Title
Novel and potential physiological roles of vacuolar-type H+-ATPase in marine organisms

Permalink
https://escholarship.org/uc/item/7qk3b2kr

Journal
JOURNAL OF EXPERIMENTAL BIOLOGY, 219(14)

ISSN
0022-0949

Author
Tresguerres, M

Publication Date
2016-07-15

DOI
10.1242/jeb.128389

Peer reviewed
Novel and potential physiological roles of vacuolar-type H⁺-ATPase in marine organisms

Martin Tresguerres*

ABSTRACT
The vacuolar-type H⁺-ATPase (VHA) is a multi-subunit enzyme that uses the energy from ATP hydrolysis to transport H⁺ across biological membranes. VHA plays a universal role in essential cellular functions, such as the acidification of lysosomes and endosomes. In addition, the VHA-generated H⁺-motive force can drive the transport of diverse molecules across cell membranes and epithelia for specialized physiological functions. Here, I discuss diverse physiological functions of VHA in marine animals, focusing on recent discoveries about base secretion in shark gills, potential bone dissolution by Osedax bone-eating worms and its participation in a carbon-concentrating mechanism that promotes coral photosynthesis. Because VHA is evolutionarily conserved among eukaryotes, it is likely to play many other essential physiological roles in diverse marine organisms. Elucidating and characterizing basic VHA-dependent mechanisms could help to determine species responses to environmental stress, including (but not limited to) that resulting from climate change.

KEY WORDS: pH, Soluble adenylyl cyclase, Carbonic anhydrase, Carbon-concentrating mechanism, Ocean acidification, Osedax

Introduction
The vacuolar-type H⁺-ATPase (VHA) is a multi-subunit enzyme that is ubiquitous in eukaryotic cells. VHA uses the energy from ATP hydrolysis to drive H⁺ transport against an electrochemical gradient, generating a potential gradient in the process (Mindell, 2012; Wagner et al., 2004). Together with the concomitant transport of a counter ion (typically Cl⁻), this process acidifies lysosomes, endosomes, multivesicular bodies and clathrin-coated, Golgi-derived and synaptic vesicles, among other intracellular compartments (Stevens and Forgac, 1997). VHA-mediated acidification in these vesicular compartments is essential for macromolecule digestion and processing, receptor-mediated endocytosis and the coupled transport of small molecules such as nutrients and neurotransmitters (Beyenbach and Wieczorek, 2006; Mindell, 2012; Stevens and Forgac, 1997). VHA subunit structure and function is best known in yeast (Benlekbir et al., 2012; Zhao et al., 2015). VHA has two regions, a V₀ domain that spans the lipidic membrane and functions as the H⁺ turbine, and a catalytic cytoplasmic V₁ domain (Fig. 1). For detailed information about VHA structure and molecular function in animals, the reader is referred to several excellent recent reviews (Beyenbach and Wieczorek, 2006; Cotter et al., 2015; Droy and Nelson, 2006; Marshansky et al., 2014; Nelson and Harvey, 1999; Stevens and Forgac, 1997; Wagner et al., 2004).

As well as being located in the membranes of vesicular compartments, VHA is also present in the plasma membrane of specialized epithelial cells, where it secretes H⁺ for systemic acid/base (A/B) regulation, ion regulation, osmoregulation and extracellular acidification or alkalization for the purposes of NH₃ excretion, carbonate dissolution and nutrient uptake. The physiological roles of VHA in insects (Baumann and Walz, 2012; Onken and Moffett, 2009; Wieczorek et al., 2009) and mammals (Brown et al., 2009; Jefferies et al., 2008; Saroussi and Nelson, 2009; Wagner et al., 2004) are periodically reviewed. Similarly, recent comprehensive reviews have discussed the involvement of VHA in H⁺ secretion and NaCl absorption in freshwater crustaceans, fish and amphibians (Evans et al., 2005; Larsen et al., 2014; Perry and Gilmour, 2006). The purpose of this Commentary is to describe some recent discoveries and hypotheses on the roles of VHA in marine organisms, where it is less well studied, and to place VHA function into the broader context of organismal physiology. Specifically, this Commentary focuses on transepithelial base secretion and acid reabsorption in shark gills, acid secretion by Osedax bone-eating worms and symbosomic acidification in corals. The final section presents a discussion of the potential functions of VHA in relation to ocean acidification (OA), an increasingly important topic in marine science.

Acid/base regulation in elasmobranchs: VHA-dependent epithelial HCO₃⁻ secretion and H⁺ reabsorption
Cellular and epithelial HCO₃⁻ secretion is essential for various physiological functions, such as systemic A/B regulation by the gill and kidney, calcification in bones by osteoblasts (see Glossary) (as well as in the fish intestine, mollusk mantle and, potentially, coral skeleton) and alkaline fluid secretion by the pancreas, intestine and mosquito midgut. The transport of ions against a concentration gradient must be powered by ATPases; however, no HCO₃⁻ ATPases have yet been molecularly identified in animals. Instead, some cells power HCO₃⁻ secretion using VHA, which removes H⁺ generated from the reaction catalyzed by carbonic anhydrase (CA; CO₂+H₂O⇌HCO₃⁻+H⁺) into the internal compartment (e.g. blood or hemolymph). This ‘pills’ the reaction to the right, promoting the generation of HCO₃⁻, which is then secreted through anion exchangers or channels into the opposite compartment (the surrounding water or lumen of the organ). Additionally, VHA activity hyperpolarizes the cell membrane and generates an electrical driving force across the membrane of the epithelial cells, which can drive the movement of anions such as Cl⁻ and HCO₃⁻ through channels and electroneutral exchangers and cotransporters.

The gill of elasmobranch fishes (sharks, rays and relatives) is an excellent model with which to study the cellular mechanisms of A/B regulation: gills are the only organ responsible for elasmobranch
Together, C, H, E and G form the peripheral stalk that connects Vo and V1 subunits, D and F are rotor subunits, and E and G are stator subunits.

**VHA is composed of cytosolic domain V1 (subunits A and B are catalytic subunits in the V1 domain are written in uppercase letters. A and B are catalytic subunits, and membrane domain Vo (subunits a, c, c, d and e in white). By convention, subunits in the V0 domain are written in lowercase letters, and subunits in the V1 domain are written in uppercase letters. A and B are catalytic subunits, D and F are rotor subunits, and E and G are stator subunits. Together, C, H, E and G form the peripheral stalk that connects V0 and V1 (reviewed in Cotter et al., 2015). In yeast, VHA subunit stoichiometry follows the formula $A_3B_3CDE_3FG_3Hadecxc_{yc′}c,c′,d$ and $e$ in white). By contrast, mammals use both lungs and kidneys (reviewed in Boron and Boulpaep, 2012), and their renal cells have multiple A/B regulatory cell types, which are difficult to isolate and cannot withstand large A/B fluctuations in vivo. The gills of teleost fishes also have several cell subtypes, and serve a dual purpose in A/B regulation and NaCl transport for osmoregulation (Evans et al., 2005). Elasmobranch gills contain two types of specialized acid- and base-secreting cells that regulate the blood A/B status (Piermarini and Evans, 2001; Reilly et al., 2011; Roa et al., 2014). Base-secreting cells are ‘VHA-rich’, express abundant intracellular CA (Tresguerres et al., 2007b) and apical pendrin-like (see Glossary) anion exchangers (Piermarini et al., 2002; Reilly et al., 2011; Roa et al., 2014), and are mitochondrion-rich (Roa et al., 2014). Normally, fish metabolism is net acidic because of CO2 release from the blood into the cloaca, and has been extensively used as a model system for epithelial NaCl secretion.**

**β-intercalated cell**

Kidney cell specialized for HCO₃⁻ secretion (via pendrin) and H⁺ reabsorption (via VHA) in the distal collecting duct of the mammalian nephron.

**Ec dysial space**

Space that is formed between the hypodermis and the cuticle before the exoskeleton is absorbed as part of the moulting process in crustaceans (and ecdysozoan animals in general).

**Etching cells**

These cells are believed to secrete acid, thus allowing certain sponges to bore into calcium carbonate substrates.

**Osteoblast**

Specialized cells in vertebrate animals that synthesize bone.

**Pendrin**

Electroneutral anion exchanger protein; its main physiological function is epithelial absorption of chloride in exchange for bicarbonate. Encoded in humans by the SLC26A4 gene (Solute carrier family 26, member 4), it owes its name to Pendred syndrome, an autosomal recessive hearing disease caused by mutations in pendrin.

**Pillar cells**

Gill epithelial cells that connect the two epithelial sheets of gill lamellae. The space delimited between two pillar cells and lamellar cells forms a channel through which blood flows in a counter-current manner compared with the water flowing over the gill.

**Rectal gland**

A diverticulum of the distal intestine of elasmobranch fishes essential for osmoregulation. The rectal gland epithelium secretes excess NaCl from blood into the cloaca, and has been extensively used as a model system for epithelial NaCl secretion.

**Ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCo)**

This enzyme catalyzes the first major step of carbon fixation in the Calvin–Benson cycle in photosynthetic organisms.
In air-breathing vertebrates, compensation of blood alkalosis largely results from a hypoventilatory adjustment that retains CO₂ and secretion of HCO₃⁻ into the small intestine by the pancreas and duodenal glands (reviewed in Wang et al., 2001). However, aquatic animals rely heavily on the active secretion of H⁺ or HCO₃⁻ across the gills to compensate for A/B stress (reviewed in Evans et al., 2005). To secrete excess blood HCO₃⁻, elasmobranch base-secreting gill cells are activated through a mechanism that involves CAs and the A/B sensor soluble adenylyl cyclase (sAC) (Fig. 2). First, blood plasma HCO₃⁻ is dehydrated into CO₂ by an extracellular CA isoform reported to be associated with the cell membrane of pillar cells (see Glossary) and by an intracellular CA isoform in red blood cells (Gilmour et al., 2007, and references therein). CO₂ then diffuses into base-secreting cells and is subsequently rehydrated by an intracellular CA isoform into HCO₃⁻ and H⁺ (Tresguerres et al., 2007b). The elevation in the concentration of intracellular HCO₃⁻ ([HCO₃⁻]i) stimulates sAC, which, potentially through the cAMP–PKA pathway, triggers the microtubule-dependent insertion of VHA-containing vesicles from the cytoplasm into the basolateral membrane (Tresguerres et al., 2006c, 2010; Roa and Tresguerres, 2016). Postprandial alkalosis also results in the insertion of pendrin into the apical membrane (Roa et al., 2014), although it is not known whether this is also mediated by sAC. From its basolateral location, VHA ‘pumps’ H⁺ into the blood and energizes HCO₃⁻ secretion to seawater in exchange for Cl⁻ through apical pendrin, counteracting blood alkalosis. In the basolateral membrane, Cl⁻ probably exits the VHA-rich cell into the blood through currently unidentified channels; this step would be essential to maintain a low [Cl⁻]i, and dissipates the transmembrane electrical force generated by VHA (Stevens and Forgac, 1997). Therefore, HCO₃⁻ secretion in base-secreting cells is likely to be driven by a combination of increased [HCO₃⁻]i and reduced [Cl⁻]i, both powered by VHA and assisted by CA, which supplies H⁺ and HCO₃⁻ at a sufficiently fast rate.

VHA translocation is gradual and dynamic rather than an ‘on/off’ process (Tresguerres et al., 2011). This is illustrated in the immunostained gill section in Fig. 2, which shows various stages of VHA translocation in the same gill filament. VHA-containing vesicles are likely to be constantly shuttled between the cytoplasm and the basolateral membranes, thus adjusting the intracellular pH (pHi) of the VHA-rich cells and, consequently, maintaining systemic A/B homeostasis. Therefore, mild and short-lasting alkaline stress can be corrected by the translocation of pre-existing VHA, but the homeostatic response to more extreme alkaline stress additionally involves upregulation of VHA synthesis (Tresguerres et al., 2006c).

The mechanism for sensing and counteracting blood alkalosis in VHA-rich gill cells is likely to be applicable to other epithelial base-secreting cells. For example, β-intercalated cells (β-ICs; see Glossary) in the mammalian nephron also express VHA, pendrin and sAC (Pâunescu et al., 2008); however, functional studies with β-ICs are notoriously difficult as a result of the low abundance of these cells in this type, the lack of cell cultures and their compromised viability upon exposure to stressful conditions (Breton and Brown, 1998; Gong et al., 2010). Sculpin, a species of marine teleost fish, also have a subpopulation of gill ionocytes that express high levels of VHA in cytoplasmic vesicles or possibly in tubulofilaments of the basolateral membrane (Catches et al., 2006). VHA in these cells presumably mediates H⁺ reabsorption and energizes apical HCO₃⁻ secretion as in elasmobranchs; however, this has not yet been confirmed experimentally. Hagfish gills seem to contain a single type of A/B regulatory ionocyte (Tresguerres et al., 2006b), which can perform either acid or base secretion depending on the blood A/B physiological needs. However, hagfish ionocytes have an extensive basolateral tubulovesicular system (Barrels and Welsch, 1986), as found in teleost fish gill ionocytes, which makes it difficult to determine the subcellular location of proteins. Nonetheless, immunohistochemistry and western blots on samples

Fig. 2. VHA translocation in shark gill base-secreting cells. (1) Sharks feed opportunistically on a variety of fish and invertebrate prey. (2) H⁺/K⁺-ATPase (HKA, do not confuse with VHA) is involved in the secretion of HCl into the stomach lumen to aid in food digestion. (3) H⁺ secretion is accompanied by HCO₃⁻ secretion into the blood, inducing alkalosis. BT, bicarbonate transporter. (4) HCO₃⁻ travels in blood plasma and in red blood cells (RBC) to the gills. Inside RBCs, intracellular carbonic anhydrase (CA) hydrates HCO₃⁻ into CO₂. (5) In addition, extracellular CA located in the cell membrane of pillar cells (6) hydrates plasma HCO₃⁻ into CO₂. (6) CO₂ from both sources diffuses into VHA-rich base-secreting cells, where intracellular CA rehydrates it into HCO₃⁻ and H⁺. (7) Intracellular HCO₃⁻ stimulates soluble adenylyl cyclase (sAC), which (8) triggers the translocation of cytoplasmic vesicles containing VHA (blue icon) to the cell basolateral membrane. VHA then secretes H⁺ into the blood. (9) A putative basolateral channel brings Cl⁻ from VHA-rich cells into the blood. (10) Intracellular HCO₃⁻ is secreted to seawater in exchange for Cl⁻ via apical pendrin (Pd)-like anion exchangers. The combined action of H⁺ reabsorption by VHA and HCO₃⁻ secretion by pendrin corrects blood alkalosis. (11) Immunostaining of the VHA A subunit (brown) in gills of Pacific dogfish Squalus acanthias in early stages of blood alkalosis. VHA-rich cells display various stages of VHA translocation from fully cytoplasmic (asterisk) to fully basolateral (arrowhead). Arrows indicate the apical opening of two VHA-rich cells. Some lamellae blood spaces are labeled with pink circles, and © indicate some of the pillar cells. Based on Gilmour et al. (2007), Roa et al. (2014) and Tresguerres et al. (2005, 2006c, 2007b, 2010).
enriched in gill cell membranes from control and base-infused hagfish suggest that VHA is present both in cytoplasmic vesicles and in the basolateral membrane, and that – similar to results from sharks – VHA inserts into the basolateral membrane and corrects blood alkalosis (Tresguerres et al., 2007a). Cells with basolateral VHA are also found in gills from squid (Hu et al., 2014), crab (Tresguerres et al., 2008) and bone-eating Osedax worms (Tresguerres et al., 2013) (see below). However, it is not known whether VHA energizes HCO$_3^-$ secretion in any of these cells.

Apical pendrin and basolateral Cl$^-$ transport across channels results in Cl$^-$ loading in the blood of sharks. For example, sharks fed a meal corresponding to ∼5% of their body mass excrete HCO$_3^-$ at an average rate of ∼230 µmol kg$^{-1}$ h$^{-1}$ (with peak rates of >600 µmol kg$^{-1}$ h$^{-1}$ in some cases), with a total of ∼10.5 mmol kg$^{-1}$ HCO$_3^-$ excreted to seawater during the 2–48 h postprandial period (Wood et al., 2007). Although this is a significant amount of HCO$_3^-$ from an A/B perspective, the equivalent Cl$^-$ loading is negligible when considering that there is a constant concentration gradient that favours Cl$^-$ diffusion from seawater (∼500 mmol l$^{-1}$) into the blood (∼270 mmol l$^{-1}$).

Furthermore, continuous infusion of NaHCO$_3$ at a rate of 1 mmol kg$^{-1}$ h$^{-1}$ for 24 h did not significantly affect plasma [Cl$^-$] (Tresguerres et al., 2005), demonstrating that the Cl$^-$ load associated with HCO$_3^-$ secretion is efficiently handled by rectal gland NaCl secretion. However, the Cl$^-$ uptake that results from HCO$_3^-$ secretion has implications for ionicregulation in freshwater fishes. For example, the rates of Cl$^-$ uptake in larval (Bayaa et al., 2009) and adult zebrafish (Boisen et al., 1992) and rainbow trout (reviewed in Goss et al., 1994) have been reported as 200–700 µmol kg$^{-1}$ h$^{-1}$, and are therefore comparable to the rates of HCO$_3^-$ secretion (and thus Cl$^-$ uptake) in post-fed dogfish sharks. Additionally, basolateral VHA has been reported in gill ionocytes from freshwater stingray (Piermarini and Evans, 2001; Piermarini et al., 2002), bull shark (Reilly et al., 2011), killifish (Katoh et al., 2003) and rainbow trout (Tresguerres et al., 2006a), as well as in skin ionocytes from medaka embryos (Lin et al., 2012). An early study found evidence for Cl$^-$/HCO$_3^-$ exchange in the osmoconforming hagfish, and proposed that the driving force for the evolution of ion uptake in freshwater fish was A/B regulation (Evans, 1984). Here, I propose that one of the cellular mechanisms that allowed the ancestors of modern-day freshwater fish to invade freshwater was the translocation of VHA to the basolateral membrane, possibly as a result of food abundance that caused a prolonged (or continuous) alkaline tide. Over evolutionary time, mutations in genes encoding components of the sensing or translocation mechanisms could have resulted in VHA being permanently located in the basolateral membrane and acting primarily for Cl$^-$ uptake rather than HCO$_3^-$ secretion (but with the two processes remaining linked). Unfortunately, this idea cannot be experimentally tested.

**Potential role of VHA in bone dissolution by Osedax worms**

*Osedax* is a genus of mouthless and gutless marine worms that live on the seafloor and feed on the bones of dead vertebrates (Rouse et al., 2004). The posterior end of *Osedax* branches out and penetrates deep into the bone, resembling the roots of a tree, and these roots host symbiotic heterotrophic bacteria. *Osedax* worms derive their nutrition from collagen and lipids trapped in the bones (Goffredi et al., 2005; Rouse et al., 2004), which they release by secreting acid using VHA that is present at high levels in the apical membrane of root epithelial cells (Tresguerres et al., 2013). The proposed *Osedax* feeding mechanism is a multistep process that starts with VHA-mediated secretion of H$^+$ onto the bone, which dissolves the calcium phosphate matrix and releases collagen and lipids (Fig. 3). In order to produce H$^+$, CO$_2$ is hydrated to H$^+$ and HCO$_3^-$ by CA. The CO$_2$ is generated by aerobic metabolism – *Osedax* has a robust circulatory system that ensures sufficient O$_2$ delivery to the root epithelium (Huusgaard et al., 2012), and
epithelial cells that are rich in mitochondria and CA (Tresguerres et al., 2013). This implies that as H+ is secreted onto the bone, an equimolar amount of HCO$_3^-$ is secreted into the worm’s tissues. This excess HCO$_3^-$ load is likely transported in the blood to the gills and then secreted to the surrounding seawater. Because a subset of gill epithelial cells has immunostaining that is consistent with basalateral VHA localization (Tresguerres et al., 2013), HCO$_3^-$ might be secreted following the cellular mechanism described above for shark gills.

As the bone calcium phosphate matrix is dissolved, lipids and collagen are released and digested, with digestion of the latter likely aided by proteolytic enzymes (cf. Goffredi et al., 2005). The released nutrients must then be absorbed across the root epithelium and transported to the symbiotic bacteria hosted in Osedax roots, which then metabolize these nutrients into diverse compounds. Finally, these metabolites must be transferred back to the worm or possibly the worm digests the bacteria, or both (Katz et al., 2011).

Further research is needed to definitively characterize the Osedax feeding mechanism; however, this task is not trivial because Osedax typically live at depths of between 300 and 3000 m [although some Osedax species may be found at depths as shallow as 30 m off the coast of Sweden (Huusgaard et al., 2012)].

Acid secretion by VHA is a characteristic likely shared by other marine invertebrates that bore into the substrate. For example, VHA is highly abundant in the brush border of cells in the accessory boring organ from the snail _Nucella lamellosa_ (Clelland and Saleuddin, 2000), which drills through the calcium carbonate shells of its prey. And, although VHA has not yet been described in boring sponges, the etching cells (see Glossary) of these organisms have abundant vacuoles and CA activity (Pomponi, 1980), consistent with the presence of VHA. Furthermore, the proposed mechanism by which _Osedax_ might dissolve bone and absorb nutrients has striking similarities to the mechanism of bone resorption by vertebrate osteoclasts, which involves acid secretion by apical VHA (Blair et al., 1989; Li et al., 1999), the formation of a ‘resorptive pit’, the degradation of the bone organic matrix by proteolytic enzymes (Gowen et al., 1999), the absorption of bone-derived substances and the removal of HCO$_3^-$ (reviewed in Väänänen et al., 2000). If confirmed, this would represent a remarkable example of convergent evolution between an epithelium specialized for feeding (_Osedax_ roots) and cells specialized for bone remodelling (osteoclasts).

**VHA and a carbon-concentrating mechanism in the coral symbiosome**

Reef-building corals are cnidarian animals with endosymbiotic _Symbiodinium_ dinoflagellate algae living inside their gastrodermal cells. Photosynthesis by _Symbiodinium_ produces abundant carbohydrates, most of which are transferred to the animal host to be used as fuel to sustain metabolism (Falkowski et al., 1984; Muscatine et al., 1984). Because the ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo; see Glossary) enzyme of dinoflagellates has a low affinity for its CO$_2$ substrate (Rowan et al., 1996), CO$_2$ must be present at high concentrations in order for _Symbiodinium_ to photosynthesize. Thus, a carbon-concentrating mechanism (CCM) is necessary to sustain photosynthesis and prevent photorespiration (Reinfelder, 2011). However, this presents a physiological challenge, as there are no known molecular mechanisms to actively transport gases such as CO$_2$ and, at the pH of seawater and coral cell cytoplasm, the most abundant dissolved inorganic carbon (DIC) species is, by far, HCO$_3^-$. Recently, VHA in the host-derived symbiosome membrane has been shown to promote _Symbiodinium_ photosynthesis (Barott et al., 2015). The proposed mechanism (Fig. 4) involves acidification of the symbiosome space by VHA to pH~4, which presumably energizes the parallel transport of HCO$_3^-$ and favours the chemical speciation of DIC into CO$_2$. The evidence in support of this mechanism includes a high abundance of VHA in symbiosome membranes of gastrodermal cells, alkalinization of the symbiosome space from pH~4 to pH~5 in response to bafilomycin (a specific

---

**Fig. 4. VHA-dependent carbon-concentrating mechanism (CCM) in coral.**

Top: Diagram of coral tissues. The red box indicates the location of gastrodermal cells hosting _Symbiodinium_. Middle: VHA immunostaining (red) in the symbiosome membrane of coral gastrodermal cells (arrow). Bottom: Proposed coral CCM. The question marks indicate aspects of the mechanism that are speculative. (1) CO$_2$ (potentially derived from coelenteron and adjacent cells, or from metabolic processes) enters the gastrodermal cell. (2) Intracellular carbonic anhydrase (CA) catalyzes the hydration into H$^+$ and HCO$_3^-$. (3) VHA (blue) in the symbiosome membrane ‘pumps’ the H$^+$ into the symbiosome space, acidifying it to pH~4. (4?) Putative bicarbonate transporters (BT?) in the cell membrane could import HCO$_3^-$ into the cytoplasm, and a different subset of BTs in the symbiosome membrane could import HCO$_3^-$ into the symbiosome space. (5?) H$^+$ and HCO$_3^-$ combine to form CO$_2$ in the symbiosome space, which first diffuses inside _Symbiodinium_ and eventually reaches the site of RuBisCo through unknown mechanisms (???). (6) _Symbiodinium_ photosynthesis produces oxygen, as well as carbohydrates that are translocated to the coral host cell through (???) unknown mechanisms. Based on Barott et al. (2015) and Tresguerres et al. (in press).
inhibitor of VHA) in isolated cell experiments, and a significant decline in O₂ production in coral branches exposed to bafilomycin (Barott et al., 2015). This mechanism has been reported for two coral species belonging to the two different coral clades, robust and complex (Barott et al., 2015), so it is likely to apply to most, if not all, reef-building corals. Furthermore, given that most marine photosynthesizing organisms require a CCM (Reinfelder, 2011), and that VHA is evolutionarily conserved in eukaryotic cells, the mechanism described for carbon concentrating in corals may be widespread. For example, VHA mRNA abundance was upregulated in coccolithophores (unicellular eukaryotic phytoplankton with external calcium carbonate plates) exposed to low CO₂ levels (Bach et al., 2013); however, the functional relevance of this finding is not yet known.

Many questions about the potential coral CCM remain unanswered, including those about the source(s) of HCO₃⁻ and H⁺, the existence and identities of HCO₃⁻- and additional H⁺-transporting proteins in the various biological membranes of coral host cells and Symbiodinium, and whether VHA–symbiosome acidification actually promotes carbon fixation (and not just O₂ production). The role of the P-type H⁺-ATPase [the plasma membrane H⁺-ATPase typical of plants and fungi that is expressed by symbiotic but not free-living Symbiodinium (Bertucci et al., 2010)], is also unknown. Furthermore, the symbiosome space is equally acidic during both light and dark periods, at least under the isolated cell conditions that have been tested thus far (Barott et al., 2015). This raises the possibility that symbiosome acidification is not only required under light conditions, when photosynthesis is occurring, but is also involved in additional metabolic exchanges across the symbiosome membrane, such as the transport of NH₄⁺ and photosynthates, or the regulation of the Symbiodinium cell cycle.

Role of VHA in calcification

Although the mechanisms for making calcium carbonate skeletons and the chemical composition of such structures may vary greatly from organism to organism, some basic unifying principles seem to apply to all: Ca²⁺ and DIC must be transported into a confined space (intra- or extra-cellular), nucleating compounds (macromolecules or other ions) must be present and H⁺ must be removed to create a high pH environment. Conceivably, VHA could be involved in removing H⁺ from the site of calcification and providing the driving force for DIC (and maybe Ca²⁺) transport. Evidence for the role of VHA in calcification was provided by studies on sternal epithelial cells from the terrestrial isopod Porcellio scaber: VHA exhibits a switch in VHA polarity coincided with a reversal of the H⁺ gradient between the ecdysial space (see Glossary) and the hemolymph. Although the latter maintains a constant pH of ~7.6, the former has an acidic pH of ~6.9 during skeleton resorption and an alkaline pH of ~8.2 during skeleton deposition (Ziegler, 2008).

Basolateral VHA is also present in marine teleost intestinal epithelial cells (Guffey et al., 2011), which secrete HCO₃⁻ into the intestinal lumen to promote CaCO₃ precipitation and water absorption (Wilson et al., 2002), and it secretes an equimolar amount of H⁺ into the blood (Grosell and Genz, 2006). Other calcifying cells from invertebrate animals that express VHA include those in molluscan mantle (Barron et al., 2012; Li et al., 2016; Mann and Jackson, 2014) and hemocytes (Barron et al., 2012). However, it is currently unknown whether VHA is involved in promoting calcification in any of those cells. Coral calcifying cells have been proposed to also use VHA for intracellular pH regulation and for removing H⁺ from the site of calcification (Allemand et al., 2004); however, expression of VHA in those cells has not yet been determined.

There is also indirect evidence for the involvement of VHA in calcification in coccolithophores, as VHA was found on the coccolith vesicle membrane and is hypothesized to alkalinate the coccolith vesicle to promote coccolith formation (Corstjens et al., 2001). However, this mechanism would require pumping of H⁺ from an endomembrane into the cytosol, which has never been reported in any system to date (Mackinder et al., 2010), or the action of as yet unidentified electrogenic exchangers such as Na⁺/2H⁺, taking advantage of the voltage set by VHA across the coccolith vesicle membrane to remove H⁺ and alkalinate the vesicle. This would be similar to the alkalization mechanism described in the insect midgut (reviewed in Wieczorek et al., 1999).

VHA in relation to ocean acidification

The partial pressures of gases in the atmosphere and in the ocean are in equilibrium. As atmospheric CO₂ levels rise as a result of anthropogenic activity, CO₂ in the ocean also increases, which is predicted to decrease the surface ocean pH at a rate faster than the most rapid events in the geological past (Caldeira and Wickett, 2003). Understandably, studies on the responses of marine organisms to OA have recently greatly increased in number (at the time of this writing, a search for ‘ocean acidification’ returned 285 peer-reviewed papers in PubMed and over 7200 results in Google Scholar for publications since 2015). However, a solid understanding about the basic physiological mechanisms of these responses is in most cases lacking, preventing proper interpretation of results. A priori, VHA could be involved in at least three processes directly related to OA: (1) systemic and intracellular A/B regulation, (2) CCM and (3) calcification. The potential roles of VHA in each of these processes are discussed in more detail below.

A/B regulation

Because VHA secretes H⁺, a popular assumption is that VHA activity may be necessary to counteract acidosis in systemic fluids under conditions of OA (e.g. Deigweber et al., 2010; Harms et al., 2014; Hu et al., 2011; Kaniewska et al., 2012; Tseng et al., 2013). However, thermodynamic and energetic considerations of intra- and extracellular pH and [Na⁺] favour H⁺ secretion via Na⁺/H⁺ exchangers (NHEs) instead of VHA in marine environments. Briefly, the [Na⁺] in seawater (~500 mmol l⁻¹) is several-fold higher than that inside cells (10–100 mmol l⁻¹) (Willmer et al., 2004) because of the action of Na⁺/K⁺-ATPase (NKA), which actively transports Na⁺ out of cells while pumping K⁺ in. Conversely, the H⁺ concentration is much lower in seawater (pH~8.0, 10 nmol l⁻¹) than in cells (pH~7.4, 40 nmol l⁻¹) or in internal extracellular fluids such as blood (pH~7.70, 20 nmol l⁻¹). Even during unrealistically extreme OA scenarios, the Na⁺ gradient would be great enough to drive H⁺ secretion via NHEs. In fact, H⁺ secretion based on NKA and NHEs is favoured in any aquatic environment with [Na⁺] equal to or higher than 5 mmol l⁻¹ (Parks et al., 2008). A few studies have reported downregulation of VHA mRNA in gills from fish exposed to OA (e.g. Esbaugh et al., 2012; Tseng et al., 2013), which, as pointed out in a recent review (Heuer and Grosell, 2014), was most likely due to downregulation of branchial HCO₃⁻ secretion to seawater and H⁺ reabsorption into the blood.
As explained above, VHA in the symbiosome of reef-building corals’ gastrodermal cells is important for promoting O₂ production by symbiotic *Symbiodinium*, probably as part of a CCM (Barott et al., 2015). As an elevated level of CO₂ diminishes the need for an energy-consuming CCM, the abundance of symbiosomal VHA would potentially be reduced during OA. Consistent with this hypothesis, reduced VHA mRNA abundance has been reported in two previous studies on corals (Kaniewska et al., 2012, 2015); however, based on the transcriptomics approach used, it was not possible to determine in which cell type(s) these changes took place. Thus, the hypothesized reduction in the level of symbiosomal VHA in response to OA still requires experimental validation.

**Calcification**

OA may affect biological calcification in at least three, not mutually exclusive, ways: (1) by reducing the seawater concentration of CO₃⁻ [which some people believe is the ‘building block’ of skeletons (e.g. Cohen and Holcomb, 2009; Kleypas and Langdon, 2006), although others (Bach, 2015; Cyronak et al., 2015; Tresguerres et al., in press) disagree], (2) by increasing dissolution of already deposited calcium carbonate skeletons (Andersson et al., 2009; Eyre et al., 2014) and (3) by limiting the rate of H⁺ removal from the calcification site (carbonate skeletons (Andersson et al., 2009; Eyre et al., 2014) and (3) others (Bach, 2015; Cyronak et al., 2015; Tresguerres et al., in press) Cohen and Holcomb, 2009; Kleypas and Langdon, 2006), although differing in efficiency of only 5%. However, as mentioned above, VHA is unlikely to pump H⁺ to seawater for the purposes of systemic or pH regulation; if anything, it is involved in CCM or calcification. To calculate the change in VHA energetic demand, it is necessary to first establish the cellular and subcellular localization of VHA, the concentration of relevant ions in the different compartments and how (or whether) these parameters change in response to OA.

**Experimental considerations**

The potential simultaneous VHA localization in multiple cell types and organelles, together with its potential involvement in various cellular processes, greatly complicates studying responses to environmental stress using common proteomics and transcriptomics techniques. For example, under OA conditions, an organism could plausibly downregulate VHA in one location for CCM, but upregulate it in a separate compartment to allow calcification [such a situation is plausible in coccolithophores (Bach et al., 2013) and coral, among others]. However, analyses of VHA mRNA or protein abundance in whole tissues/organisms would not discriminate between the various pools of VHA, and therefore could lead to erroneous interpretation of the organism’s response to OA. Furthermore, as shown in the base-secreting shark gill cells, VHA (and most other proteins) can be regulated by post-translational means such as insertion or removal from the cell membrane, or phosphorylation, which are not detectable by transcriptomic and regular proteomic techniques. To understand the responses of VHA (and any other protein) to OA (and to any other source of stress), the localization of VHA in specific cells and subcellular compartments should be first determined, for example, using immunohistochemistry. After this basic information is obtained, systematic experiments should determine its physiological role. Only after the basic mechanism has been characterized can -omics techniques be confidently used.

**Conclusions**

Although VHA always pumps H⁺ across a biological membrane, its ultimate physiological role is determined by its subcellular localization in apical, basolateral or intracellular membranes, and its colocalization and interaction with other proteins. In conjunction with regulatory signalling cascades, this allows the VHA to mediate diverse functions such as A/B regulation, bone dissolution and carbon concentrating for photosynthesis, among others. Given the great biodiversity found in the oceans, future studies are likely to unveil novel VHA functions in marine organisms. Basic information about VHA presence, localization, function and regulation is essential for us to be able to understand potential responses to environmental challenges.

**Acknowledgements**

I would like to thank Dr Horst Onken (Wagner College, USA) and Dr Carlos Luquet (CEAN-CONICET, Argentina) for providing feedback about epithelial VHA function, Dr Greg Rouse ( Scripps Institution of Oceanography, UCSD, USA) for ideas and insightful discussions about VHA in Osedax, and Dr Davey Kline and Ms Jinae Roa (Scripps Institution of Oceanography, UCSD, USA) for general feedback on the manuscript. I also thank one anonymous reviewer for insightful comments about VHA molecular structure and function. This Commentary is based on a presentation on a previous 2016 conference paper. I am also grateful to Dr Horst Onken (Wagner College, USA) for providing feedback on an earlier draft.

**Energetic considerations**

Depending on the coupling efficiency between ATPase activity and H⁺ transport, the action of modulator proteins, the dissipation of membrane potential, the H⁺ permeability and other unidentified factors (reviewed in Stevens and Forgac, 1997), VHA can transport H⁺ against concentration gradients (ΔpH) of over 1000-fold (Davies et al., 1994; Kettner et al., 2003; Mindell, 2012; Müller et al., 1996). Logically, the ATP demand for H⁺ pumping increases proportionally to ΔpH, which might suggest that a drop in ocean pH associated with OA would result in an increased energy requirement for VHA activity. The ATP requirement of VHA at different values of ΔpH has been experimentally calculated in isolated yeast vesicles (Kettner et al., 2003) and plant vacuoles (Davies et al., 1994), and the ratio of 0.7H⁺/ATP per ΔpH unit is very consistent in those two distantly related organisms. For example, at ΔpH=4, yeast VHA can pump ~2 H⁺ for every ATP that is hydrolyzed (2H⁺/ATP), and at ΔpH=0 the ratio is over 4H⁺/ATP (Kettner et al., 2003). Based on these values, OA is unlikely to significantly affect the VHA-dependent energy budget of an organism. In a scenario where ΔpH=0.3 (equivalent to a drop in ocean pH from 8.0 to 7.70), the VHA would use 1 ATP to pump ~3.8 H⁺ instead of ~4, which represents a reduction in efficiency of only 5%. However, as mentioned above, VHA is unlikely to pump H⁺ to seawater for the purposes of systemic or pH regulation; if anything, it is involved in CCM or calcification. To calculate the change in VHA energetic demand, it is necessary to first establish the cellular and subcellular localization of VHA, the concentration of relevant ions in the different compartments and how (or whether) these parameters change in response to OA.
at the session ‘Osmoregulation: From magnesium to mosquitoes: a tribute to Klaus W. Beyenbach’, at the General Meeting of the Society for Experimental Biology (Prague, Czech Republic, 2015).

Competing interests
The authors declare no competing or financial interests.

Funding
M.T. was supported by grants from the National Science Foundation (NSF) [IOS 1354181 and EF 1220641] and a Sloan Research Fellowship from the Alfred P. Sloan Foundation [BR2013-103].

References


Mann, K. and Jackson, D. J. (2014). Characterization of the pigmented shell-forming proteome of the common g الروve snail Cepaea nemoralis. BMC Genomics 15, 249.


COMMENTARY


