The Journal of Experimental Biology 212, 1762-1772
Published by The Company of Biologists 2009
doi:10.1242/jeb.028803

Review

Regulation of the V-ATPase in kidney epithelial cells: dual role in acid–base homeostasis and vesicle trafficking

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Accepted 26 January 2009

Summary

The proton-pumping V-ATPase is a complex, multi-subunit enzyme that is highly expressed in the plasma membranes of some epithelial cells in the kidney, including collecting duct intercalated cells. It is also located on the limiting membranes of intracellular organelles in the degradative and secretory pathways of all cells. Different isoforms of some V-ATPase subunits are involved in the targeting of the proton pump to its various intracellular locations, where it functions in transporting protons out of the cell across the plasma membrane or acidifying intracellular compartments. The former process plays a critical role in proton secretion by the kidney and regulates systemic acid–base status whereas the latter process is central to intracellular vesicle trafficking, membrane recycling and the degradative pathway in cells. We will focus our discussion on two cell types in the kidney: (1) intercalated cells, in which proton secretion is controlled by shuttling V-ATPase complexes back and forth between the plasma membrane and highly-specialized intracellular vesicles, and (2) proximal tubule cells, in which the endocytotic pathway that retrieves proteins from the glomerular ultrafiltrate requires V-ATPase-dependent acidification of post-endocytotic vesicles. The regulation of both of these activities depends upon the ability of cells to monitor the pH and/or bicarbonate content of their extracellular environment and intracellular compartments. Recent information about these pH-sensing mechanisms, which include the role of the V-ATPase itself as a pH sensor and the soluble adenylyl cyclase as a bicarbonate sensor, will be addressed in this review.

Key words: H^+-ATPase, soluble adenylyl cyclase, intercalated cells, proton secretion, acidification, pH sensing.

Introduction

The V-ATPase is a ubiquitous enzyme that is best known for its role in acidifying various intracellular organelles in all eukaryotic cells (e.g. endosomes, lysosomes, trans-Golgi network), and its name stems from the fact that it was originally characterized by its role in acidifying the yeast vacuole (Beyenbach and Wieczorek, 2006; Forgac, 2007; Nelson and Harvey, 1999). However, the V-ATPase is also expressed at very high density in the plasma membranes of several specialized cells that are involved in extracellular pH regulation, *via* proton transport, in different organs including the kidney, the inner ear, the epididymis and bone (Brown and Breton, 1996; Forgac, 2007; Wagner et al., 2004) (see also Shum et al., 2009). Furthermore, analogous cell types, together known as ‘mitochondria-rich’ (MR) cells (Brown and Breton, 1996), are also present in lower organisms. These include flask cells in insect mid-gut (Russell et al., 1990; Wieczorek et al., 1999) and amphibian kidneys (Brown, 1978; Jonas, 1981), some types of ionocytes in fish gills and epidermis (Hwang and Lee, 2007), and carbonic-anhydrase-rich cells in the turtle and amphibian urinary bladders (Al-Awqati et al., 1976; Rosen, 1972; Schwartz et al., 1982; Stetson and Steinmetz, 1985) and amphibian epidermis (Brown and Breton, 1996; Brown, 1978; Rosen and Friedley, 1973; Brown and Ilic, 1978). Much of what we know about the function of V-ATPase-rich cells in proton secretion has been derived from studies on these ‘model’ organisms, especially the turtle and toad bladder (Steinmetz, 1986).

This review will address the function and regulation of V-ATPases and proton secretion in the kidney by intercalated cells (IC), which are present in the late distal tubule, the connecting segment and the collecting duct (Wagner et al., 2004). However, in addition to its role in the plasma membrane of IC, the V-ATPase is also expressed quite abundantly at the cell surface and on intracellular membranes of other cell types in the nephron (Brown et al., 1988b). In particular, the proximal tubule depends, in part, on the activity of apical V-ATPase to achieve bicarbonate reabsorption, a major role of this tubule segment (Gluck et al., 1996; Nakhoul and Hamm, 2002). Proximal tubule cells and IC have distinct and important roles to play in whole body acid–base regulation: defective IC function leads to systemic distal renal tubule acidosis (dRTA) whereas proximal tubule defects lead to proximal RTA (pRTA) (DuBose, 2004).

Importantly, however, the V-ATPase also has a vital function in the endocytotic/lysosomal degradative pathway in proximal tubule epithelial cells and, probably, in all cell types (Marshansky et al., 2002). Recent work from our group has identified a novel role for transmembrane V-ATPase subunits as endosomal pH sensors that are involved in the recruitment of cytosolic downstream trafficking proteins to acidified vesicles, ensuring their appropriate and correct progression along the degradative pathway from early endosomes to lysosomes (Hurtado-Lorenzo et al., 2006). Thus, the V-ATPase not only generates intravesicular acidification but also senses and responds to the acidic pH by mobilizing vesicle coat components,

which are critical for vesicle trafficking, from the cytosol to the endosomal membrane.

Structural organization of the V-ATPase

The V-ATPase is an extremely large and complex aggregation of subunits whose final size approaches 900 kDa (Beyenbach and Wieczorek, 2006; Forgac, 2007; Nelson and Harvey, 1999; Wagner et al., 2004). The enzyme is formed of two distinct domains: the V_o domain that contains transmembrane-spanning subunits, and the V_1 domain whose subunits have no transmembrane domain but are anchored to the membrane *via* interaction with components of the V_o domain. The precise arrangement of many of the subunits in relation to one another is not entirely clear but a schematic representation of the complete structure is illustrated in the inset to Fig. 1B. Variations of this diagram can be found in other reviews in this volume, in which more details concerning the structure of this complex enzyme are also described (Saroussi and Nelson, 2009; Wieczorek et al., 2009). In brief, the V_1 domain is composed of eight cytosolic subunits (named A–H) whereas the transmembrane V_o domain contains five subunits (named a, d, e, c and c″). Important functional roles have been attributed to almost all of the subunits, largely by genetic analysis and knockout mutations in yeast. The major bulk of the V_1 domain is composed of three copies of subunit A that alternate with three copies of subunit B to form the ATPase catalytic site. This hexameric structure can be easily seen in conventional electron micrographs in the form of dense, stud-like projections that emanate from the cytosolic side of the plasma membrane of proton-secreting MR cells (Fig. 1A) (Brown et al., 1987a). Rapid-freeze, deep-etch analysis clearly shows the remarkable arrays of these V_1 domains that characterize both the plasma membrane and vesicles in specialized proton-secreting epithelial cells (Fig. 1B) (Brown et al., 1987a). Early electron microscopy (EM) studies in insect epithelia were the first to describe these structures (Gupta and Berridge, 1966), which were later referred to as ‘portasomes’ (Harvey et al., 1983a; Harvey et al., 1983b). The V-ATPase bears a strong morphological resemblance to the previously described mitochondrial F_0F_1-ATP synthase, not surprisingly in view of the remarkable conservation of structure and subunit arrangement between these two enzymes that has occurred throughout evolution (Cross and Muller, 2004; Nelson and Nelson, 1989).

Of importance for this discussion is that the B-subunit in the V_1 domain as well as the a-subunit in the V_o domain exist as alternative isoforms that are differentially expressed not only in specific cell types but also in different membrane domains and organelles within the same cell (Hemken et al., 1992; Hurtado-Lorenzo et al., 2006; Kawasaki-Nishi et al., 2001; Pietrement et al., 2006; Sun-Wada et al., 2003; Sun-Wada et al., 2004; Toyomura et al., 2000). The potential roles of the various B- and a-isoforms will be described in more detail below, in relation to proton extrusion by IC and acidification of the endocytotic pathway in proximal tubule cells, respectively.

A- and B-IC phenotypes in the kidney

Depending on the cellular localization of the V-ATPase and anion transport proteins, IC have been grouped into different subtypes. So-called A-type intercalated cells (A-IC) are present in all collecting duct regions and are identified by the presence of apical V-ATPase and basolateral anion exchanger AE1 (Alper et al., 1989) (Figs 2–6). B-type intercalated cells (B-IC), however, are identified as a population of V-ATPase-rich IC in the cortical collecting duct that do not express basolateral AE1 (Alper et al., 1989) but that express the V-ATPase in various locations, including on the basolateral plasma membrane, and express the apical anion exchanger, pendrin (Brown et al., 1988a; Royaux et al., 2001) (Figs 2–6). B-IC but not A-IC also express the PDZ-binding protein NHERF1 (sodium/hydrogen exchanger regulatory factor), which interacts directly with the C-terminus of the B1 isoform of the V-ATPase B-subunit (Breton et al., 2000). The specific role of NHERF1 in B-IC remains unclear, however. In addition, while many B-IC have either a basolateral, diffuse cytoplasmic or even bipolar distribution of the V-ATPase, some B-IC have predominantly apical V-ATPase, giving rise to a population of IC that look like A-IC with respect to the V-ATPase localization but have no basolateral AE1 (Alper et al., 1989; Brown et al., 1988a). These cells, which can have apical pendrin, are similar to the so-called non-A and non-B cells that have been described by others (Wall and Pech, 2008). A-IC and B-IC were proposed to be

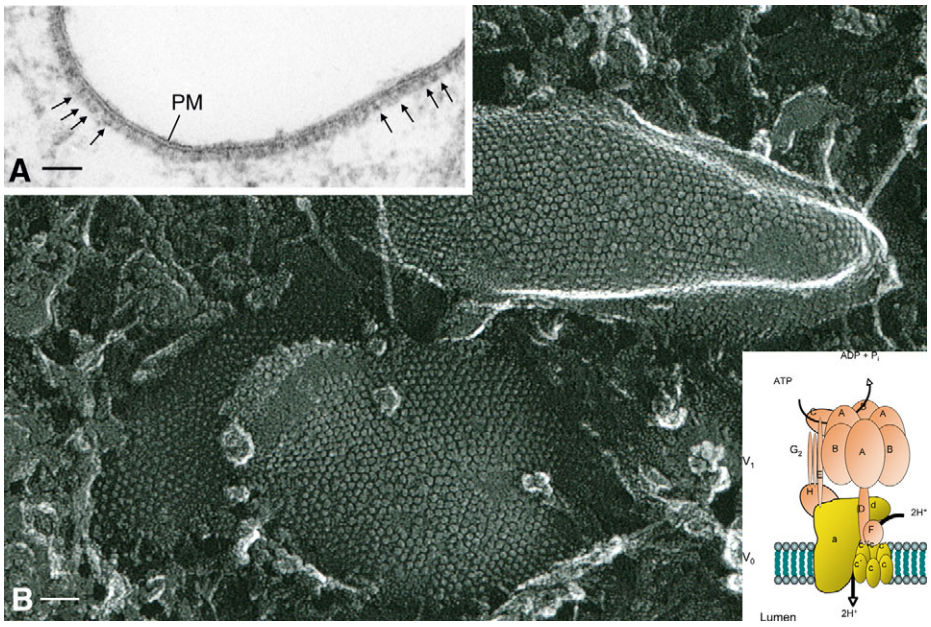

Fig. 1. Structure of the V-ATPase. The large cytoplasmic oriented V_1 sector of the V-ATPase holoenzyme can be visualized by conventional (A) and rapid-freeze, deep-etch (B) electron microscopy (EM). By conventional EM, the V-ATPase appears as dense projections (arrows) attached to the cytoplasmic side of the apical plasma membrane (PM) of this A-type intercalated cell (A-IC) from a rat kidney. Note that almost all of the membrane in this section is coated with the V-ATPase. Panel B shows the underside of a mitochondria-rich (MR)-cell from toad urinary bladder, coated with arrays of stud-like projections that correspond to V-ATPase V_1 sectors and to the projections seen by thin section EM in panel A. Each projection is about 10 nm in diameter. The bottom right inset shows a schematic representation of the subunits that comprise the transmembrane V_o sector and the cytoplasmic V_1 sector of the holoenzyme. See text for more details. Scale bar=50 nm.

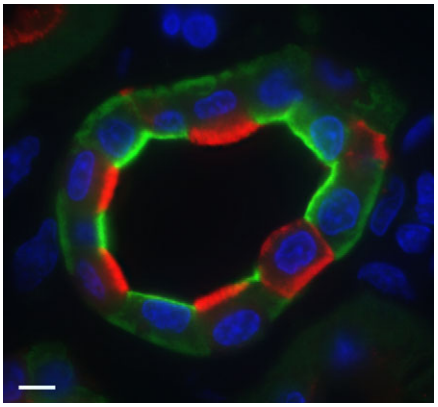

Fig. 2. Cryostat section of cortical collecting duct from a PLP (paraformaldehyde, lysine, periodate)-fixed rat kidney immunostained to reveal aquaporin 2 (AQP2) (green: anti-AQP2 C-terminus, raised in goat, followed by donkey anti-goat IgG coupled to Alexa 488) and the V-ATPase (red: anti-V-ATPase A-subunit C-terminus, raised in rabbit, followed by donkey anti-rabbit IgG coupled to CY3). Principal cells contain a tight apical band of AQP2 in this tissue and also show a weaker staining at their basolateral pole. Intercalated cells (IC) have either a strong apical staining only [A-type intercalated cells (A-IC)] or a basolateral/bipolar staining for the V-ATPase [B-type intercalated cells (B-IC)]. Nuclei are stained blue with DAPI. Scale bar=5 μm.

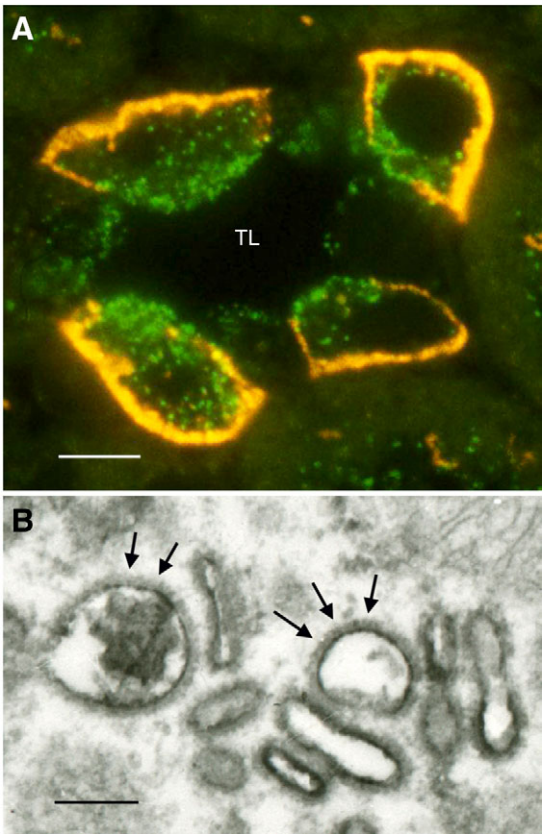

Fig. 3. A-type intercalated cells (A-IC) show a very high rate of apical endocytosis. The cells in panel (A) are from a rat that was injected with 25 mg of FITC-dextran in 1 ml PBS as an endocytotic marker. Cells in this cryostat section of a PLP (paraformaldehyde, lysine, periodate)-fixed medullary collecting duct are identified as A-IC by the basolateral immunostaining for the anion exchanger AE1 (orange color). Anti-AE1 antibodies raised in rabbit were a gift from Dr Seth Alper, Beth Israel Deaconess Medical Center, Boston, MA, USA (Alper et al., 1989). All of the A-IC contain many vesicles labeled with FITC-dextran – they are most abundant at the apical pole but some labeled endosomes are also found in the basolateral region of the cells. Panel (B) is an electron micrograph showing endocytotic uptake of luminal horseradish peroxidase (HRP) into a similar population of intercalated cell vesicles (but seen here at higher magnification than in the fluorescence image in panel A) that are coated with stud-like projections characteristic of the V-ATPase (arrows). The vesicles contain variable amounts of an amorphous, electron-dense diaminobenzidine reaction product that indicates the presence of internalized HRP in these vesicles. The rat from which these images were derived was injected *via* the jugular vein with HRP (6 mg 100g^{-1} body mass) and fixed by vascular perfusion with 3% paraformaldehyde/1% glutaraldehyde 15 min later (Brown et al., 1987b). TL=tubule lumen. Scale bar=5 μm (A) and 0.2 μm (B).

functional mirror images with A-IC responsible for acid secretion into the tubule lumen and B-IC being involved in bicarbonate secretion into the lumen (Al-Awqati, 1996). The various subcellular locations of the V-ATPase in AE1 negative, pendrin positive B-IC may represent cells in various states of functional transition. Whether A-IC and B-IC are variants of the same cell type but that change polarity and expression of some membrane proteins or whether they represent two distinct cell types has long been discussed. The fact that the apical and basolateral anion exchange proteins are not the same (pendrin *versus* AE1, respectively) clearly indicates that A-IC and B-IC are not simple mirror images of each other that are produced by transcytosis of V-ATPase and an anion exchanger into opposite poles of the cell. Changes in protein expression are also necessary.

Response of IC to acidosis and alkalosis

Many studies have shown that IC respond to systemic acidosis or alkalosis by reorganizing the polarized distribution of the V-ATPase in their plasma membranes. Functional studies were supported by conventional EM observations to follow the relocation of membrane studs (previously referred to as 'portasomes' – see above and Fig. 1) from intracellular vesicles to the plasma membrane and *vice versa* (Madsen and Tisher, 1986; Schwartz and Al-Awqati, 1986). Subsequently, these structures were identified as components of the V-ATPase (Brown et al., 1987a), and specific antibodies were then used to follow phenotypic changes in IC under different acid–base conditions (Bastani et al., 1994; Bastani et al., 1991; Sabolic et al., 1997). Furthermore, work on isolated perfused tubules exposed to acid–base changes in the basolateral bathing medium yielded similar results (Satlin and Schwartz, 1989). Together, data from the kidney and other model epithelia, such as the turtle and toad urinary bladders (Stetson et al., 1985; Stetson and Steinmetz, 1985) and more recently the epididymis (Breton and Brown, 2007; Breton et al., 1996), showed that regulation of proton secretion occurs in large part *via* the regulated trafficking of V-ATPase molecules between an intracellular compartment and the cell surface.

Acidosis results in V-ATPase accumulation in the apical plasma membrane of IC. By contrast, alkalosis causes apical pumps to be endocytosed into sub-apical vesicles of A-IC (Bastani and Haragsim, 1996; Sabolic et al., 1997). This rearrangement would have the desired effect of increasing apical acid secretion and basolateral bicarbonate extrusion during systemic acidosis and inhibiting this process during systemic alkalosis. Acidosis was reported to result in the removal by endocytosis of the apical Cl^-/HCO_3^- transporter in B-IC, prior to its molecular identification

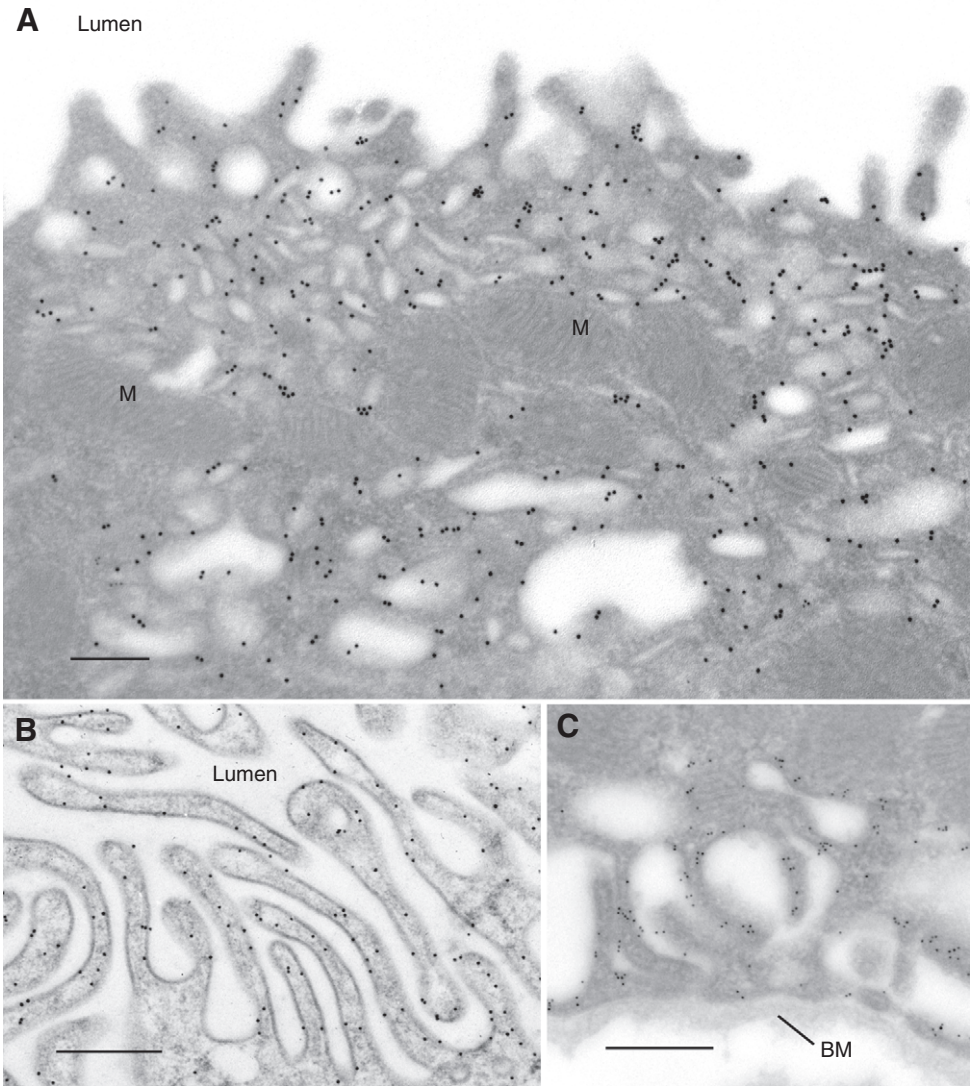

Fig. 4. Immunogold electron microscopy (EM) showing extensive labeling for the A-subunit (70 kDa) of the V-ATPase in intercalated cells (IC). Antibodies against the C-terminus of the A-subunit were raised in rabbit and affinity purified prior to use. Rat kidney was fixed by vascular perfusion using PLP (paraformaldehyde, lysine, periodate) and embedded at low temperature in Lowicryl HM20 resin prior to cutting ultrathin sections for immunolabeling using the immunogold procedure. (A) The apical cytoplasm of an 'unstimulated' A-type intercalated cell (A-IC) from an outer medullary collecting duct with many apical vesicles whose membranes are extensively labeled with anti-V-ATPase antibodies/IgG-gold particles. Note that mitochondria (M) are unlabeled. (B) Heavily labeled apical microvilli characteristic of a 'stimulated' A-IC. (C) The basolateral region of a B-type intercalated cells (B-IC) from the cortical collecting duct illustrating the extensive V-ATPase labeling of the basolateral plasma membrane that can occur in B-IC (but not in A-IC). Scale bar=0.25 μm. BM, basement membrane.

as pendrin (Satlin and Schwartz, 1989). Interestingly, alkalosis induces the endocytotic retrieval of V-ATPase from the apical membrane of all IC but specifically stimulates the accumulation of V-ATPase in the basolateral membrane only of B-IC. Thus, during alkalosis, not only is apical proton secretion by A-IC and B-IC reduced but apical bicarbonate secretion (*via* pendrin) and basolateral proton extrusion (*via* the V-ATPase) are increased in an attempt to correct the alkalemia.

These phenotypic changes can be detected over relatively short time frames (less than 6 h) in the kidney (Sabolic et al., 1997) and in perfused tubules (Satlin and Schwartz, 1989). While the molecular mechanisms underlying V-ATPase redistribution remain poorly understood, exo- and endocytosis of acid–base transporters is certainly involved. A-IC in particular have a very high rate of apical endocytosis that can be detected by introducing various probes, including FITC-dextran or horseradish peroxidase into the collecting duct lumen (Fig. 3). The vesicles involved in this trafficking process have been identified as V-ATPase-coated vesicles using immunogold EM (Fig. 4). Immunogold staining also reveals the V-ATPase associated with apical plasma membranes in stimulated A-IC (Fig. 4B) and basolateral membranes in B-IC (Fig. 4C).

In the past few years, the Al-Awqati laboratory has addressed the question of A-IC and B-IC interconversion, and has provided evidence that this process involves a cell differentiation event that is mediated by a protein called hensin (Al-Awqati et al., 1998). This protein is secreted into the extracellular environment under certain conditions (e.g. acidosis), where it polymerizes and binds to cell surface integrins that in turn induce terminal differentiation signals resulting in transformation of B-IC into A-IC (Al-Awqati, 2003; Schwartz and Al-Awqati, 2005). This can occur within a few hours and requires protein synthesis. Antibodies against hensin, when applied to isolated perfused tubules *in vitro*, can prevent this conversion and inhibit the acidosis-induced increase in apical proton secretion by IC (Schwartz et al., 2002). Thus, in this model, B-IC are seen as the 'precursors' of A-IC and the transition from one to another can be induced by acidosis. How rapidly, or indeed whether, this terminal differentiation process can be reversed, to allow the kidney to respond to alkalosis, for example, is not yet clear.

Role of B-subunit isoforms in IC function

As mentioned above, several V-ATPase subunits have more than one isoform or splice variant. Of particular importance for IC, and other specialized proton-secreting cells, is the high level of expression of the B1-isoform of the 56 kDa subunit in these cells (Nelson et al., 1992; Puopolo et al., 1992). The other B-subunit

isoform, known as B2, is more ubiquitously expressed and is generally associated with intracellular compartments, such as endosomes and lysosomes. It was, therefore, originally believed that the B1-isoform was uniquely associated with V-ATPase complexes at the plasma membrane whereas the B2-isoform complexed with V-ATPase on intracellular organelle membranes. The importance of the B1-isoform in IC function was highlighted by the discovery of human subjects in whom the B1 protein is mutated due to a genetic defect in the *Atp6v1b1* gene (Karet et al., 1999; Stover et al., 2002). These patients all have dRTA and many of them also suffer from sensorineural deafness. The hearing phenotype is presumably a result of defective function of the inner ear, in which several cell types, but notably cells called interdental cells, express high levels of the B1-subunit (Stankovic et al., 1997). These cells are involved in maintaining the high potassium levels in the endolymph, which is essential for the function of cochleal hair cells. The B1-subunit isoform is also highly expressed in epididymal clear cells (Breton and Brown, 2007) (see also Shum et al., 2009) but the reproductive status of the B1-deficient human subjects has not yet been assessed due to their young age at the time of diagnosis.

Based on these data from human patients, a mouse knockout of the B1-subunit was made and examined. Unexpectedly, these mice had only a mild baseline acidification defect, with a slightly alkaline urine but normal blood pH, so there was no detectable acidemia in these animals (Finberg et al., 2005). However, when subjected to an acid load, the B1-knockout mice were affected much more than the wild-type mice and could not efficiently excrete the excess acid load, implying a defect in urinary acidification. Upon further examination, it was discovered that the A-IC in the transgenic mice expressed abundant apical plasma membrane V-ATPase but that the enzyme complex contained the B2-subunit in place of the deleted B1-subunit (Paunescu et al., 2007). Thus, under some conditions, the normally intracellular B2-isoform can incorporate into pumps that are subsequently inserted into the plasma membrane (Fig. 5A,B). This so-called 'isoform replacement' appears to be sufficient to allow the A-IC to function relatively normally under baseline conditions, so no acidosis ensues. However, upon acid loading, the alternative B2-containing pumps seem to be less effective in secreting an acid load. This could be due to reduced numbers of pumps at the plasma membrane or could indicate that the B2-containing complex is a less effective proton pump, at least when present at the cell surface. Furthermore, plasma membrane expression of B2 was also detected in epididymal clear cells in these mice (Da Silva et al., 2007) and, more recently, increased apical staining was described in their olfactory mucosa (Paunescu et al., 2008b). It should be noted, however, that there is a precedent for a role of B2 in proton pumping at the cell surface, because this subunit is the major B-isoform found in plasma membrane V-ATPases in osteoclasts (Lee et al., 1996; van Hille et al., 1994) and proximal tubule epithelial cells (Brown et al., 1988b; Nelson et al., 1992). Interestingly, angiotensin II failed to stimulate V-ATPase activity as monitored by proton extrusion in IC from B1-deficient mice, implying that the B1-isoform is normally involved in the functional response to this hormone (Pech et al., 2008; Rothenberger et al., 2007). By contrast, as mentioned above, patients with B1 mutations do develop dRTA and, frequently also have sensorineural deafness. The reason for this difference between mice and men is not known. It could simply be due to the more acidic diet ingested by human subjects compared with mice or it could have a molecular explanation. In the mice, the B1 protein is absent and this might allow B2 to take its place in the holoenzyme. In the B1-deficient humans, most of the mutations would allow at least some B1 protein to be made, which might compete with B2 for incorporation into the holoenzyme but this remains to be determined. It has, however, been shown in cultured cells that mutated B1 protein can severely interfere with the trafficking and function of the V-ATPase holoenzyme (Yang et al., 2006).

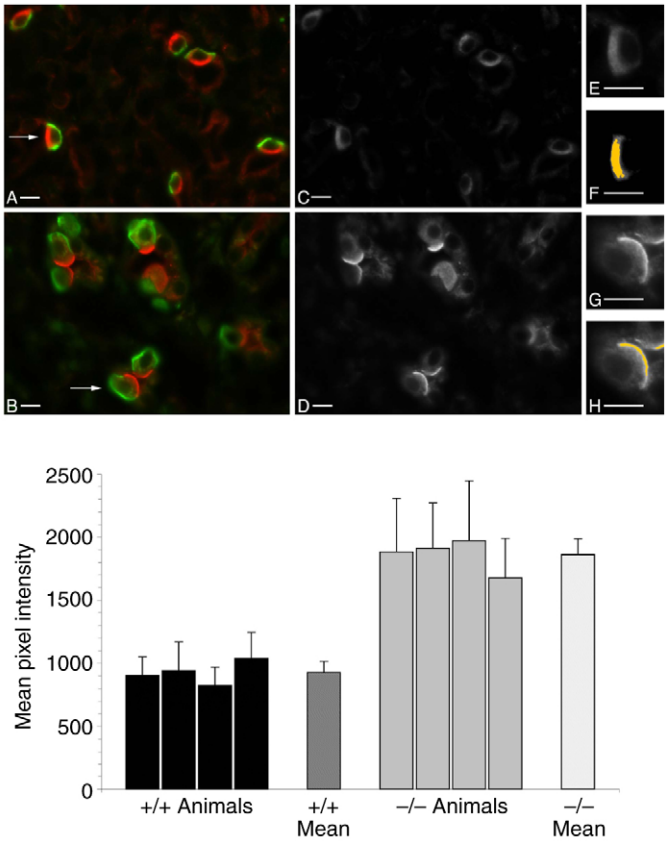

Fig. 5. This figure shows the localization of the B2-isoform (red) of the V-ATPase in intercalated cells (IC) from PLP (paraformaldehyde, lysine, periodate)-fixed mouse kidney inner medulla, identified by basolateral staining for AE1 (green). Anti-B2-isoform specific antibodies were raised in chicken against the C-terminal 10 amino acids of the B2-isoform, which differs completely from the B1-isoform sequence in this region (Paunescu et al., 2004). In control animals, this isoform is diffusely located in the apical pole of the cells (A) whereas in mice that lack the B1-isoform, the B2 is now found in a tight apical band at the apical plasma membrane (B). Panels C and D show V-ATPase staining alone in a raw image that was used for quantification of apical fluorescence. The raw image was subjected to a thresholding step that highlighted the V-ATPase apical staining (see insets E–H). The mean pixel intensity measured in these apical regions is shown in histogram form below. The four B1-null mice (–/– animals) examined here all have a significantly greater apical B2 staining in their A-type intercalated cells (A-IC) than the four wild-type mice (+/+ animals). Mean values from the four mice in each group are shown in a separate column. Values are given as means ± s.d., and wild-type and B1-deficient mice values were significantly different (P<0.001, ANOVA and Student's t-test). Thus, under some circumstances, the normally intracellular B2-isoform can associate with plasma membrane V-ATPase holoenzymes and become concentrated at the apical pole of IC.

Recycling of the V-ATPase in IC

Regulation of proton secretion by vesicle recycling was originally suggested using turtle bladder MR cells as model acid secreting

cells (Dixon et al., 1986; Gluck et al., 1982; Stetson and Steinmetz, 1983). In this tissue, cells that are homologous to A-IC and B-IC, i.e. with opposite polarities of proton secretion and bicarbonate transporters, were also described based on functional and morphological EM studies (Stetson et al., 1985). Subsequent work showed the existence of proton-translocating cells of opposite functional polarity in the kidney (Brown et al., 1988a; Schwartz et al., 1985) and, as described above, numerous studies have shown that exo- and endocytosis of specialized V-ATPase-containing vesicles is a central mechanism for regulating proton secretion by IC (Al-Awqati, 1996; Brown and Sabolic, 1993; Schwartz and Al-Awqati, 1985) (Fig. 3). A similar recycling process also occurs in epididymal proton-secreting clear cells (Breton and Brown, 2007; Shum et al., 2009). This process involves calcium, SNARE proteins and the regulatory protein Munc 18b, microtubules and actin (Brown et al., 1992; Holliday et al., 2000; Nicoletta et al., 2004; Schwartz et al., 2007; van Adelsberg and Al-Awqati, 1986; Wagner et al., 2004). A direct interaction of the V-ATPase subunits B1, B2 and C with actin has been demonstrated (Chen et al., 2004; Holliday et al., 2000; Vitavska et al., 2005; Vitavska et al., 2003) but the role played by actin in V-ATPase recycling remains unclear. Other studies have shown that the V-ATPase can also interact indirectly with actin *via* the PDZ protein NHERF1 (Breton et al., 2000). The B1- but not the B2-isoform of the V-ATPase has a C-terminal DTAL PDZ-binding motif that binds to NHERF1 and allows interaction with actin *via* MERM (merlin, ezrin, radixin, moesin) proteins. Interestingly, in the proximal tubule of the kidney, proton secretion is achieved by apical V-ATPase complexes that contain the B2-isoform, which has a different C-terminal sequence that does not associate with NHERF1 (Breton et al., 2000). As in this tubule segment the apical membrane contains abundant NHERF1 in a complex with other apical transporters and receptors (Donowitz et al., 2005; Shenolikar and Weinman, 2001), it is possible that the presence of B2 in the V-ATPase complexes has evolved to allow the V-ATPase to recycle rapidly and prevent it from being tightly anchored at the apical membrane *via* association with NHERF1. As mentioned earlier, however, the NHERF1 protein is detectable only in B-IC, implying a specific function in this IC subtype that remains to be determined (Breton et al., 2000).

Sensing of extracellular acid–base status by IC: role of the soluble adenylyl cyclase (sAC) and cAMP signaling

A major unresolved issue in renal physiology is how extracellular acid–base status is sensed by renal epithelial cells to initiate their homeostatic response to these stimuli. Among the factors that have been suggested are, not surprisingly, pH, CO_2 and bicarbonate as well as a number of potential hormonal stimuli (Wagner et al., 2004). Indeed early studies by the Schwartz and Al-Awqati team showed that basolateral CO_2 elevation, together with an initial increase in calcium, stimulates proton secretion by proximal tubules and collecting duct IC (Schwartz and Al-Awqati, 1985; Schwartz and Al-Awqati, 1986). This occurs, at least in part, by inducing the apical insertion of V-ATPase in these cell types. Some candidate proteins including the Pyk2 tyrosine kinase in the proximal tubule have been implicated in pH sensing (Li et al., 2004), and a family of G-protein coupled receptors can generate cAMP or IP3/calcium signals in response to acidic pH in some cell types, including osteoblasts (Ludwig et al., 2003). However, the sensing and signaling mechanism in renal cells remains poorly understood. The recent work on proton-secreting cells in the epididymis described in an accompanying review by Shum et al. (Shum et al., 2009) revealed that apical V-ATPase accumulation is stimulated by luminal HCO_3^- *via* a soluble adenylyl cyclase (sAC)-mediated increase in intracellular cAMP (Pastor-Soler et al., 2003). The cyclase activity of sAC is stimulated directly by HCO_3^- ions, and calcium can further modify its activity (Chen et al., 2000; Litvin et al., 2003). This makes sAC quite distinct from transmembrane adenylyl cyclases (ACs) that are often associated with G-protein coupled receptors, and that are activated by forskolin (sAC is insensitive to this compound). sAC could also act as a CO_2 sensor, because an increase in CO_2 levels would produce intracellular HCO_3^- *via* the action of cytosolic carbonic anhydrases that are highly enriched in IC and epididymal clear cells (Brown and Breton, 1996).

We have recently shown that sAC is highly expressed in IC and, moreover, that its localization partially overlaps with that of the V-ATPase (Paunescu et al., 2008a). Thus, in A-IC, sAC is mainly apical whereas in B-IC, sAC is bipolar (Figs 6 and 7). In the apical pole of B-IC, sAC staining overlaps with that of the anion exchanger pendrin (Fig. 7). Furthermore, sAC immunoprecipitates in a complex with the V-ATPase (Paunescu et al., 2008a). These findings are consistent with the presence of HCO_3^--stimulated adenylyl cyclase activity previously described in rat kidney (Mittag et al., 1993). sAC-regulated cAMP signaling may, therefore, constitute a general sensing mechanism for regulating V-ATPase-mediated proton transport. Interestingly, the electroneutral Na^+/HCO_3^- transporter NBC3 has been reported to associate with the V-ATPase in IC (Pushkin et al., 2003), suggesting the existence of a HCO_3^--regulated signaling complex involving the V-ATPase. Thus, the presence of sAC could allow IC to respond to an elevation of apical (luminal) HCO_3^-, such as would occur during a pRTA with a defect in HCO_3^- reabsorption leading to increased delivery to the apical surface of IC.

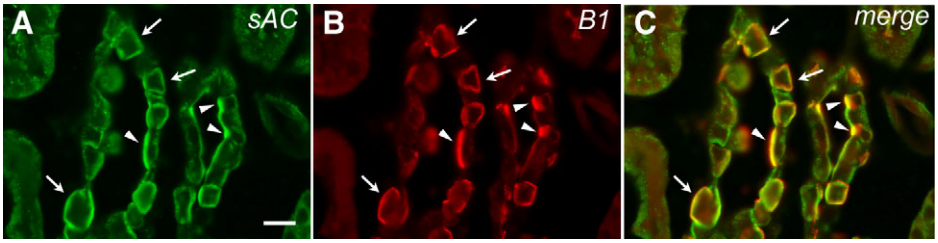

Fig. 6. The soluble adenylyl cyclase (sAC) (green) detected in a cryostat section of rat kidney cortex, using a monoclonal antibody raised against the N-terminal catalytic regions of the enzyme (Paunescu et al., 2008a), is highly expressed in both A-type intercalated cells (A-IC) and B-type intercalated cells (B-IC), where its intracellular localization closely resembles that of the V-ATPase (red: detected using an antibody against the C-terminus of the 56 kD B1 V-ATPase-subunit) (Paunescu et al., 2008a). In A-IC, identified by apical V-ATPase staining (A – arrowheads), sAC is co-localized apically with the V-ATPase (B and C – arrowheads) and in B-IC, identified by bipolar V-ATPase staining (A – arrows), sAC also has a bipolar distribution (B and C – arrows). Adapted from Paunescu et al. (Paunescu et al., 2008). Scale bar=5 μm.

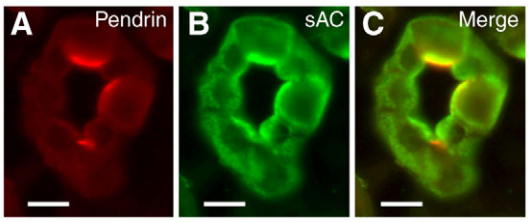

Fig. 7. Double staining of a rat cortical collecting duct for pendrin (A) and soluble adenylyl cyclase (sAC) (B) shows apical colocalization in pendrin positive B-type intercalated cells (B-IC) (C). However, some sAC staining in B-IC is also present in the basolateral pole, and sAC is also present in pendrin negative cells but at lower levels. Anti-pendrin antibodies raised in rabbit were kindly provided by Dr Ines Rouaux (NIH) (Paunescu et al., 2008a). Scale bar=5 μm.

This would trigger an increase in HCO_3^- uptake by IC and stimulation of sAC, leading to increased apical accumulation of V-ATPase in an appropriate response to the metabolic acidosis that would result from renal bicarbonate losses. Alternatively, increased basolateral CO_2 that occurs in acidosis might also trigger increased apical proton secretion by IC upon its conversion to HCO_3^- by the action of intracellular CAII, although it is not yet clear how apical *versus* basolateral exocytosis/endocytosis of V-ATPase would be differentially regulated in A-IC and B-IC under these conditions. Perhaps the basolateral plasma membrane of A-IC is more permeable to CO_2 than that of B-IC.

The downstream signaling cascade activated by cAMP could include protein kinase A (PKA) and/or the exchange proteins Epac 1 and 2. Epacs are guanine nucleotide-exchange factors (GEFs) for the small GTPases Rap1 and Rap2 (de Rooij et al., 1998; Kawasaki et al., 1998). However, we showed recently that Epac activation is probably not involved in the recycling of V-ATPase in epididymal clear cells (Pastor-Soler et al., 2008). By analogy with other systems involving the cAMP/PKA cascade, protein phosphorylation is likely to play a role in V-ATPase trafficking. It is not yet known if direct phosphorylation of any V-ATPase subunits occurs during V-ATPase recycling. The brain isoform of the V-ATPase B-subunit (presumably the B2-isoform) was reported to be phosphorylated *in vitro* by AP50, part of the AP-2 clathrin assembly protein AP-2 (Myers and Forgac, 1993). Some V-ATPase subunits (A, B1, B2 and C) do contain putative phosphorylation sites (Sun-Wada et al., 2004) but phosphorylation of V-ATPase subunits in mammals has not yet been demonstrated *in vivo*. However, V-ATPase-subunit phosphorylation has been described in insects (Voss et al., 2007). It is also possible that, V-ATPase recycling *via* PKA could be indirectly regulated by assembly and disassembly of the actin cytoskeleton. The calcium-activated, actin-remodeling protein, gelsolin, is highly expressed in kidney IC and epididymal clear cells and plays a key role in the regulation of V-ATPase recycling in clear cells (Beaulieu et al., 2005).

V-ATPase as a pH sensor in the endosome/lysosomal degradative pathway

The importance of V-ATPase-mediated vesicle acidification in intracellular organelle trafficking and function has been known for many years (Mellman, 1992). Generating an acidic pH that is necessary for receptor/ligand dissociation or for lysosomal enzyme activity is a well-established role of the V-ATPase. How vesicle acidification regulates other key intracellular trafficking events including the budding, coating and possibly fusion of intracellular carrier vesicles is less well understood. While degradative pathways exist in most cell types, kidney proximal tubule cells have a particularly extensive apical endocytotic apparatus that is involved in the reabsorption and degradation of filtered proteins, and is also part of the extensive recycling of many critical apical plasma membrane proteins (Christensen, 1982; Christensen and Birn, 2002; Marshansky et al., 2002). Disruption of the V-ATPase-mediated vesicle acidification process in proximal tubule cells using a variety of inhibitors, including bafilomycin, FCCP and NH_4Cl, as well as Na^+/H^+ exchanger 3 (NHE3) inhibition (Gekle et al., 1999), leads to diminished reabsorption of albumin (Marshansky et al., 2002) and other proteins, such as IgG light chains (Batuman and Guan, 1997) (Fig. 8). Thus, proximal tubule cells are ideally suited for the dissection of the molecular mechanisms by which vesicle acidification regulates these trafficking pathways.

Acidification-dependent recruitment of cytosolic proteins to vesicles

One clue as to how vesicle acidification might influence vesicle trafficking came from studies that reported a pH-dependent interaction of Arf small GTPases with purified pancreatic microsomal vesicles (Zeuzem et al., 1992a; Zeuzem et al., 1992b). These small GTPases are known to have critical roles in the regulation of many vesicular transport processes in eukaryotic cells (Donaldson, 2005). Further studies extended these findings to show

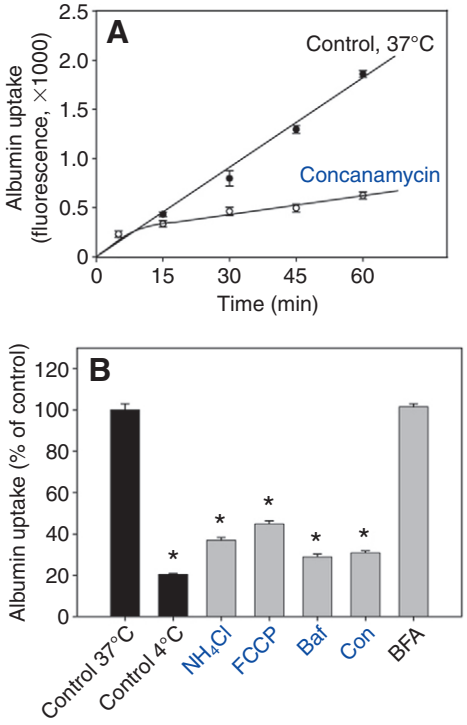

Fig. 8. Panel (A) shows that proximal tubule (PT) cells in culture internalize FITC-albumin in a time-dependent manner, and that the rate of internalization is significantly reduced upon inhibition of the V-ATPase by concanamycin (1 μmol l^{-1}). Panel (B) shows that in addition to V-ATPase inhibition [in this case using 1 μmol l^{-1} bafilomycin (Baf) or concanamycin (CON)], non-specific disruption of the endosomal pH gradient using FCCP (10 μmol l^{-1}) and NH_4Cl (20 mmol l^{-1}) also inhibit albumin uptake by PT cells. By contrast, brefeldin A (BFA – 50 μmol l^{-1}) has no effect on albumin endocytosis. Figure modified from Hurtado-Lorenzo et al. (Hurtado-Lorenzo et al., 2006). *P<0.05.

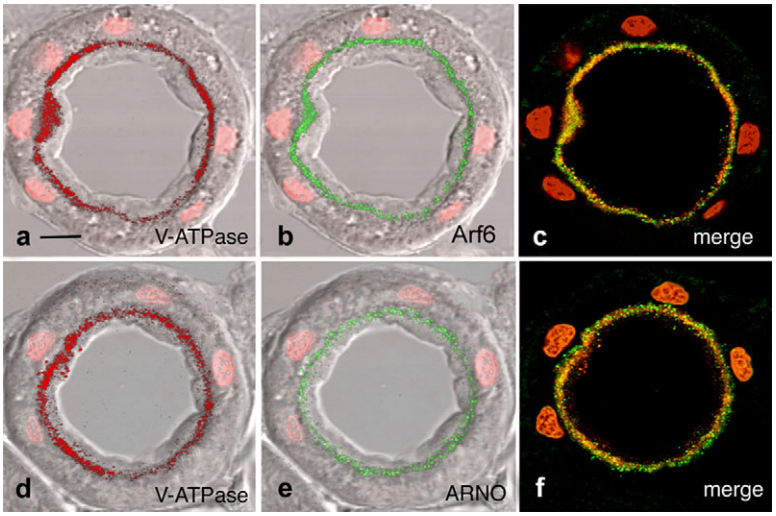

Fig. 9. Confocal sections of rat proximal tubules showing partial colocalization of the V-ATPase (a: red) with the small GTPase ARF6 (b: green) in sub-apical vesicles (c: merge of a and b), and colocalization of the V-ATPase (d: red; detected using antibodies against the 31 kD E-subunit, raised in rabbit) with the GDP/GTP exchange factor ARNO (e: green) in a similar sub-apical location (f: merge of d and e). Images a, b, d and e show immunofluorescence staining superimposed upon a transmitted light image. Arf6 and ARNO were detected using mouse monoclonal antibodies as previously described (Brown and Marshansky, 2004). Scale bar=5 μm.

the acidification-dependent recruitment of two major vesicle coat proteins, β-COP and ε-COP, as well as the small GTPase Arf1 (Aniento et al., 1996; Clague et al., 1994; Gu and Gruenberg, 2000) onto early endosomes purified from BHK cells. Recently, we demonstrated that in proximal tubule epithelial cells, Arf6 and its cognate GDP/GTP exchange factor ARNO are co-localized with V-ATPase on early endosomes (Maranda et al., 2001) (Fig. 9). Importantly, this study went on to show that both ARNO and Arf6 (but not Arf1) are recruited from the cytosol to endosomal membranes upon V-ATPase-driven endosomal acidification (Fig. 10). Based on these combined studies, the presence of a pH-sensing protein (PSP) was proposed but the nature of the PSP and the mechanism of its pH-dependent interaction with small GTPases remained elusive. More recently, the existence of a PSP in yeast vacuoles was also suggested (Shao and Forgac, 2004).

Identification of the V-ATPase a2-subunit as a pH-sensing protein

Based on a number of predictions about the nature of the putative pH-sensing protein, Marshansky and his colleagues formulated the testable hypothesis that one of more subunits of the V-ATPase itself could serve this function (Marshansky, 2007; Marshansky and Futai, 2008). Indeed we were able to show that the transmembrane a2-subunit of the V-ATPase was specifically located in early endosomes in the proximal tubule and that the N-terminus of this subunit associates with the Arf6 GEF (ARNO) in a pH-dependent manner (Hurtado-Lorenzo et al., 2006). Thus, a2–ARNO interaction was stimulated by endosomal acidification. In addition, the transmembrane c-subunit specifically interacted with Arf6 but, interestingly, not with Arf1. Inhibition of endosomal acidification by bafilomycin does not affect initial ligand internalization or trafficking through the recycling

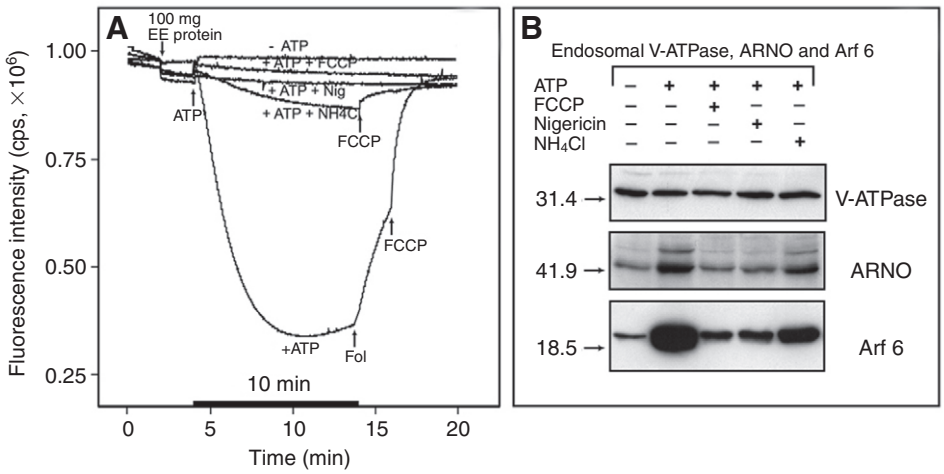

Fig. 10. Acidification-dependent recruitment of ARNO and Arf6 to isolated, purified proximal tubule early endosomes *in vitro*. Panel (A) shows an assay in which isolated endosomes were loaded with the pH indicator dye acridine orange, and acidification in response to added ATP was followed in a fluorimeter. A decrease in fluorescence intensity represents endosomal acidification. ATP addition activates the V-ATPase and induces a strong initial acidification that is reversed by the inhibitor folimycin (Fol, 1 μmol l^{-1}) and even more so by the uncoupling agent FCCP (1 μmol l^{-1}). When FCCP, the ionophore nigericin or NH_4Cl (1 mmol l^{-1}) are added to the endosomes prior to ATP addition, acidification is inhibited or greatly reduced. Panel (B) shows a 'protein recruitment assay' in which isolated proximal tubule endosomes were incubated with cytosol in the presence of various inhibitors of acidification. After a few minutes of incubation, endosomes were pelleted and subjected to western blotting using antibodies against the V-ATPase E-subunit, ARNO and Arf 6. The main message of this panel is that incubation with ATP alone (maximal acidification condition) resulted in a large increase in the amount of both ARNO and Arf6 associated with the endosomes. This 'recruitment' of these cytosolic proteins to endosomes was greatly reduced under all conditions in which acidification was also reduced, either in the absence of ATP (minimal acidification condition) or when acidification was inhibited by FCCP, nigericin or NH_4Cl. Modified from Maranda et al. (Maranda et al., 2001).

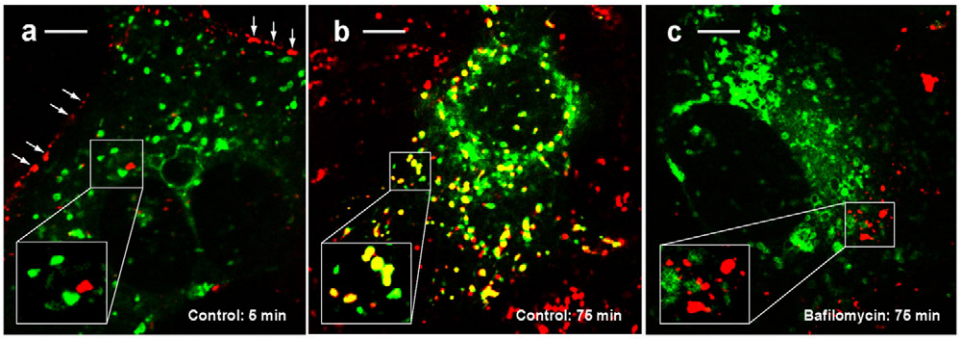

Fig. 11. Inhibition of acidification by bafilomycin prevents the delivery of internalized albumin-Alexa594 from early to late endosomes. Proximal tubule cells in culture were transfected with a vector expressing Rab7-EGFP, a marker of late endosomes. They were then pulsed for 5 min with albumin-Alexa594 and chased for about 75 min. (a) After 5 min, albumin (red) is delivered from plasma membrane (arrows) to early endosomes (insert, red vesicles) and after 75 min chase (b), albumin was delivered to late endosomes (insert, yellow vesicles). In striking contrast, in bafilomycin (0.5 μmol l^{-1})-treated cells, albumin is still confined to early endosomes (insert, red vesicles) even after 75 min chase (c). No yellow vesicles are detectable. These images are single frames taken from real-time movies of the chase period imaged by spinning disk confocal microscopy. Modified from Hurtado-Lorenzo et al. (Hurtado-Lorenzo et al., 2006). Scale bar=1 μm.

pathway but selectively affects the degradative pathway by preventing delivery of albumin-Alexa594 from early to late endosomes (Fig. 11), as shown also in earlier studies using an *in vitro* trafficking system (Clague et al., 1994). The identification of the V-ATPase as a pH sensor that recruits critical downstream trafficking components to endosomes in an acidification-dependent manner provides a mechanistic explanation for a phenomenological observation, i.e. that inhibiting vesicular acidification disrupts intracellular trafficking in the endosomal pathway, a finding that has long puzzled cell biologists.

Conclusion

The V-ATPase plays a central role in many aspects of cellular function in the kidney as well as in other cells and organs. These range from proton secretion in order to acidify the extracellular milieu, to intracellular acidification of vesicles and regulation of a variety of processes that range from lysosomal degradation, ligand receptor dissociation and intracellular trafficking *via* the recruitment of specific coat proteins to transport vesicles. The holoenzyme is comprised of many different subunits, each having different functions that are involved not only in the activity of the enzyme but also to its intracellular regulation and targeting. It is becoming increasingly apparent that the V-ATPase itself has many features that are characteristic of other 'coat' proteins that are involved in vesicle trafficking. In specialized proton-secreting cells such as IC, the apparent absence of other well-known coat proteins such as clathrin and caveolin from V-ATPase trafficking vesicles (Breton et al., 1998; Brown and Orci, 1986) as well as COP proteins (S.B. and D.B., unpublished data) suggests that indeed the V-ATPase itself may play a central role in directing its own trafficking within these cells. The abundance of subunits and alternative isoforms of several of the V-ATPase subunits provides this enzyme with the potential to be custom-assembled to achieve its final functional location within different cells. Dissecting the multiple states of the holoenzyme that are theoretically and practically possible will be an important task in the years that lie ahead.

List of abbreviations

AC	adenylate cyclase
AE	anion exchanger
A-IC	A-type intercalated cells
AQP2	aquaporin 2
B-IC	B-type intercalated cells
dRTA	distal renal tubule acidosis
EM	electron microscopy
FCCP	carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone
GEF	guanine nucleotide-exchange factor
HRP	horseradish peroxidase
IC	intercalated cells
MR	mitochondria-rich cells
NHERF	sodium/hydrogen exchanger (NHE) regulatory factor
PKA	protein kinase A
PLP	paraformaldehyde, lysine, periodate
pRTA	proximal renal tubule acidosis
PSP	pH-sensing protein
sAC	soluble adenylate cyclase

We thank all of our colleagues who have made important contributions to the work from the MGH Program in Membrane Biology that is included in this review. We have received critical support for this work from several grants from the NIH over the years, including R37 DK42956 (D.B.), PO1 DK38452 (D.B., S.B. and V.M.), HD40793 (S.B.) and KO1 DK73266 (T.G.P.). Deposited in PMC for release after 12 months.

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