydrolysis to electrochemical potential differences of protons across diverse biological membranes *via* the primary active transport of H^+. In turn, the transmembrane electrochemical potential of H^+ is used to drive a variety of (i) secondary active transport systems *via* H^+-dependent symporters and antiporters and (ii) channel-mediated transport systems. For example, expression of Cl^- channels or transporters next to the V-type H^+ ATPase in vacuoles of plants and fungi and in lysosomes of animals brings about the acidification of the endosomal compartment, and the expression of the H^+/neurotransmitter antiporter next to the V-type H^+ ATPase concentrates neurotransmitters in synaptic vesicles.

First found in association with endosomal membranes, the V-type H^+ ATPase is now also found in increasing examples of plasma membranes where the proton pump energizes transport across cell membranes and entire epithelia. The molecular details reveal up to 14 protein subunits arranged in (i) a cytoplasmic V_1 complex, which mediates the hydrolysis of ATP, and (ii) a membrane-embedded V_0 complex, which translocates H^+ across the membrane. Clever experiments have revealed the V-type H^+ ATPase as a molecular motor akin to F-type ATPases. The hydrolysis of ATP turns a rotor consisting largely of one copy of subunits D and F of the V_1 complex and a ring of six or more copies of subunit c of the V_0 complex. The rotation of the ring is thought to deliver H^+ from the cytoplasmic to the endosomal or extracellular side of the membrane, probably *via* channels formed by subunit a. The reversible dissociation of V_1 and V_0 complexes is one mechanism of physiological regulation that appears to be widely conserved from yeast to animal cells. Other mechanisms, such as subunit–subunit interactions or interactions of the V-type H^+ ATPase with other proteins that serve physiological regulation, remain to be explored. Some diseases can now be attributed to genetic alterations of specific subunits of the V-type H^+ ATPase.

Key words: proton pump, molecular motor, V_0 complex, V_1 complex, subunit, endosomal membrane, plasma membrane, primary active transport, secondary active transport, channel-mediated transport, epithelial transport, actin, pathophysiology, genetic mutation.

A brief historical perspective

Thirteen years ago *The Journal of Experimental Biology* dedicated an entire volume to V-ATPases, providing a permanent record of the proceedings of a symposium held in Telluride, Colorado in June 1992 (Harvey and Nelson, 1992). On first inspection, the absence of a prologue on the history of V-ATPases suggests a casual omission. On reflection, nearly every investigator who had made first observations of what eventually turned out as the V-ATPase had come to Telluride, and a historically perspective may have seemed too early and less exciting than the science each had come to report. Efraim Racker, who was to have been the keynote speaker, would have offered at least some anecdotes. But his passing away in September 1991 was a loss, and not to this symposium alone.

Today, a historical perspective seems timely and appropriate. Accordingly, we begin these pages with our attempt to review the history of the V-ATPase as we see it, and with an apology to all those who have not been mentioned and cited.

V-type H^+ ATPases, also known as H^+ V-ATPases and V-ATPases, were not discovered in a single ‘Eureka’ experiment; they were gradually uncovered independently in various

laboratories working with animals, plants and fungi. In animal cells, adrenal medullary chromaffin granules provided the first evidence for the existence of a proton ATPase in a vacuolar system when Kirshner (1962) showed that uptake of catecholamines is an ATP-dependent process. But it would take another 13 years before Radda and coworkers demonstrated the existence of a proton pump in the membrane of chromaffin granules (Bashford et al., 1975). Thereafter, reports accumulated on the identification of ATP-driven proton transport and/or respective ATPase activity in the membrane of organelles such as clathrin-coated vesicles, platelet dense granules, lysosomes and chromaffin granules (Apps and Reid, 1977; Cidon and Nelson, 1983; Dean et al., 1984; Forgac et al., 1983; Harikumar and Reeves, 1983; Ohkuma et al., 1982; Xie et al., 1983).

In plants, the finding of a salt- and ionophore-stimulated ATPase in microsomes of turnips signaled the advent of a new transport pump (Rungie and Wiskich, 1973). An anion-stimulated ATPase activity was also observed in vacuolar membranes of rubber trees and red beets which, curiously, was not inhibited by vanadate, the classical inhibitor of P-type pumps (D'Auzac, 1975; Walker and Leigh, 1981). Moreover, these studies suggested that a proton pump acidifies vacuolar and lysosomal compartments. By the early 1980s, several laboratories working with isolated microsomal or vacuolar vesicles had independently attributed the anion-stimulated and vanadate-insensitive ATPase activity to an electrogenic proton pump. These laboratories included those of Hager (Hager et al., 1980), Sze (Churchill and Sze, 1983), Spanswick (DuPont et al., 1982) and Taiz (Mandala et al., 1982).

Interest in the newly found proton pump grew with reports of a vacuolar ATPase in fungi such as yeast (Kakinuma et al., 1981) and *Neurospora* (Bowman and Bowman, 1982). By the second half of the eighties, the purification of vacuolar ATPases from animals, plants and fungi had revealed their multisubunit composition (Arai et al., 1987; Bowman et al., 1986; Moriyama and Nelson, 1987; Randall and Sze, 1986; Uchida et al., 1985; Xie and Stone, 1986). In view of (1) the location of vacuolar ATPases in organellar membranes, where they mediate proton transport, (2) the subunit similarity among V-ATPases from diverse sources, (3) common inhibitor profiles, and (4) the absence of a covalent phosphorylated intermediate in the reaction cycle, the vacuolar ATPases were acknowledged, alongside F-type and P-type ATPases (F- and P-ATPases), as a third family of ion-motive ATPases, to be called V-type ATPases (Pedersen and Carafoli, 1987) or V-ATPases (Nelson, 1989). Since the eukaryotic V-ATPases all transport protons, they are also called H^+ V-ATPases or V-type H^+ ATPases.

To date, impressive progress has been made in elucidating the structural, functional and regulatory properties of V-type H^+ ATPases. The discovery of bafilomycin as a specific potent inhibitor enabled the detection of this new proton pump in a variety of unexpected locations and with unforeseen physiological activities (Bowman et al., 1988). The amino acid sequences of the complete set of subunits of several V-type ATPases have now been deduced from cDNA cloning, and much has been learned about the interactions between subunits, the regulation of enzyme activity, and the assembly and targeting during biogenesis (Nishi and Forgac, 2002).

Electron microscopic images of the V-type H^+ ATPase were obtained before they were found to show parts of this proton pump. As early as 1966, Gupta and Berridge (Gupta and Berridge, 1966) had observed repeating structures on the cytoplasmic surface of the plasma membrane in the ion-transporting epithelium of the blowfly rectum. Similar structures were later seen at the apical plasma membrane of goblet cells in the caterpillar midgut of *Cecropia* (Anderson and Harvey, 1966). But it would take until 1983 before these structures were noted to resemble the catalytic sector of the mitochondrial ATP synthase and called 'portasomes' (Harvey et al., 1983). Portasomes were subsequently observed in vesicular membranes of many eukaryotic cells such as the vacuolar membrane of *Neurospora* (Dschida and Bowman, 1992), acidosomes of *Dictyostelium* (Nolta et al., 1991), tonoplasts of several higher plants (Klink and Lüttge, 1991; Moore et al., 1991; Taiz and Taiz, 1991), bovine chromaffin granules (Moriyama et al., 1991), and even in plasma membranes (Brown et al., 1987), where they were called 'studs' (Fig. 1). Since then, additional light has been shed on the topology of V-ATPases. Transmission electron microscopy has provided the low-resolution structures of the holoenzyme and its subcomplexes (reviewed by Wilkens et al., 2005). High-resolution structural analysis by X-ray crystallography of several subunits and subcomplexes of eukaryotic V-ATPases and their bacterial Na^+-pumping relatives are now in progress (Drory et al., 2004; Iwata et al., 2004; Murata et al., 2005).

The V-type H^+ ATPase is now thought to be present in virtually every eukaryotic cell. The proton pump occupies intracellular membranes such as those of clathrin-coated vesicles, synaptic vesicles, endosomes, storage vesicles, Golgi vesicles, secretory vesicles, lysosomes and their partner organelle in plants and fungi, the central vacuole (Stevens and Forgac, 1997). Best known are the contributions made by the V-type H^+ ATPase to the acidification of intracellular compartments. In lysosomes and in vacuoles of plants and fungi, a pH of about 5 serves the breakdown of macromolecules by up to 40 types of acid hydrolases including proteases, glycosidases, lipases, nucleases and phosphatases. A further function of the vacuole aided by the V-type H^+ ATPase is the regulation of cytosolic pH and the uptake of cations such as Na^+, Ca^{2+} and Cd^{2+} *via* H^+-driven antiport (Dietz et al., 2001). The acidification of endocytotic and exocytotic organelles mediated by the V-type H^+ ATPase in animal cells provides optimal pH values for diverse functions. For instance, in the endocytotic pathway the endosomal pH of 6 causes the release of ligands such as transferrin or low density lipoprotein from their respective receptors (Johnson et al., 1993). In the exocytotic pathway, peptides and proteins in acidic secretory vesicles are often proteolytically processed from pro-proteins by acid proteases to yield, for example, enkephalin or insulin.

Next to the V-type H^+ ATPase, synaptic vesicles possess

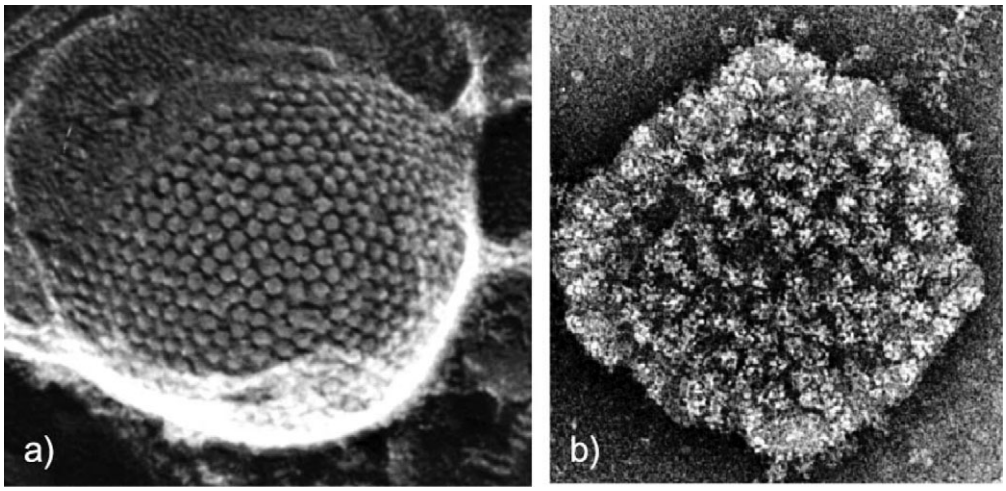

Fig. 1. Electron micrographs of 'studs', the globular headpieces of the V_1 complex of the V-type H^+ ATPase. (a) Stereoelectron micrograph of a vesicle from the apical region of a mitochondria-rich cell after rapid freezing and freeze-drying of apical membrane segments of toad urinary bladder (courtesy of D. Brown, Boston). (b) Negative stained electron micrograph of a vesicle after purification of goblet cell apical membranes from the midgut of the tobacco hornworm (courtesy of M. Huss and H. Wieczorek, Osnabrück). The diameter of 'studs' (portasomes) is approximately 10 nm. The average density is about 16 800 studs μm^{-2} of membrane in the toad urinary bladder (Brown et al., 1987) and about 5000 μm^{-2} in the tobacco hornworm.

transporters for glutamate (SLC17), monoamines and acetylcholine (SLC18), and γ-aminobutyrate (SLC32). What these families of vesicular solute-linked carriers (SLC) share in common is H^+-dependent transport of neurotransmitter (Hediger et al., 2004; Parsons, 2000). The uptake of monoamines and γ-aminobutyrate is driven by the outward proton concentration difference, and the uptake of anionic glutamate is driven by the membrane voltage, positive inside (Moriyama et al., 1992). Both proton concentration difference and membrane voltage are generated by the V-type H^+ ATPase.

The V-type H^+ ATPase also supports important functions in protozoans. In *Paramecium*, the proton pump is located in the decorated spongiome of radial arms that extend from the contractile vacuole complex (Fok et al., 1995). Concanomycin, an inhibitor of V-type H^+ ATPases, significantly decreases the rate of fluid uptake by the contractile vesicle complex, suggesting that the proton pump serves volume regulation in *Paramecium* (Gronlien et al., 2002). In the malaria parasite *Plasmodium*, the V-type H^+ ATPase occurs not only in the membranes of cell organelles but also in the plasma membrane, where it may be involved, among other functions, in energizing the secondary transport of diverse solutes (Moriyama et al., 2003). What is more, the *Plasmodium*-encoded V-type H^+ ATPase is exported to the cytoplasm of the host erythrocyte and targeted to the plasma membrane, where it has a role in maintaining the intracellular pH of the infected erythrocyte (Marchesini et al., 2005).

Located in plasma membranes of cells, the V-type H^+ ATPase can acidify the extracellular compartment that serves a number of roles: the resorption of bone by osteoclasts (Schlesinger et al., 1997), the maturation and storage of sperm in the epididymal lumen (Breton et al., 1996), the reabsorption of bicarbonate in renal proximal tubules (Wagner et al., 2004), the urinary acidification in the distal nephron (Al Awqati, 1996), and the regulation of pH in the inner ear (Stankovic et al., 1997). Even frog skin, the hallmark epithelium of Na^+/K^+-ATPase driven epithelial transport, has been found to use the plasma membrane V-type H^+ ATPase to secrete H^+ and absorb Na^+ across the epithelium (Ehrenfeld and Klein, 1997). Freshwater crustaceans, amphibians and fish employ the V-type H^+ ATPase in osmoregulation (Kirschner, 2004). Located in the plasma membrane, the proton pump is implicated in transepithelial Cl^- absorption across the gill of freshwater crab (Weihrauch et al., 2004) and in transepithelial Na^+ absorption across the gill of freshwater fish *via* channels and/or carriers that are ultimately dependent on the V-type H^+ ATPase (Kirschner, 2004; Wilson et al., 2000).

Metastasizing cells are thought to use the V-type H^+ ATPase in the plasma membrane to acidify the extracellular fluid, with the effect of destroying normal tissue in advance of the invading tumor (Sennoune et al., 2004). The fusion of viral and endosomal membranes that delivers the viral genome to the cytoplasm is dependent on the V-type H^+ ATPase (Perez and Carrasco, 1994).

When anion channels are absent in membranes inhabited by the V-type H^+ ATPase, acidification is much reduced (Harvey, 1992). Under this condition the proton pump generates large membrane voltages at small ΔpH, driving a diversity of electrogenic secondary active transport systems such as nH^+/cation antiport or nH^+/oligopeptide symport (Grinstein and Wieczorek, 1994; Leibach and Ganapathy, 1996).

Molecular architecture and mechanistic interpretations

V-type H^+ ATPases and F-ATPases share structural and functional similarities (Nishi and Forgac, 2002). In general, F-ATPases produce ATP and V-ATPases consume ATP in eukaryotic cells. In principle, the function of both ATPases is reversible (Hirata et al., 2000). ATP is synthesized when the ionic electrochemical potential is greater than the free energy of ATP hydrolysis. In contrast, when the free energy of ATP hydrolysis is greater than the ionic electrochemical

potential, the hydrolysis of ATP drives the uphill transport of ions.

The V- and F-ATPases are multisubunit proteins of up to 14 different polypeptides, which assemble as two major ring structures: (1) a peripheral V_1 or F_1 complex (400–600 kDa) that interacts with ATP, ADP and inorganic phosphate, and (2) an integral membrane V_0 or F_0 complex (150–350 kDa) that mediates the transport of H^+ or Na^+. In the case of eukaryotic V-type H^+ ATPases, the V_1 complex is invariably present in the cytoplasm such that the pump transports H^+ into vesicles and vacuoles when expressed in endosomal membranes and into the extracellular fluid when expressed in the plasma membrane.

By convention, the subunits of V_1 and V_0 complexes are distinguished with large and small letters respectively (Fig. 2). The V_1 complex consists of: (1) a globular headpiece with three alternating copies of subunits A and B that form a ring, (2) a central rotational stalk composed of single copies of subunits D and F, and (3) a peripheral stalk made of subunits C, E, G and H. Subunits A and B mediate the hydrolysis of ATP at three reaction sites associated with subunit A. Both the central rotational stalk and fixed peripheral stalk connect the V_1 complex with the V_0 complex (Fig. 2). The fixed peripheral stalk holds the V_1 complex in place, aided in part by subunits B and C of the V_1 complex that bind to actin. Subunit C alone is capable of binding monomeric actin as well as cross-linking and stabilizing actin filaments. The proton-transporting V_0 complex consists of six or more c subunits, also forming a ring structure (Fig. 2). As many as 10 subunits form a concave ring structure in the eubacterial V-type Na^+ ATPase of *Enterococcus hirae* (Murata et al., 2005).

A functional model that is widely accepted considers the V-type H^+ ATPase to consist of a stationary and a mobile part, the stator and rotor, respectively. The rotor consists of subunits D, F and the ring of subunits c (Fig. 2B). The remaining structures are considered the stator. The function of the V_0 subunits d and e remains enigmatic.

How rotation of the rotor mediates the linear transfer of H^+ across the membrane is hypothetically constructed in Fig. 2, based on the innovative models proposed for the F-ATPases (Feniouk et al., 2004; Junge et al., 1997; Junge et al., 2001) and V-ATPases (Grabe et al., 2000; Murata et al., 2005). Fundamental to the model are (1) two H^+ half-channels across the membrane, provided by subunit a in close proximity to the c-ring, (2) a H^+ binding site on each c subunit of the c-ring, and (3) the rotation of the c-ring driven by the hydrolysis of ATP (Fig. 2). The inner half channel of subunit a is thought to allow cytoplasmic H^+ to access and bind to one subunit of the c-ring. After the nearly 360° rotation of the c-ring, clockwise when viewed from the cytoplasm (Meier et al., 2005), H^+ can unbind and exit the membrane through the outer half channel (Fig. 2B). Variations of this model have been proposed. For example, inlet and outlet half channels are thought to be located in stator and rotor, respectively, in the F_1F_0-ATPase of

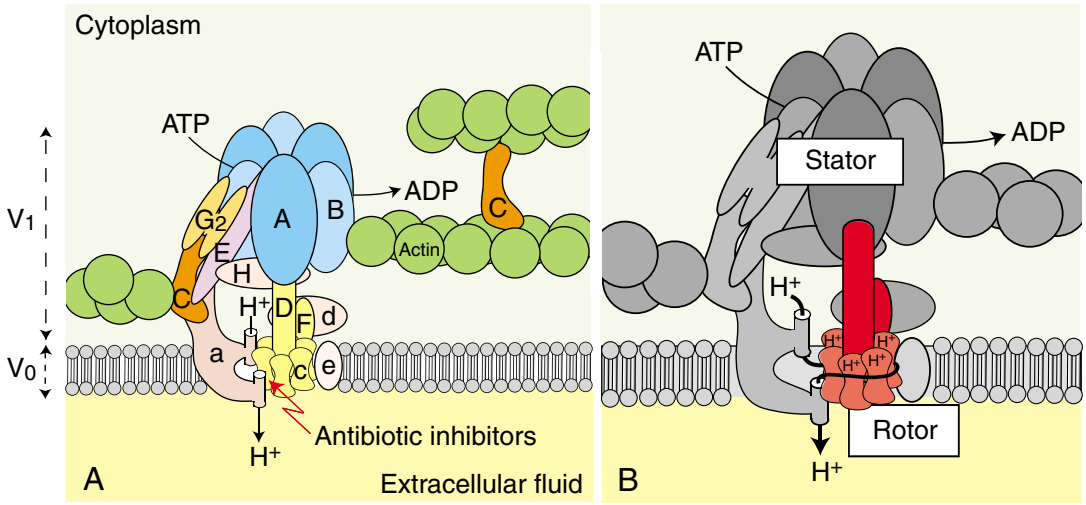

Fig. 2. Model of the V-type H^+ ATPase expressed in a eukaryotic cell membrane. (A) Molecular model. The peripheral V_1 complex consists of eight different subunits identified with capital letters A–H. Subunit G exists as the dimer G_2. The integral membrane V_0 complex consists of at least four different subunits identified with small letters (a,c,d,e). Subunit c and its isoforms c′ and c′′ form a H^+-binding rotor ring. Actin binds to subunits B (Holliday et al., 2000) and C (Vitavska et al., 2003). (B) Mechanistic model. V_0 and V_1 complexes are joined by a central rotating shaft (subunits D,F) and a peripheral stationary shaft (subunits C,E,G,H,a). The central shaft of the V_1 complex and the c-ring of the V_0 complex form the rotor (red). The remainder is considered the stator (grey). Hydrolysis of ATP brings about rotation of the central shaft together with the c-ring of the V_0 complex. Subunit a hypothetically provides two H^+ half channels that are offset. Rotation of the c-ring conveys H^+ from the inner half channel to the outer half channel *via* an intermediary H^+ binding step to one subunit c. The pleomacrolides bafilomycin and concanomycin, as well as the recently discovered macrolactone archazolid, are highly specific inhibitors that bind to the c subunits in the V_0 complex (Huss et al., 2002; Huss et al., 2005). Adapted from various sources (Inoue and Forgac, 2005; Murata et al., 2005; Wilkens et al., 2005).

the anaerobic bacterium *Propionigenium modestum* (Xing et al., 2004), and another model (Aksimentiev et al., 2004) proposes swiveling motions of individual helices of subunit c as well as the rotation of the entire c-ring.

As to the number of protons transported per ATP consumed, coupling ratios determined for V-type H^+ ATPases agree on $2H^+$/1ATP (Tomashek and Brusilow, 2000). The 2:1 functional stoichiometry is consistent with the structural stoichiometry of six binding sites for H^+ on the c-ring of the V_0 complex and three sites binding sites for ATP on the V_1 complex (Fig. 2). Moreover, F-ATPases can be observed to hydrolyze three ATP molecules with each revolution of the rotor (Yasuda et al., 1998). Nevertheless, coupling ratios must be neither integral nor constant (Junge and Nelson, 2005; Murata et al., 2005; Tomashek and Brusilow, 2000).

Clever experiments have visualized the rotation of the central stalk in the bacterial ATP synthase (Noji et al., 1997). In brief, the catalytic F_1 complex was immobilized upside down *via* a His-tag on a coverslip, and a fluorescent actin filament was attached to the central stalk *via* streptavidin. Adding ATP triggered the rotation of the fluorescent actin filament and the stalk. Furthermore, the reversibility of this motor was demonstrated by constructing a 'molecular sparkler' (Itoh et al., 2004). Here, a magnetic bead rather than fluorescent actin was attached to the central stalk. The bead was then rotated using an external magnet. The medium contained luciferin and luciferase such that one photon was emitted upon each capture and hydrolysis of ATP newly formed with each rotation. Rotation of the magnetic bead in one direction increased the number of chemiluminescent photons beyond those observed upon rotation in the opposite direction, proving vectorial ATP synthesis. The rotation of a eubacterial V_1 complex has now also been visualized with the aid of flurorescent actin filaments (Imamura et al., 2003), leaving little doubt that rotational catalysis is the mechanism of both F- and V-ATPases *in vivo*.

Energizer of endosomal membranes, plasma membranes and whole epithelia

The V-type H^+ ATPase was first considered an energizer of endosomal membranes before this proton pump was found in plasma membranes. As shown in Fig. 3A, the partnership formed by the V-type H^+ ATPase and an organic anion channel in vacuoles of plants is responsible for voltage-dependent malate transport into the vacuole (Hafke et al., 2003). In subapical membrane vesicles of the renal proximal tubule, the V-type H^+ ATPase colocalizes with ClC-5 that once was thought to be a voltage-gated Cl^- channel (Jentsch et al., 2002). Today ClC-5, ClC-4, and possibly other endosomal Cl^- transporters are considered electrogenic Cl^-/H^+ exchangers (Scheel et al., 2005). The collaboration between the V-type H^+ ATPase and the Cl^-/H^+ exchanger ClC-5 is thought to be fundamental to (1) the recycling of apical membrane proteins such as megalin, the Na^+/P_i cotransporter, the Na^+-dependent D-glucose cotransporter SGLT, and other transporters, and (2) the endocytotic reabsorption of filtered proteins in renal proximal tubules (Fig. 3B). Loss of function mutation in the Cl^-/H^+ exchanger ClC-5 produces the signs of Dent's disease and Fanconi-like syndrome, which include not only the renal loss of low molecular mass proteins, but also calcium, phosphate, glucose, salt and water (Jentsch et al., 2002).

A defective Cl^-/H^+ exchanger is expected to hyperpolarize the endocytotic membrane, bringing its voltage towards the electromotive force of the V-type H^+ ATPase with the effect of reducing both H^+ and Cl^- transport into the vesicle. Vesicular acidification would thereby be reduced, with possible negative effects on trafficking the vesicle in the endocytotic pathway.

In synaptic regions of neurons, the pas-de-deux of the V-type H^+ ATPase and the H^+/neurotransmitter antiporter of the VMAT-type (vesicular monoamine transporter, SLC18) (Eiden et al., 2004) is responsible for accumulating and storing neurotransmitters such as serotonin, dopamine, adrenaline,

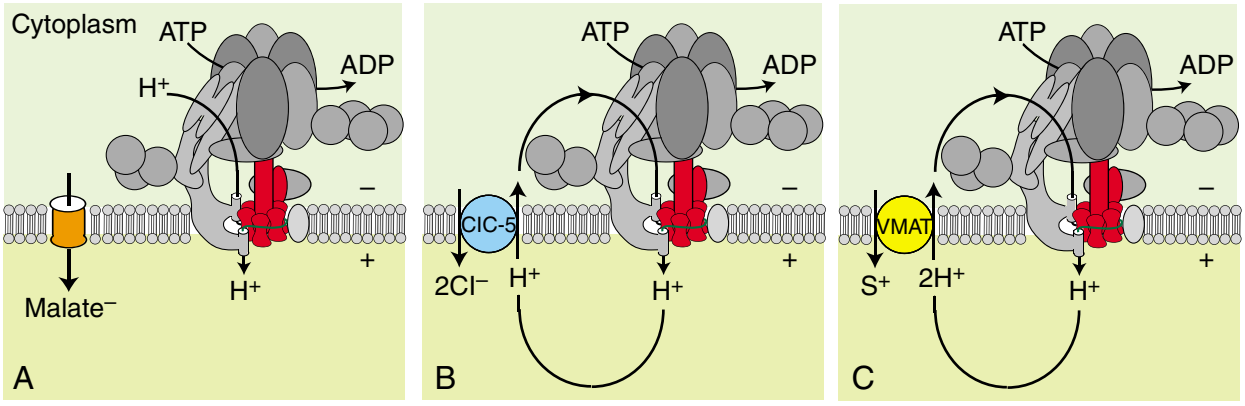

Fig. 3. Utility of the V-type H^+ ATPase expressed in endosomal membranes. Large transmembrane H^+ electrochemical potentials generated by the V-type H^+ ATPase drive the electrophoretic uptake of malate in vacuoles of plants (A), electrophoretic Cl^- transport across endocytotic membranes *via* the Cl^-/H^+ antiporter ClC-5 (B), and the uptake of the neurotransmitter serotonin (S^+) in synaptic vesicles *via* VMAT, a member of the SLC18 family of solute-linked-carriers (C). See www.bioparadigms.og/slc/menu.asp for the HUGO classification of solute-linked-carriers.

noradrenaline and histamine (Fig. 3C). Here the V-type H^+ ATPase generates (1) a vesicular ΔpH about 1.4 pH lower than cytoplasm pH, and (2) a membrane voltage of 40 mV (positive inside). Since VMAT exchanges 2 H^+ ions for each serotonin (Parsons, 2000), the ΔpH concentrates serotonin (S^+) 630-fold in the vesicle:

$$\ln([S^+]_i / [S^+]_o) = 2 \ln([H^+]_i / [H^+]_o) ,$$

and the membrane voltage concentrates serotonin 4.8-fold in the vesicle:

$$40 \text{ mV} = 26 \text{ mV} \ln([S^+]_i / [S^+]_o) ,$$

where 26 mV is the product R*T*/zF (R, gas constant; *T*, temperature; z, valence; F, Faraday constant). Together, chemical and electrical potentials yield a total 3000-fold concentration difference. If the serotonin concentration in the cytoplasm is 10 μmol l^{-1}, then the vesicular serotonin concentration can reach a maximal value of about 30 mmol l^{-1} as the $2H^+/S^+$ antiporter goes to electrochemical equilibrium. Due to intravesicular association, the serotonin concentration may reach values up to 100 mmol l^{-1} in the vesicle.

The observation of electrogenic H^+ secretion dependent on metabolism in the turtle urinary bladder gave the first hint of an ATP-dependent proton pump in a plasma membrane (Al Awqati, 1978). The characterization of this proton pump in membrane fractions of the turtle bladder (Gluck et al., 1982) and mammalian kidney (Gluck and Al Awqati, 1984) revealed striking functional similarities with proton pumps of vacuolar membranes. Striking structural similarities with the V-type H^+ ATPase of yeast were observed upon the isolation of the kidney proton pump (Gluck and Caldwell, 1987; Gluck and Caldwell, 1988). Antibodies prepared against the isolated kidney proton pump confirmed its location in the apical plasma membrane of epithelial cells (Brown et al., 1987).

Wieczorek et al. (1991) were the first to recognize that the V-type H^+ ATPase can energize secondary active transport across the plasma membrane. As shown in Fig. 4A, the V-type H^+ ATPase (and not the Na^+/K^+ ATPase) was found to power the active transport of K^+ *via* nH^+/K^+ antiport in a highly purified preparation of the apical membrane of the midgut of the tobacco hornworm *Manduca sexta*. In mammals, the association of the V-type H^+ ATPase with Cl^- channels in the ruffled membrane of osteoclasts (Fig. 4B) is known to secrete the strong acid HCl that serves the digestion and remodeling of bone (Chatterjee et al., 1992; Cleiren et al., 2001; Schlesinger et al., 1997). In renal proximal tubules, H^+ secreted into the tubule lumen by the apical membrane V-type H^+ ATPase is thought to account for 40% of HCO_3^- reabsorption *via* the formation of CO_2 (Wagner et al., 2004). In addition, the transmembrane H^+ electrochemical potential drives H^+-oligopeptide cotransport *via* PEPT1 and PEPT2 (Fig. 4C). In the renal medulla, the V-type H^+ ATPase contributes to urinary acidification when the proton pump is expressed in the apical membrane of α-intercalated cells, and it contributes to urinary alkalinization when the proton pump is expressed in the basolateral membrane of β-intercalated cells (Brown and Breton, 1996).

The laboratory of Beyenbach has extended the concept of energizing plasma membranes by the V-type H^+ ATPase to energizing whole epithelia (Beyenbach, 2001). Malpighian tubules of the yellow fever mosquito *Aedes aegypti* express the V-type H^+ ATPase at the apical membrane of principal cells (Fig. 5). The tubules have no measurable activity of the Na^+/K^+ ATPase. Instead, most ATPase activity stems from the V-type H^+ ATPase (Weng et al., 2003). The electromotive force of the proton pump (E_p, Fig. 5) serves largely to polarize the apical membrane to voltage that on average is 111 mV (negative inside). The small transmembrane proton concentration difference (ΔpH 0.16) supports H^+ transport from cell to tubule lumen, i.e. opposite to the gradient needed to drive outward Na^+ and K^+ (cat^+) transport *via* exchange transport with H^+ (Petzel et al., 1999). Since electroneutral

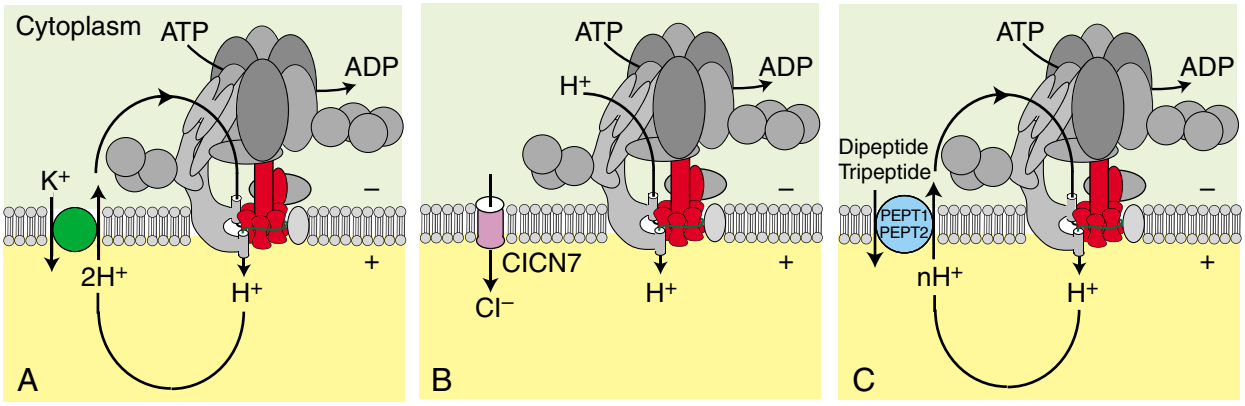

Fig. 4. Utility of the V-type H^+ ATPase expressed in plasma membranes. (A) Coupling of proton secretion to K^+ secretion *via* electrogenic $2H^+/K^+$ antiport in apical membranes of goblet cells in insect midgut (Azuma et al., 1995). (B) Secretion of strong acid across the ruffled border membrane (apical membrane) into the lacunar space of osteoclasts, serving the acid digestion of bone (Chatterjee et al., 1992; Cleiren et al., 2001). (C) Coupling of proton secretion to oligopeptide absorption in the apical brush border membrane of the renal proximal tubule (Lee and Kim, 2004; Wagner et al., 2004).

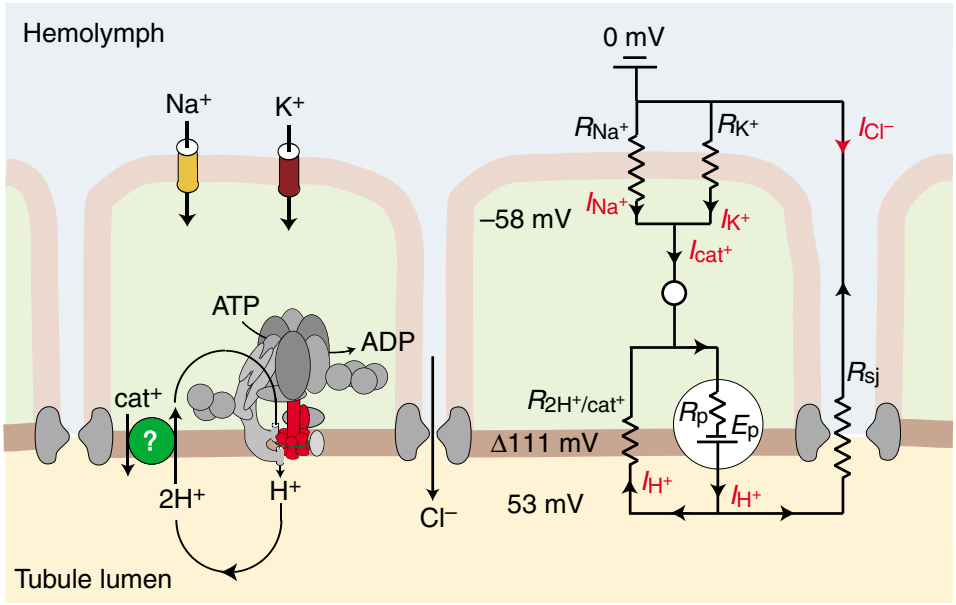

Fig. 5. The V-type H^+ ATPase powers transepithelial NaCl and KCl secretion in Malpighian tubules of the yellow fever mosquito. Only conductive transport pathways are shown to illustrate diverse voltage-dependent transport mechanisms driven by the V-type H^+ ATPase located at the apical membrane. Intraepithelial current generated by the proton pump is carried by H^+ across the apical membrane, by Cl^- through the paracellular pathway (septate junctions, sj), and by K^+ and Na^+ across the basolateral membrane. Under control conditions, K^+ channels account for as much as 64% of the conductance of the basolateral membrane (Beyenbach and Masia, 2002). Claudin-like proteins are hypothesized to define the Cl^- selectivity of the paracellular, septate junctional pathway (Beyenbach, 2003). The $2H^+/cat^+$ antiporter in the apical membrane remains to be identified. Mosquito natriuretic peptide and its second messenger cyclic AMP decrease the resistance to Na^+ entry across the basolateral membrane (Beyenbach, 2001). The diuretic peptide leucokinin decreases the resistance of the paracellular pathway for Cl^- (Beyenbach, 2003). E_p, electromotive force of the V-type H^+ ATPase; R, resistance; I, current; p, pump, cat^+, cation. E_p and R_p form a proton current generator.

antiport is insensitive to membrane voltage, an electrogenic antiporter that exchanges $2H^+$ for each Na^+ or K^+ would overcome the outward directed proton gradient and take advantage of the high apical membrane voltage generated by the V-type H^+ ATPase (Fig. 5). At the prevailing pH and voltage difference across the apical membrane, an antiport stoichiometry of $2H^+/cat^+$ would generate luminal K^+ and Na^+ concentrations approximately 40 times higher than their respective concentrations in the cell:

$$RT \ln([cat^+]_o / [cat^+]_i) + zFV = nRT \ln([H^+]_o / [H^+]_i) + nzFV ,$$

where n is the stoichiometry of H^+/cat^+ antiport, V is the apical membrane voltage (111 mV), o and i are outer and intracellular concentrations respectively, and z, F, R and T have their usual meaning.

The apical membrane voltage generated by the V-type H^+ ATPase also drives paracellular Cl^- transport and the entry of K^+ into the cell across the basolateral membrane. In brief, positive current carried by H^+ across the apical membrane must return to the cytoplasmic face of the pump. Positive pump current returning to the peritubular side of the epithelium is carried by Cl^- passing in the opposite direction through the paracellular pathway as the mechanism of paracellular Cl^- secretion (Pannabecker et al., 1993). Positive current carried by K^+ and Na^+ across the basolateral membrane completes the electrical circuit (Masia et al., 2000; Sawyer and Beyenbach, 1985). Similar patterns of coupling the electromotive force of the proton pump located at the apical membrane to transport across the basolateral membrane are likely to be found in other epithelia.

Regulation of the V-type H^+ ATPase

A 800 kDa protein as complex as the V-type H^+ ATPase is expected to respond to a diversity of factors, including regulatory inputs from physiological feedback loops. The process of molting has provided the first glimpse at physiological regulation of the V-type H^+ ATPase that links growth of the whole organism to transepithelial transport across the intestine. Growing tobacco hornworms *Manduca sexta* support their voracious appetite with a midgut that enigmatically secretes K^+ into the lumen akin to the mechanism of K^+ secretion in insect Malpighian tubules illustrated in Fig. 5. Large transepithelial, lumen-positive voltages across the midgut (>100 mV) attest to the high activity of the V-type H^+ ATPase at the apical membrane that provides the electrical driving force for K^+ secretion *via* $2H^+/K^+$ antiport. In the midgut of larvae that have ceased feeding during molt, transepithelial voltage goes to zero, and the hydrolysis of ATP as well as ATP-dependent proton transport drop to less than 15% of control, indicating the inactivation of the V-type H^+ ATPase. Parallel immunocytochemical studies demonstrated the loss of the V_1 complex from the apical plasma membrane that was subsequently found to reflect the physical separation of V_1 and V_0 complexes, as shown by gel electrophoresis and immunoblot (Sumner et al., 1995). Alone, the native V_1 complex does not hydrolyze ATP in the presence of cytosolic Mg^{2+} concentrations (Gräf et al., 1996), and free V_0 complexes normally do not allow the passage of protons (Beltran and Nelson, 1992; Zhang et al., 1992). The dissociation of the holoenzyme was subsequently confirmed in yeast, where

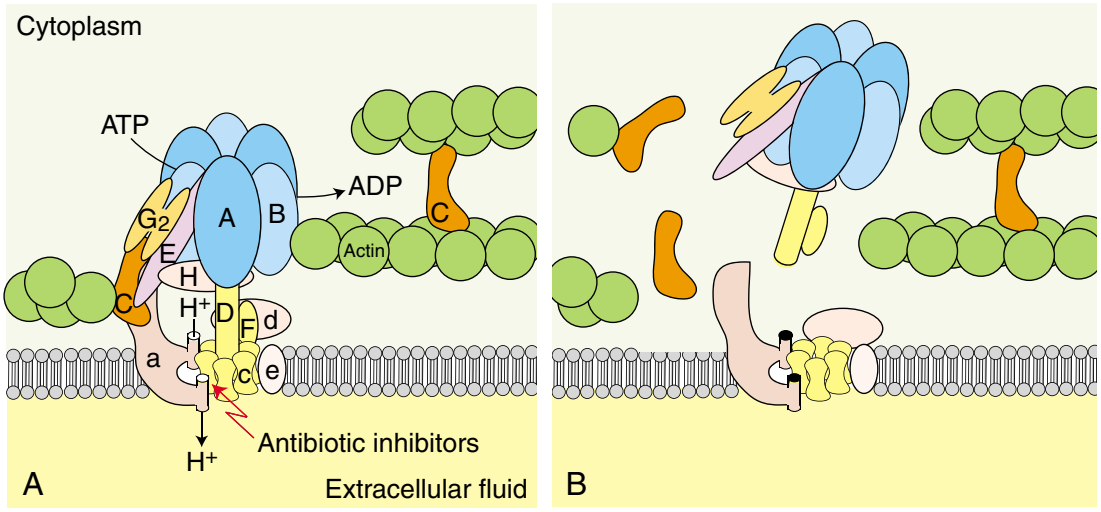

Fig. 6. Dissociation of the V-type H^+ ATPase into V_1 and V_0 complexes. (A) The intact holoenzyme hydrolyzes ATP, transports H^+, and generates voltage across the membrane; (B) intestinal inactivity during molt or starvation in caterpillars and glucose deprivation in yeasts causes the holoenzyme to dissociate. Concomitantly, ATP hydrolysis and proton transport collapse and membrane voltage goes to zero. (Adapted from Vitavska, 2005.)

glucose deprivation for as little as 5 min triggered the dissociation of 70% of holoenzyme (Kane, 1995). The addition of glucose reversed the process, bringing about the reassembly of V_0 and V_1 complexes. Holoenzyme association/dissociation is now thought a universal regulatory mechanism of V-ATPases (Fig. 6).

Today we are still far from knowing the molecular details of reversible V_1–V_0 dissociation. What we presently understand best has largely been gleaned in studies of yeast, but also in studies of mammalian kidneys and insect salivary glands. In yeast cells, the glucose-induced reassembly of V_1 and V_0 appears to be assisted by the heterotrimeric protein RAVE (Regulator of the H^+ ATPase of Vacuolar and Endosomal membranes) binding to the free V_1 complex (Smardon et al., 2002). Though glucose-induced, the RAVE-mediated reassembly is not glucose-dependent, because RAVE binds to V_1 whenever it is present in the cytosol. In contrast, glucose dramatically increases the interaction of the V-ATPase with the glycolytic enzyme aldolase (Lu et al., 2004). It appears that aldolase uses three different sites to bind respectively to subunits B and E of the V_1 complex and to subunit a of the V_0 complex, thereby facilitating the assembly of the holoenzyme. Thus, glucose stimulates the aldolase-mediated assembly of holoenzyme. In this role aldolase acquires the additional function as glucose sensor for regulating the activity of V-type H^+ ATPase activity (Lu et al., 2004).

Other mechanisms for triggering the assembly of holoenzyme may abound. For example, in renal epithelial cells, glucose activates phosphatidylinositol 3-kinase dependent signaling and the assembly of V_1 and V_0 complexes (Sautin et al., 2005). Still, the molecular details for enhancing the reassembly of holoenzyme by RAVE, aldolase and kinase(s) remain to be elucidated. Also intriguing are the molecular mechanisms that couple glucose withdrawal to V_1–V_0 dissociation, since apparently conventional signal transduction pathways are not activated by glucose depletion (Parra and Kane, 1998).

Though hormones can activate the assembly of holoenzyme, the molecular details are unknown. For example, it has been known for decades that transepithelial secretion of a KCl-rich primary saliva in the blowfly *Calliphora* is stimulated by serotonin (Berridge et al., 1976). Apparently, the serotonin-induced increase in cyclic AMP (cAMP) activates an electrogenic K^+ transport mechanism (Berridge et al., 1976) that today is thought to derive from the V-type H^+ ATPase working in parallel with a K^+/H^+ antiporter (Wieczorek et al., 1999). Immunofluorescent labeling of different V-ATPase subunits, as well as measurements of enzyme activity, have shown that serotonin recruits V_1 subunits from the cytosol, consistent with the assembly of the V_1V_0 holoenzyme (Zimmermann et al., 2003). In Malpighian tubules of insects, where intracellular second messengers of diuresis and antidiuresis have been identified, it remains unknown whether cAMP, cGMP, Ca^{2+} and NO affect the disassembly/reassembly of the V_1V_0 holoenzyme.

Subunit C of the V_1 complex is unique among V-ATPase subunits in that it is released from the V_1 complex upon its dissociation from the V_0 complex (Gräf et al., 1996; Kane, 1995; Merzendorfer et al., 2000; Vitavska et al., 2003). Recent studies suggest that subunit C may play a central role in holoenzyme disassembly/reassembly. Subunit C appears to bridge the V_1 and V_0 complexes, binding to subunits E and G of the V_1 complex and to subunit a of the V_0 complex (Inoue and Forgac, 2005). Subunit C is thus a good candidate for modulating the stability of the V_1V_0 holoenzyme. Indeed, the structural changes observed in subunit C in yeast and

Arabidopsis may depend on the ATP/ADP ratio (Armbrüster et al., 2005). Since this ratio can be influenced by the availability of glucose, subunit C might also serve indirectly as a glucose sensor, responding to changing concentrations of glucose with conformational changes, which in turn affect the stability of the V_1V_0 holoenzyme.

Subunit C of the caterpillar midgut of *Manduca sexta* binds with high affinity to actin filaments, either as an isolated protein, as subunit of the V_1V_0 holoenzyme, or reconstituted into the V_1 complex (Vitavska et al., 2003). Morevoer, subunit C, occurring in micromolar concentrations in the cytosol, cross-links actin filaments and even binds monomeric G-actin (Vitavska et al., 2005). F-actin crosslinking is likely to stabilize actin filament bundles in the apical microvilli of goblet cells of *Manduca sexta*. In addition, subunit C may play an important role in controlling the dynamics of the actin cytoskeleton because it binds F-actin and G-actin (Vitavska et al., 2005). Furthermore, F-actin binding to subunits B and C of the membrane-embedded V_1V_0 holoenzyme could serve to stabilize the stator (Fig. 2). In the intact cell, this hypothetical novel function of F-actin may strengthen the stator to withstand the torque generated by the rotor.

Subunit knockouts, gene mutations and some diseases

Because of their involvement in basic cellular mechanisms, V-ATPases are crucial components of virtually every eukaryotic cell. It is widely appreciated that an intact V-type H^+ ATPase is required for the normal function of the Golgi complex, endoplasmic reticulum, vacuoles and endocytotic and exocytotic vesicles. The indispensability of V-type ATPases was first shown in yeast which, with the exception of subunit a, possesses one gene only for each subunit. Disruption of single-copy genes encoding the V_1 subunit B or the V_0 subunit c resulted in the inability of yeast cells to survive at physiological pH (Nelson and Nelson, 1990). Since then, the same lethal effect has been demonstrated for every knock-out of a V-ATPase single-copy gene in yeast (Nelson, 2003). A lethal effect after knockout of the gene encoding subunit A was also observed for *Neurospora* (Ferea and Bowman, 1996). Later on it was shown that, as in yeast, lethality occurred only at physiological pH values slightly above 7; at acidic pH values (<pH 6) *Neurospora* was able to survive (Bowman et al., 2000).

Inactivation of single-copy genes encoding subunit A in *Neurospora*, subunit B in *Drosophila*, and subunit c in mice also resulted in lethal phenotypes (Davies et al., 1996; Ferea and Bowman, 1996; Inoue et al., 1999).

When more than one gene encodes a V-ATPase subunit, different subunit isoforms are usually found at different locations. In yeast, one of the two isoforms of subunit a is targeted to the vacuolar membrane, whereas the second isoform is targeted to the late Golgi apparatus (Kawasaki-Nishi et al., 2001). In multicellular higher eukaryotes, different isoforms often show cell-type or tissue-specific locations. For example, in mammals different isoforms of several subunits have been selectively identified in the kidney (Oka et al., 2001; Smith et al., 2002; Sun-Wada et al., 2003a; Sun-Wada et al., 2003b), inner ear (Dou et al., 2003), brain (Murata et al., 2002), osteoclasts (Manolson et al., 2003), alveolar cells (Sun-Wada et al., 2003a; Sun-Wada et al., 2003b) and the acrosome (Sun-Wada et al., 2002). Genetic defects in a tissue-specific isoform must not necessarily result in a lethal phenotype, but it may give rise to inherited disorders.

Mutations in the genes encoding the kidney-specific isoforms B1 and a4 are partly responsible for inheritable forms of distal renal tubular acidosis, characterized by elevated H^+ and Cl^- concentrations in the plasma due to the impaired renal excretion of acid (Karet et al., 1999; Smith et al., 2000). Gene mutations of subunits B1 and a4 in the cochlea can result in sensorineural deafness, evidently due to impaired contractile responses of hair cells (Karet et al., 1999; Stover et al., 2002). Mutations in the gene encoding subunit a3 lead to one type of infantile malignant autosomal recessive osteopetrosis, a disease where the bone progressively hardens due to reduced osteoclast activity (Frattini et al., 2000; Susani et al., 2004). In contrast to the tissue-specific isoforms B1 and a4, a3 is found in all mammalian tissues so far examined (Nishi and Forgac, 2000). In osteoclasts, a3 is part of the V-type H^+ ATPase inhabiting the ruffled border membrane (Fig. 4B) while a1 appears to be restricted to endomembranes, leading to the suggestion (Toyomura et al., 2000) that V-ATPases housing the a3 isoform in transport vesicles may interact with microtubules to be carried to the ruffled border membrane. Thus, a defect in the gene encoding a crucial site in subunit a3 may impair the targeting of the holoenzyme from the endosomal system to the ruffled border in osteoclasts.

Concluding thoughts

The V-type H^+ ATPase is widely distributed in prokaryotes and eukaryotes. Molecular studies have elucidated the structure and function of this proton pump in some detail. Interactions between various subunits of the pump and other cellular nanostructures are now emerging. What lags behind is our understanding of the regulation of the proton pump in intact cells. Questions remain about the physiological connections between growth of the whole organism and the regulation of pump activity at the molecular level. Also largely unexplored are the collaborations that the V-type H^+ ATPase enters with other membrane transport proteins – pumps, carriers and channels – and how these give rise to diverse functions that range from protein sorting to membrane trafficking, exocytosis, endocytotic digestion, the recycling of neurotransmitters, and the energizing of membrane and epithelial transport systems. Regulators beyond the usual second messengers that dictate pump activity *via* kinases, phosphatases or other enzymes are largely unknown. Molecular, genetic and proteomic studies will undoubtedly illuminate structural and functional properties in further detail. Equally important are ‘classical’ studies at the level of membranes, tissues and organisms. We probably would not

know about the reversible dissociation of the holoenzyme as a universal regulatory mechanism of this proton pump had not the molting caterpillar first shown us how it was done. Accordingly, the study of general physiology will continue to identify the relevant biological reactions from the gamut of chemical reactions proteins can execute, and the study of comparative physiology will continue to subscribe to the August Krogh principle, where nature is found to provide an ideal study system for any particular question in biology (Krebs, 1975).

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