

# Physiological implications of the regulation of vacuolar H<sup>+</sup>-ATPase by chloride ions

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Vacuolar H<sup>+</sup>-ATPase is a large multi-subunit protein that mediates ATP-driven vectorial H<sup>+</sup> transport across the membranes. It is widely distributed and present in virtually all eukaryotic cells in intracellular membranes or in the plasma membrane of specialized cells. In subcellular organelles, ATPase is responsible for the acidification of the vesicular interior, which requires an intraorganellar acidic pH to maintain optimal enzyme activity. Control of vacuolar H<sup>+</sup>-ATPase depends on the potential difference across the membrane in which the proton ATPase is inserted. Since the transport performed by H<sup>+</sup>-ATPase is electrogenic, translocation of H<sup>+</sup>-ions across the membranes by the pump creates a lumen-positive voltage in the absence of a neutralizing current, generating an electrochemical potential gradient that limits the activity of H<sup>+</sup>-ATPase. In many intracellular organelles and cell plasma membranes, this potential difference established by the ATPase gradient is normally dissipated by a parallel and passive Cl<sup>-</sup> movement, which provides an electric shunt compensating for the positive charge transferred by the pump. The underlying mechanisms for the differences in the requirement for chloride by different tissues have not yet been adequately identified, and there is still some controversy as to the molecular identity of the associated Cl<sup>-</sup>-conducting proteins. Several candidates have been identified: the CIC family members, which may or may not mediate nCl<sup>-</sup>/H<sup>+</sup> exchange, and the cystic fibrosis transmembrane conductance regulator. In this review, we discuss some tissues where the association between H<sup>+</sup>-ATPase and chloride channels has been demonstrated and plays a relevant physiologic role.

Key words: H<sup>+</sup>-ATPase; Chloride channels; Cl<sup>-</sup>/H<sup>+</sup> Antiporter; Intracellular organelles; Plasma membranes

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## General considerations

Vacuolar H<sup>+</sup>-ATPase (V-ATPase) is a large multi-subunit protein that mediates ATP-driven vectorial H<sup>+</sup> transport across membranes. The enzyme is composed of two functional domains, a catalytic and cytoplasmic V<sub>1</sub> domain (640 kDa), which is the site of ATPase activity, and a membrane-bound V<sub>0</sub> domain (240 kDa), which includes the proton translocation pathway. Together the V<sub>1</sub> and V<sub>0</sub> domains form a protein complex of approximately 900 kDa (1-3). The V<sub>1</sub> domain is a large complex composed of 8 subunits, designated by the capital letters A-H, organized

into a hexameric head of nucleotide binding (subunits A and B) that is attached to the V<sub>0</sub> sector by a central and a peripheral stalk (Figure 1). V<sub>0</sub> comprises single copies of a, c, c', c'', d, and e. Some of these subunits, in turn, are encoded by different genes and have more than one isoform (4). Like F-ATPases, the V-ATPases operate by a rotary mechanism in which ATP hydrolysis in V<sub>1</sub> drives rotation of a central stalk connected to the ring of proteolipid subunits (c, c', and c''). Movement of the ring of proteolipids past subunit a is believed to drive the active transport of protons across the membrane (4).

V-ATPase is distributed widely and found in virtually all

eukaryotic cells in intracellular membranes or in the plasma membrane of specialized cells (3). In subcellular organelles, including lysosomes, coated vesicles and endo/exocytic vesicles, ATPase is responsible for the acidification of the vesicular interior, which requires an acidic intraorganellar pH to maintain optimal enzyme activity (2). Intracellular V-ATPases are important for receptor-mediated endocytosis and intracellular trafficking, protein processing and degradation, coupled transport of small molecules and ions, and the entry of various pathogens into cells (4). An important model for the study of intracellular organelle H-ATPase has been the yeast cell, which has structure and functions similar to those of mammalian cells (5,6). However, there are important differences, e.g., the presence of a smaller number of subunit isoforms, a property involving a smaller degree of molecular variability, thus contributing to a simpler general structure that represents an advantage by being a more workable model. In addition, genetic deletion of several subunits is lethal in mammals and insects, but not in yeast and other fungi, permitting the study of their functional role (6).

The V-ATPase expressed in the plasma membrane transports protons to the outside of the cell (4), which is intricately related to specialized cell function, as exemplified in osteoclasts (7), and in renal tubular cells (1,8-11).

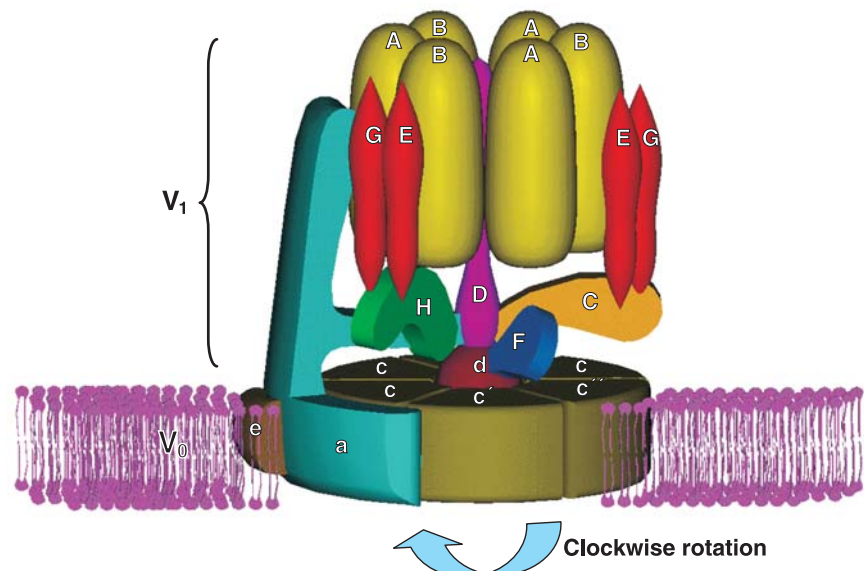
There are several mechanisms for the regulation of V-ATPase, such as recruitment by vesicular trafficking and sorting into and from the membrane, assembly and disassembly of  $V_0$  and  $V_1$  domains, transcription and/or translation, kinetic regulation, changes in V-ATPase content, and chloride dependence (1,3). This review will focus on the

regulation of V-ATPase by chloride channels (or chloride transporters) in physiologic and pathophysiologic conditions, identifying the importance of each channel and the mechanisms whereby the anion controls the proton ATPase activity.

### Chloride dependence of $H^+$ secretion: involvement of chloride channels and/or a chloride/proton exchanger

Control of V-ATPase depends on potential difference across the membrane into which the proton ATPase is inserted (2). Since the transport performed by  $H^+$ -ATPase is electrogenic, translocation of  $H^+$ -ions across membranes by the pump creates a lumen-positive voltage in the absence of a neutralizing current, creating an electrochemical potential gradient that limits the activity of  $H^+$ -ATPase. In many intracellular organelles and at cell plasma membranes, this potential difference established by the ATPase gradient is normally dissipated by a parallel and passive  $Cl^-$  movement, which provides an electric shunt compensating for the positive charge transferred by the pump (2,3). Several lines of evidence for this interaction support this view. The phenomenon has been demonstrated in most intracellular organelles that are acidified by proton ATPases, such as lysosomes (12), endosomes (13) and the trans-Golgi network (14,15). Furthermore, dependence of plasma membrane  $H^+$ -ATPase on  $Cl^-$  has also been observed in several studies, even in a much more complex way, due to expression of several other conductances or electrogenic transporters in both apical and

**Figure 1.** Model of vacuolar  $H^+$ -ATPase. The V-ATPase complex is composed of a peripheral domain ( $V_1$ ), responsible for ATP hydrolysis, and an integral domain, responsible for proton translocation across the membrane. The  $V_1$  domain is a hexamer of A and B subunits. The  $V_0$  domain is composed of a ring of proteolipid subunits (c, c' and c''), adjacent to subunits a and e. The  $V_1$  and  $V_0$  domains are connected by a central stalk, composed of subunits D and F of  $V_1$  and subunit d of  $V_0$ , and multiple peripheral stalks, composed of subunits C, E, G, H, and a. Modified from Ref. 4, with permission.



basolateral membrane domains (3).

The role of chloride channels in  $H^+$ -ATPase function is not explained only by the establishment or dissipation of a potential difference across cellular and intracellular membranes, but some evidence has supported a direct, specific role of  $Cl^-$  in stimulating  $H^+$ -ATPase activity (16). Kaunitz et al. (17) have shown that acidification of microsomes from the rat renal medulla depends on chloride channels. However, even after dissipating the potential difference across endosome membranes with valinomycin, ATPase still depends on the presence of  $Cl^-$  ions. These findings suggest a chloride binding site in an intramembrane pump sector.

### Chloride channels

The underlying mechanisms for the differences in chloride requirements in different tissues have not been identified, and there is still some controversy as to the molecular identity of the associated  $Cl^-$ -conducting proteins. Some candidates have been identified, such as CIC family members and the cystic fibrosis transmembrane conductance regulator (CFTR).

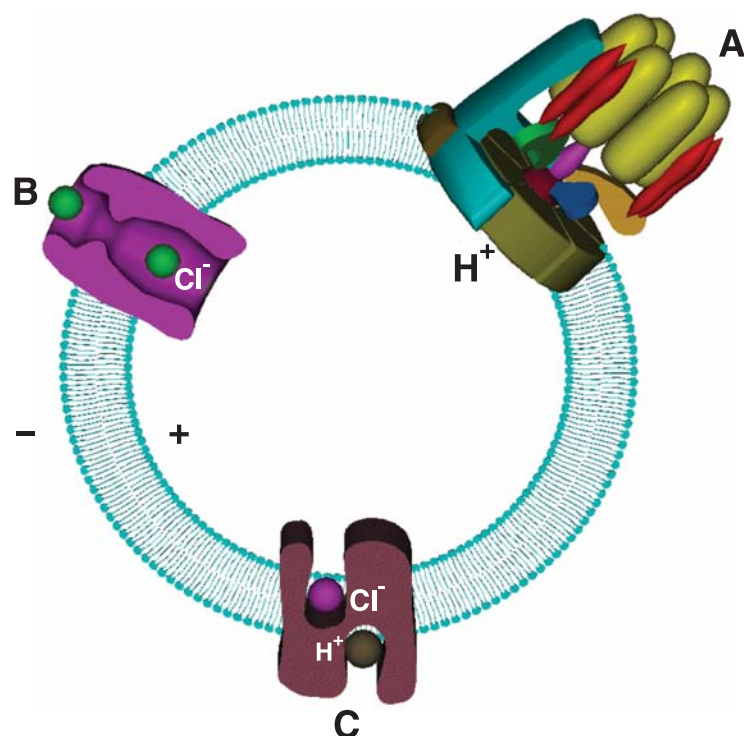
Chloride channels are classified into the following categories: 1) the often voltage-gated CIC family, which includes the Gef1 protein of the yeast *Saccharomyces cerevisiae* (Gef1p). The latter has been thought to provide the compensatory transport of  $Cl^-$  anions to the lumen of

the Golgi, thereby regulating the pH of this compartment (18); 2) CFTR; 3) ligand-gated GABA and glycine-activated  $Cl^-$  channels; 4) calcium-activated chloride channels; 5) swelling-activated chloride channels, or volume-regulated anion channels (19,20). The CIC chloride channel family was initially identified by expression cloning of the voltage gated  $Cl^-$  channel (CIC-0) from the electrical organ of the *Torpedo* (20). Since this initial cloning of the CIC family, nine mammalian CICs have been cloned by using homology-based cloning methods (19).

The CFTR  $Cl^-$  channel was identified as the protein encoded by the gene whose mutation results in cystic fibrosis, a lethal autosomal recessive disease common in populations of Caucasian or northern European descent (21). A characteristic of the disease is the severely impaired  $Cl^-$  transport across several epithelia. In the airway of cystic fibrosis patients, fluid and electrolyte secretion is inhibited because of the defect in CFTR. The protein is a plasma membrane cAMP-regulated  $Cl^-$  channel that belongs to the family of ATP-binding cassette proteins and is gated in response to binding and hydrolysis of ATP (22).

### Chloride/proton antiporter

Accardi and Miller (23) have provided evidence that the bacterial CIC homologue (CIC-ec1 protein, which promotes  $H^+$  extrusion activated an extremely acid medium) is not an ion channel as originally thought, but instead acts as



**Figure 2.** Counter-ion conductance involved in the generation of a proton gradient by vacuolar  $H^+$ -ATPase in a hypothetical organelle. The neutralizing current is thought to be mediated by chloride channels, which mediate or not  $nCl^-H^+$  exchange. A, Vacuolar  $H^+$ -ATPase; B, chloride channels; C,  $Cl^-H^+$  antiporter.

an H<sup>+</sup>-Cl<sup>-</sup> exchange transporter with a likely stoichiometric ratio of 2 Cl<sup>-</sup>/H<sup>+</sup>. This conclusion leads to a reinterpretation of the activation of Cl<sup>-</sup> currents by low pH in terms of proton transport rather than proton-dependent channel gating. The findings also suggest that some eukaryotic ClC channels (except ClC-0, ClC-1 and ClC-2, which show no indications of H<sup>+</sup> permeability) could also act as an exchanger. Indeed, a number of data have suggested a chloride/proton antiporter activity in several mammalian ClC proteins, such as ClC-4 and ClC-5, as well as ClC-7 (24-26). Figure 2 shows that due to its electrogenicity, the exchanger may produce a Cl<sup>-</sup> current into a subcellular vesicle, reducing the PD caused by electrogenic H<sup>+</sup> transport. Thus, if the coupling of Cl<sup>-</sup> to H<sup>+</sup> were 2:1, the exchanger would have properties similar to those of a Cl<sup>-</sup> channel, shorting out electrical gradients opposed to H<sup>+</sup> ion transport. Of course, in this way part of the H<sup>+</sup> transported, e.g., into an organelle, will be opposed, reducing transport efficiency. At first sight, the advantage of a system of this kind is not apparent. An exchanger will use more metabolic energy to produce a given pH gradient, but may establish a more constant internal Cl<sup>-</sup> concentration. The absence of these Cl<sup>-</sup> gradients may contribute to the lysosomal storage disease in ClC-6 and ClC-7 KO mice (27).

The conclusion that the ClC family consists of channel and transporter subtypes, the former residing in plasma membranes and the latter in acidifying intracellular membranes, presented biophysicists with unusual behaviors. This suggests that membrane proteins with similar structures can support ion-transport mechanisms that are enormously different from a thermodynamic point of view (28).

In the following section, we will identify tissues where the association between H<sup>+</sup>-ATPase and chloride channels has been demonstrated and shown to play a relevant physiologic role.

## Chloride dependence of V-ATPase in several tissues

### Kidney

In the kidney, the role of Cl<sup>-</sup> channels in regulating H<sup>+</sup>-ATPase activity has been studied in endosomal fractions (16,27,29), brush border membrane vesicles (3) as well as in apical membranes of renal tubules (10,30-32).

Using a Cl<sup>-</sup> fluorescent indicator, Bae and Verkman (29) observed that in endocytic vesicles from rabbit proximal tubule the chloride conductance was activated by phosphorylation through a cyclic AMP (cAMP)-dependent protein kinase.

In isolated rabbit S3 proximal tubules, the apical inser-

tion of V-ATPase-containing vesicles was shown to also be dependent on chloride, being delayed in the absence of the anion (10). Furthermore, in rat proximal tubules, H<sup>+</sup>-ATPase activity was reduced after preincubation in Cl<sup>-</sup> free solution (33). Ang II (1 nM) stimulated the H<sup>+</sup>-ATPase-dependent proton secretion in isolated rat proximal tubules, and this stimulation was also shown to be dependent on chloride and on the integrity of the microtubular network (34).

In rat cortical late distal tubules, microperfusion experiments have shown that the rate of proton secretion by H<sup>+</sup>-ATPase was also dependent on chloride transport, since the inhibition of Cl<sup>-</sup> channels by 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) markedly reduced distal tubule acidification, in parallel to a small alteration in trans-epithelial potential difference (30).

Furthermore, in Madin-Darby Canine Kidney (MDCK) cells, a permanent cell line originated from the renal collecting duct, regulation of pH mediated by H<sup>+</sup>-ATPase is inhibited when Cl<sup>-</sup> is absent from the incubation solution or when NPPB is added (32). In addition, a study of MDCK-C11 cells (a sub-clone of MDCK cells that have many properties of collecting duct intercalated cells) reported that the V-ATPase responsible for part of the recovery of pHi after an acid pulse depends on Cl<sup>-</sup> for its activation (35). Cell potential difference is not an important factor for the maintenance of its activity, since its abolition by valinomycin/high K<sup>+</sup> did not modify the function of H<sup>+</sup>-ATPase.

In proximal tubule cells, the co-localization of proton pumps with ClC-5 chloride channels in apical endosomes is related to an X-chromosome-linked disease characterized by low-molecular weight proteinuria, hypercalciuria and kidney stones, called Dent's disease (13,27). This syndrome is caused by mutations of the *CLCN5* gene, predominantly expressed in kidney, which has been shown to decrease or abolish ClC-5 activity. This channel conducts outwardly rectifying chloride currents that represent a counter-ion flux to the electrogenic influx of protons into the endosomes, suggesting that ClC-5 is essential for renal endocytosis (13). These endosomes form part of the receptor-mediated endocytic pathway that transports proteins (13), and therefore the lack of ClC-5 might impair endosomal acidification, compromising the reabsorption of filtered proteins and causing the proteinuria observed in Dent's disease (27).

The *in vitro* acidification of cortical renal endosomes prepared from ClC-5 KO mice was shown to be significantly reduced. In addition, the loss of ClC-5 reduced apical fluid phase- and receptor-mediated endocytosis, as well as the endocytotic retrieval of plasma membrane proteins in a cell-autonomous manner (36). These findings support

the postulated importance of CIC-5 in endosomal acidification essential for protein reabsorption.

The alterations observed in Dent's disease are not restricted to endosomal function. Recently, Moulin and colleagues (37) examined renal biopsy specimens from patients with Dent's disease and consistently noted inversion of H<sup>+</sup>-ATPase polarity in the proximal tubules to a basolateral distribution, in contrast to its apical localization in the normal kidney. This inversion of polarity was specific for H<sup>+</sup>-ATPase and did not affect distribution of aminopeptidase, megalin, or Na<sup>+</sup>/K<sup>+</sup>-ATPase.

In the collecting duct the expression of CIC-5 colocalized with H<sup>+</sup>-ATPase in intracellular vesicles and in the plasma membrane has also been described in both  $\beta$ -intercalated cells and in acid-secreting  $\alpha$ -intercalated cells. CIC-5 is involved in the plasma membrane insertion and recycling of these vesicles (13). Mutations of CIC-5 may affect the expression of apical H-ATPase in  $\alpha$ -intercalated cells and impair urinary acidification (38). Thus, these data also suggest a role for CIC-5 in the proton secretion mechanism in these cells.

It is possible, however, that CIC-5 is not the only chloride channel playing a significant role in H<sup>+</sup>-ATPase activity in endosomes and brush border membrane from proximal tubules. CIC-5 lacks some features, for example activation by protein kinase A (since chloride conductance in endosomes from proximal tubules is stimulated by this protein), suggesting that another conductance is involved (3). Recent studies have demonstrated that CIC-4 facilitates endosomal acidification and is important but not essential for the endocytosis process because CIC-4 KO mice do not display proteinuria (39).

The partial expression of CIC-4 and CIC-5 at the cell surface allowed the detection of strongly outwardly rectifying currents that depended on anions and pH. Surprisingly, these currents reflect also an electrogenic exchange of Cl<sup>-</sup> for H<sup>+</sup>, and not Cl<sup>-</sup> channels (24,26). Indeed, recent studies have suggested that CIC-5 acts as a voltage-dependent electrogenic chloride/proton exchanger, which is thought to provide a chloride current to neutralize the increase of H<sup>+</sup> in the intraorganellar space, permitting an effective acidification process (24,26,27). This finding is incompatible with the assumed role for CIC-5 providing a shunt conductance in the membrane of intracellular organelles or in the plasma membrane to compensate for the electrogenic activity of H<sup>+</sup>-ATPase (19). Therefore, the function and characteristics of chloride currents in modulating the function of H<sup>+</sup>-ATPase need to be further investigated.

Another channel proposed to have a role in the regulation of V-ATPase is CFTR. This channel is expressed in

renal endosomes, membranes of proximal and distal tubules, and intercalated cells of collecting ducts, especially in type- $\beta$  cells (40). Barasch and colleagues (41) have demonstrated a defective acidification of intracellular organelles in cystic fibrosis, indicating a possible role for CFTR in organelle acidification dependent on V-type H<sup>+</sup>-ATPase. The positive modulation of CFTR channels by cAMP is well known (3). Recently, Tararhuch et al. (35) demonstrated that cAMP stimulates the extrusion of H<sup>+</sup> in MDCK-C11 cells in the presence of Na<sup>+</sup>-free solution. This finding suggests a participation of chloride currents through CFTR channels stimulated by cAMP, increasing the activity of H<sup>+</sup>-ATPase. In contrast, no changes in the acidification rates of several intracellular organelles were demonstrable after activation of chloride currents stimulated by cAMP (42). Thus, the role of CFTR in the activity of H<sup>+</sup>-ATPase is controversial.

#### Liver

By means of the measurement of the uptake of radioactive chloride into vesicles, Glickman et al. (14) detected a chloride conductance in parallel to an electrogenic H<sup>+</sup>-pump in isolated rat liver Golgi membranes, arguing in favor of the involvement of an electrochemical gradient.

In rat liver multivesicular bodies (an endosome intermediate between coated vesicles and lysosomes), chloride was found to increase the initial rate of vesicle acidification and to affect the relative chemical and electrical contributions of the steady-state pump proton-motive force (43).

Using highly enriched fractions of rat liver endosomes, Fuchs et al. (44) have found that ATP-dependent acidification is electrogenic and the membrane potential established by the pump can be dissipated by the influx of permeant external anions or by the efflux of internal alkali cations. Although the external replacement of permeable anions with less permeable anions (replacing Cl<sup>-</sup> with gluconate) diminished acidification, ATP-dependent H<sup>+</sup> transport was not found to be coupled to any specific anion or cation.

In clathrin-coated vesicles from rat liver, proton transport showed no specific cation requirement, but was dependent on a permeant anion, with the following hierarchy of importance: Cl<sup>-</sup> = Br<sup>-</sup> > NO<sub>3</sub><sup>-</sup> > SO<sub>4</sub><sup>2-</sup> > HPO<sub>4</sub><sup>2-</sup> = gluconate (45). Although the authors of the present study did not exclude the possibility of an electroneutral exchange or a symport mechanism, it is more likely that an electrogenic H<sup>+</sup> transport mechanism that does not depend directly on chloride but rather depends on the presence of a permeant anion is involved in this modulation (45). In addition, lysosomal H<sup>+</sup>-ATPase purified from rat

liver is activated by chloride, suggesting the presence (in the ATPase) of one or more binding sites for activating anions (46).

Although some investigators have demonstrated a role for chloride channels in the acidification of intracellular organelles in hepatocytes, the molecular identity of these channels remains poorly understood. Some studies revealed that CIC-3 protein localized abundantly in the vesicular membranes and as an intracellular Cl<sup>-</sup> channel in these vesicles, cooperating with V-ATPase to achieve H<sup>+</sup> and Cl<sup>-</sup> flux (47). Hara-Chikuma et al. (48) reported that endosomal acidification and Cl<sup>-</sup> accumulation were significantly reduced in hepatocytes from CIC-3-deficient versus wild-type mice. These investigators found a functional CIC-3 Cl<sup>-</sup> conductance in endosomes of cell homogenates from wild-type versus CIC-3-deficient hepatocytes containing fluorescently labeled endosomes. The data of this study suggest that CIC-3 Cl<sup>-</sup> conductance provides a quantitatively significant electrical shunt pathway to permit endosomal acidification by the vacuolar H<sup>+</sup> pump.

#### Central nervous system

In rat brain synaptic vesicles, Glebov et al. (49) found, through measures of acidification with the permeant weak base-acridine orange, that the ATP-dependent generation of delta pH was completely dependent on the presence of a permeant anion, and was maximal at 150 mM Cl<sup>-</sup>. The authors suggested the presence of a chloride channel whose conductance can regulate the H<sup>+</sup> transport by switching it from an electrogenic to an electroneutral (coupled entry of H<sup>+</sup> and Cl<sup>-</sup>) mode of operation (49). Furthermore, the transmembrane potential of the same synaptic vesicles, measured by means of the potential-sensitive fluorescent probe 3,3' dipropylthiocarbocyanine iodide, demonstrated that H<sup>+</sup>-ATPase is electrogenic and the level of the transmembrane potential depends on the presence of anion (Cl<sup>-</sup>) in the incubation medium (50).

In bovine brain clathrin-coated vesicles, acidification of the vesicle interior showed considerable anion selectivity (Cl<sup>-</sup> greater than Br<sup>-</sup> much greater than NO<sub>3</sub><sup>-</sup> much greater than gluconate, SO<sub>2</sub><sup>-</sup> (4), similar to HPO<sub>2</sub><sup>-</sup> (4) and mannitol), but was relatively insensitive to cation replacement as long as Cl<sup>-</sup> was present (51). On the other hand, purified H<sup>+</sup>-ATPase from otherwise intact clathrin-coated vesicles in both detergent-solubilized and reconstituted states were shown to be independent of Cl<sup>-</sup>. Furthermore, H<sup>+</sup> transport does not occur even at high Cl<sup>-</sup> concentrations unless K<sup>+</sup> and valinomycin are present to dissipate the membrane potential, indicating that the Cl<sup>-</sup> channel that provides Cl<sup>-</sup> flux in intact coated vesicles is not a component of the purified H<sup>+</sup>-ATPase (52).

Since the molecular identity of the chloride channels involved in acidification by H<sup>+</sup>-ATPase in the brain remains to be well elucidated, a number of studies have provided evidence that CIC-3 plays a critical role in acidifying synaptic vesicles (53). Disruption of CIC-3 (by generation of CIC-3-deficient mice) present in endosomal compartments of synaptic vesicles has been shown to lead to a loss of hippocampus function (characterized by postnatal growth retardation, blindness secondary to retinal degradation and behavioral abnormalities). CIC-3 deficiency in mice led to elevated intraendosomal pH, which influenced the cellular protein degradation cascade, causing phenotypes similar to human neuronal ceroid lipofuscinosis (a neurodegenerative disorder characterized by excessive accumulation of lipopigments) (53). Similarly to CIC-3, disruption of mouse CIC-6 and CIC-7 leads to neurodegeneration, with neurons displaying intracellular electron-dense deposits characteristic of lysosomal marker proteins (27). Since CIC-6 and CIC-7 have a subcellular localization, it is possible that lysosomal storage observed in the respective KO mice is a consequence of defective late endosomal or lysosomal function.

A recent study has identified CIC-4, another chloride channel in excitable tissues such as the nervous system and skeletal muscle, that may reside mainly in their sarco/endoplasmic membranes (54). An influx of chloride anions through these channels and an efflux of protons from the sarcoplasmic/endoplasmic reticulum (SR/ER) might contribute to dissipate the electrical gradient generated by the SERCA (Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPases that sequester Ca<sup>2+</sup> into the SR/ER).

#### Bone

In order to solubilize bone mineral and degrade the organic matrix of bone, osteoclasts must secrete 1-2 protons for every Ca<sup>2+</sup> liberated. This transport is a major metabolic activity of osteoclasts requiring an electrogenic H<sup>+</sup>-ATPase, a conductive chloride channel, a chloride-bicarbonate exchanger, carbonic anhydrase, and functional/morphological polarization of the cell (55). In proton-transporting membrane vesicles from avian osteoclast ruffled membrane (a special membrane facing the bone) it was found that the acidification driven by H<sup>+</sup>-ATPase has an absolute requirement for conductive anion transport with a nonlinear dependence on external chloride. The passive conductive Cl<sup>-</sup> transport was found to be due to an apparently unique Cl<sup>-</sup> channel, a mechanism participating in net vectorial HCl secretion by osteoclasts, for bone reabsorption (56). Purification of this protein and reconstitution into phospholipid membranes retained the transport of Cl<sup>-</sup>.

In 1997, Schlesinger and colleagues (57) isolated a 62-kDa Cl<sup>-</sup> channel from avian osteoclast ruffled border whose human homologue is the intracellular chloride channel 5 (CLIC5), one of a group of chloride channels expressed intracellularly and believed to act in acid secretion, but not correlated with human disease (57-59). In planar bilayers, the ruffled border channel was found to be a stilbene sulfonate-inhibitable, outwardly rectifying chloride channel. Since outward rectification of a chloride channel means that the single channel conductance is greater for chloride entering than exiting the cell and the proposed function of this channel is to allow chloride to exit the cell in parallel with protons, outward rectification may seem contradictory. The explanation for this could be that, when Cl<sup>-</sup> is exiting from the cell, the transport of H<sup>+</sup> and Cl<sup>-</sup> is electrically coupled, and the chloride current across the ruffled border represents HCl transport. When the chloride conductance is limiting, proton pump activity hyperpolarizes the ruffled border membrane, increasing the internal negative potential in proportion to the pump activity. If the ruffled border chloride channel were not rectifying, the proton pump-driven hyperpolarization would increase the chloride current and HCl transport. However, since the chloride channel is outwardly rectifying, changes in the membrane potential have a small effect on the chloride current and acid transport. So, during bone reabsorption, when the electrogenic proton pump hyperpolarizes the ruffled border membrane, the number of active chloride channels in that membrane will directly determine and limit HCl transport (57).

While CLIC5 is not related to human disease, transgenic mice deficient in a chloride channel analogue, CIC-7, have osteopetrosis (a rare inherited disorder whereby the bones harden, becoming denser) (27). This shows that CIC-7 provides the chloride conductance required for an efficient proton pumping by the H<sup>+</sup>-ATPase of the osteoclast ruffled membrane. CIC-7 is a mostly intracellular chloride channel from late endosomes and lysosomes. Due to this localization, no direct electrophysiological data on CIC-7 channel function are available, but sequence alignments imply that the channel functions as an H<sup>+</sup>/Cl<sup>-</sup> antiporter similar to CIC-4 and CIC-5 (26).

Since CLIC5 as well as CIC-7 have been immunolocalized to the ruffled border, and since both are members of families of known chloride channels and suppression of their expression leads to decreased bone resorption, it is possible that the regulation of acidification and bone re-

sorption are complex processes with multiple sites where chloride transport can exert an influence. CLIC5 and CIC-7 could be cooperating components of anion transport activity in the ruffled border membrane that supports bone resorption (59). In addition, more recently, Okamoto et al. (60) showed expression of CIC-3 channels in mouse osteoclasts, present on intracellular organelles, where CIC-3 deficiency leads to decreased organelle acidification and consequent reduced bone reabsorption activity.

## Concluding remarks

The present review shows that Cl<sup>-</sup> ions have an important function in the activity of vacuolar and membrane H<sup>+</sup>-ATPase. Colocalization of Cl<sup>-</sup> channels and this ATPase has been demonstrated in several tissues. The original idea concerning this cooperative effect suggested that Cl<sup>-</sup> ions were needed to provide a shunt current via Cl<sup>-</sup> channels of negative ions that neutralized H<sup>+</sup> ion flux into subcellular organelles, a positive voltage being able to block or limit H<sup>+</sup> flux into these organelles. More recently, it was shown that several of the membrane molecules that were considered to be Cl<sup>-</sup> channels in reality were Cl<sup>-</sup>/H<sup>+</sup> exchangers with 1:1 or 2:1 coupling, whose end effect would be similar to the function of the channels. Channels such as CFTR, CIC-1 and CIC-2 were confirmed to be channels, while CIC-4 and CIC-5 are now considered to be Cl<sup>-</sup>/H<sup>+</sup> exchangers. The lack of these channels (by lack in specific diseases or in knock-out models) may cause defects in acidification in H<sup>+</sup>-ATPase-containing structures and produce now well-defined diseases, such as, for instance, Dent's disease, which is characterized by renal tubular acidosis, increase in Ca<sup>2+</sup> excretion and kidney stone formation. This disease is related to a defect in the expression of CIC-5 channels or exchangers. Other diseases related to defects in Cl<sup>-</sup> channels have been described, including forms of neurodegeneration and osteopetrosis. The description of these diseases as well as the cloning and characterization of Cl<sup>-</sup> transporters have now firmly established the importance of these transporters for the function of vacuolar and membrane H<sup>+</sup>-ATPases in a large number of cells and tissues.

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