The Journal of Experimental Biology 204, 3251–3259 (2001)
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JEB3546

Immunochemical analysis of the vacuolar proton-ATPase B-subunit in the gills of a euryhaline stingray (*Dasyatis sabina*): effects of salinity and relation to Na^+/K^+-ATPase

Peter M. Piermarini* and David H. Evans

University of Florida, Department of Zoology, Box 118525, 223 Bartram Hall, Gainesville, FL 32611, USA

*e-mail: pmpierma@zoo.ufl.edu

Accepted 2 July 2001

Summary

In the gills of freshwater teleost fishes, vacuolar proton-ATPase (V-H^+-ATPase) is found on the apical membrane of pavement and chloride (Na^+/K^+-ATPase-rich) cells, and is an important transporter for energizing Na^+ uptake and H^+ excretion. In the gills of elasmobranch fishes, the V-H^+-ATPase has not been extensively studied and its expression in freshwater individuals has not been examined. The goals of this study were to examine the effects of environmental salinity on the expression of V-H^+-ATPase in the gills of an elasmobranch (the Atlantic stingray, *Dasyatis sabina*) and determine if V-H^+-ATPase and Na^+/K^+-ATPase are expressed in the same cells. We found that gills from freshwater stingrays had the highest relative abundance of V-H^+-ATPase and greatest number of V-H^+-ATPase-rich cells, using immunoblotting and immunohistochemistry, respectively. When freshwater animals were acclimated to sea water for 1 week, V-H^+-ATPase abundance and the number of V-H^+-ATPase-rich cells decreased significantly. Atlantic stingrays from seawater environments were characterized by the lowest expression of V-H^+-ATPase and least number of V-H^+-ATPase-rich cells. In contrast to teleost fishes, localization of V-H^+-ATPase in freshwater stingray gills was not found in pavement cells and occurred on the basolateral membrane in cells that are presumably rich in mitochondria. In freshwater stingrays acclimated to sea water and seawater stingrays, V-H^+-ATPase localization appeared qualitatively to be stronger in the cytoplasm, which may suggest the transporter was stored in vesicles. Using a double-immunolabeling technique, we found that V-H^+-ATPase and Na^+/K^+-ATPase occurred in distinct cells, which suggests there may be two types of mitochondrion-rich cells in the elasmobranch gill epithelium. Based on these findings, we propose a unique model of NaCl and acid–base regulation where the V-H^+-ATPase-rich cells and Na^+/K^+-ATPase-rich cells are the sites of Cl^- uptake/HCO_3^- excretion and Na^+ uptake/H^+ excretion, respectively.

Key words: V-H^+-ATPase, Na^+/K^+-ATPase, gills, elasmobranch, stingray, salinity, euryhaline, *Dasyatis sabina*, NaCl regulation, acid–base regulation.

Introduction

The vacuolar proton-ATPase (V-H^+-ATPase) is a multi-subunit transporter that is important for energizing a variety of active transport processes in animals (see Nelson and Harvey, 1999, for a review). V-H^+-ATPase is composed of a catalytic V_1 domain that binds ATP and a membrane-bound V_0 domain that forms a channel for protons to cross the cell or vacuolar membrane (Wieczorek et al., 2000). The B-subunit of the V-H^+-ATPase is a component of the V_1 domain and the B-subunit amino acid sequence is highly conserved across a wide range of animal taxa (Sudhof et al., 1989; Gill and Ross, 1991; Filippova et al., 1998; Niederstatter and Pelster, 2000; Perry et al., 2000). Two isoforms of the B-subunit have been identified in mammals and fish; using mammalian nomenclature, they are a ubiquitous 58 kDa brain (B2) isoform and a 56 kDa renal (B1) isoform (Nelson et al., 1992; Niederstatter and Pelster, 2000).

V-H^+-ATPase has been studied extensively in the mammalian renal collecting duct and turtle urinary bladder where immunocytochemical and ultrastructural research has demonstrated two populations of mitochondrion-rich intercalated cells that acidify or alkalinize the urine. Acidifying (α) intercalated cells are characterized by an apical cell membrane localization of the V-H^+-ATPase, whereas alkalinizing (β) intercalated cells express the V-H^+-ATPase diffusely throughout their cytoplasm and on their basolateral membrane (Stetson and Steinmetz, 1985; Brown et al., 1988a; Brown et al., 1988b; Verlander et al., 1992; Verlander et al., 1994; Brown and Breton, 1996; Steinmetz et al., 1996; Brown and Breton, 2000). In mammals, this transporter is also important for acidification of the male reproductive tract and bone reabsorption by osteoclasts (Lee et al., 1996; Brown et al., 1997).

In aquatic vertebrates, V-H^+-ATPase has been implicated in the energization of NaCl uptake. For example, in frog and

toad skin it is well established that an apical V-H^+-ATPase, localized to mitochondrion-rich cells, generates a membrane potential (inside negative) that drives Na^+ entry into the epithelium *via* an apical Na^+ channel (Harvey, 1992; Ehrenfeld and Klein, 1997), and can energize active Cl^- uptake through apical Cl^-/HCO_3^- exchangers (Larsen et al., 1996). Recently, the V-H^+-ATPase has also been considered important for driving Na^+ uptake across the gill epithelium of freshwater fishes. Similar to processes in frog skin, it was proposed that an apical V-H^+-ATPase would generate a favorable electrical gradient to drive Na^+ uptake through an apical Na^+ channel (Lin and Randall, 1993; Lin et al., 1994; Sullivan et al., 1995).

This model of ion uptake in freshwater teleosts was supported by the results of Wilson et al. (Wilson et al., 2000), who found colocalization of the V-H^+-ATPase with the epithelial Na^+ channel (ENaC) on the apical membrane of pavement cells from the leading edge of tilapia gills (*Oreochromis mossambicus*). In rainbow trout (*Oncorhynchus mykiss*), V-H^+-ATPase and ENaC were expressed in both pavement and chloride (Na^+/K^+-ATPase-rich) cells (Wilson et al., 2000). Other supporting evidence for the proposed role of V-H^+-ATPase in freshwater teleost ion uptake includes bafilomycin inhibition of Na^+ uptake in tilapia (*O. mossambicus*) and carp (*Cyprinus carpio*) (Fenwick et al., 1999), and decreased activity and localization of branchial V-H^+-ATPase when freshwater rainbow trout are acclimated to sea water (Lin and Randall, 1993; Lin et al., 1994).

Since an apical V-H^+-ATPase in freshwater teleost gills would directly pump protons into the environment, this transporter has also been hypothesized to play an important role in systemic acid–base balance. Experiments on rainbow trout have corroborated this hypothesis by demonstrating that gill V-H^+-ATPase activity, immunoreactivity and mRNA expression all increase after exposure to environmental hypercapnia (Lin and Randall, 1993; Lin et al., 1994; Sullivan et al., 1995; Sullivan et al., 1996; Perry et al., 2000). Studies on branchial V-H^+-ATPase in a true marine teleost have yet to be published, but the transporter's role in acid–base regulation of seawater teleosts is assumed to be minimal, given the favorable Na^+ gradient for Na^+/H^+ exchangers (see Claiborne, 1998; Claiborne et al., 1999).

V-H^+-ATPase has been localized and/or measured in the gills of two marine elasmobranch species, *Squalus acanthias* and *Raja erinacea* (Kormanik et al., 1997; Wilson et al., 1997). In *S. acanthias*, Wilson et al. (Wilson et al., 1997) found V-H^+-ATPase immunoreactivity in mitochondrion-rich cells of the gill interlamellar region, presumably within cytoplasmic tubulovesicles. Interestingly, we found that Na^+/K^+-ATPase was also localized to cells of the interlamellar region in gills from marine Atlantic stingrays (*Dasyatis sabina*) (Piermarini and Evans, 2000). Therefore, it is possible that V-H^+-ATPase and Na^+/K^+-ATPase colocalize to the same branchial cell type in marine elasmobranchs.

The goals of this study were to examine the effects of environmental salinity on the expression of V-H^+-ATPase in the gills of the Atlantic stingray and determine if V-H^+-ATPase and Na^+/K^+-ATPase are expressed in the same cells. To date, V-H^+-ATPase expression in the gills of an elasmobranch acclimated to different salinities has not been studied. In a previous study, we found that expression of Na^+/K^+-ATPase in the Atlantic stingray (*Dasyatis sabina*) was affected by environmental salinity (Piermarini and Evans, 2000). Gills from freshwater stingrays were characterized by a three- to fourfold higher activity and abundance of Na^+/K^+-ATPase relative to marine individuals. Since the V-H^+-ATPase is considered important for ion uptake, we hypothesized a similar trend for this transporter.

Materials and methods

Animal collection and holding conditions

The animals used in this study were identical to those used by Piermarini and Evans (Piermarini and Evans, 2000). In brief, ten Atlantic stingrays *Dasyatis sabina* were captured from the St Johns River, FL, USA (Lake Jesup or Lake George) as described by Piermarini and Evans (Piermarini and Evans, 1998). Five of the freshwater rays were left in fresh water, while the other five were gradually acclimated to sea water (30–32 ‰) over 2 weeks. In addition, five marine Atlantic stingrays were captured *via* hook and line from Cedar Key, FL, USA, transported to Gainesville, FL, USA and held in a 379 liter closed-system tank containing sea water (30–32 ‰). All animals were fed live grass shrimp (*Palaemonetes* sp.) every other day, and were starved for 48 h prior to tissue collection.

Collection of gill tissue

Details of gill tissue collection for the immunoblotting and immunohistochemistry have been described (Piermarini and Evans, 2000). In brief, stingrays were perfused through the conus arteriosus with an elasmobranch Ringer's solution (Forster et al., 1972), then gill filaments were trimmed off the arches and placed in fixative (3 % paraformaldehyde, 0.05 % glutaraldehyde, 0.05 % picric acid in 10 mmol l^{-1} phosphate buffered saline, pH 7.3) for 24 h at 4 °C. Additional filaments were snap-frozen in liquid nitrogen for immunoblot analysis and stored at –80 °C until analyzed.

Anti- V-H^+-ATPase B-subunit antibody

The antibody used in this study was developed by Filippova et al. (Filippova et al., 1998) and is a rabbit polyclonal antibody made against a 279-amino-acid region (residues 79–357) of the V-H^+-ATPase B-subunit from the insect *Culex quinquefasciatus*. This region of the insect B-subunit V-H^+-ATPase shares 91 % amino acid identity with that published for teleost V-H^+-ATPase B-subunits (Niederstatter and Pelster, 2000; Perry et al., 2000). The antibody was kindly provided by Dr William Harvey, Whitney Laboratory, University of Florida (with permission from Dr Sarjeet Gill, University of California at Riverside).

Immunoblotting of V-H$^+$-ATPase B-subunit

Immunoblots were performed on polyvinylidene difluoride (PVDF) membranes (Bio-Rad) from a previous study (Piermarini and Evans, 2000), containing 20 μg of total gill membrane protein per lane. Details of tissue preparation, electrophoresis and blotting have been described (Piermarini and Evans, 2000). Since these PVDF membranes were previously used to detect Na^+/K^+-ATPase, it was necessary to 'strip' the antibodies that were bound to the membrane.

To remove previous antibodies, PVDF membranes were soaked in 100 % methanol for 15 min and placed in a Strip-Buffer (62 mmol l^{-1} Tris-base, 2 % sodium lauryl sulfate, 0.6 % β-mercaptoethanol, pH 6.7) for 30 min at 60 °C to 'strip' previous primary and secondary antibodies off the PVDF. After 'stripping' the PVDF, it was washed three times with dH_2O (5 min each). The PVDF was reblocked with Blotto (Boehringer) for 1.5 h at 25 °C, and then transferred to the primary antibody solution (polyclonal rabbit anti-insect V-H$^+$-ATPase B-subunit diluted 1:10,000 in Blotto) and incubated overnight at 4 °C.

After primary antibody incubation, the PVDF was washed three times (15 min each) with Tris-buffered saline + 1 % Tween-20 (TTBS) with 5 % dry milk, then incubated with an alkaline-phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad; diluted 1:3000 in Blotto) for 2 h at 25 °C. The PVDF was then washed three times (15 min each) with TTBS, and a substrate solution (Bio-Rad Immun-Star ECL Kit) applied to the PVDF for 5 min at 25 °C to initiate a luminescent signal. Binding of antibody was detected by exposing Hyperfilm-ECL imaging film (Amersham) to the PVDF membrane. Film was developed according to the manufacturer's protocol. As a control, we incubated stripped membranes with normal rabbit or goat serum instead of the primary antibody and found no detectable signal.

Negatives were digitized into TIFF files using a UMAX flatbed scanner with transparency adapter, and analyzed using *NIH Image* version 1.61 (National Institutes of Health, USA). To quantify the relative abundance of the V-H$^+$-ATPase B-subunit, we measured the intensity of the immunopositive bands using densitometry and standardized all measurements to the FW condition. Therefore, all intensity measurements of FW individuals were normalized to a value of 1.0.

Immunohistochemical localization of V-H$^+$-ATPase B-subunit

Histological tissues used in this study were identical to those described previously (Piermarini and Evans, 2000). In brief, 6 μm serial sections of paraffin-embedded gill tissue were cut parallel to the long axis of the filament and placed on poly-L-lysine coated slides. Sections were deparaffinized in Hemo-De, hydrated in a graded ethanol series, and washed in 10 mmol l^{-1} phosphate-buffered saline (PBS). A hydrophobic PAP-Pen (Electron Microscopy Suppliers) was used to draw circles around the tissue sections, and then 3 % H_2O_2 was placed on the sections for 30 min to inhibit endogenous peroxidase activity. Sections were also blocked with *Biogenex* Protein Block (BPB; normal goat serum with 1 % bovine serum albumin, 0.09 % NaN_3 and 0.1 % Tween-20) for 20 min before application of the primary antibody.

The primary antibody (polyclonal rabbit anti-insect V-H$^+$-ATPase B-subunit diluted 1:10,000 in BPB) was incubated on the sections overnight at 4 °C. The antibody was rinsed off and the sections were washed in PBS for 5 min. The sections were then incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Biogenex) and a horseradish peroxidase-labeled strepavidin solution (Biogenex) for 20 min each at 25 °C. After washing with PBS for 5 min, antibody binding was visualized by applying the chromagen DAB (3,3′-diaminobenzidine tetra-hydrochloride; Biogenex) to the sections for 5 min at 25 °C. No staining was detected when non-immune rabbit serum or BPB was used instead of primary antibody.

The number of immunopositive (V-H$^+$-ATPase-rich) cells per gill lamella and per interlamellar region was counted to quantify the distribution of these cells. For each animal, three immunostained slides were chosen. On a section from each slide, the number of V-H$^+$-ATPase-rich cells was counted on 30 randomly selected lamellae and interlamellar regions. Lengths of lamellae were also measured to standardize cell counts to lamellar length. Results are expressed as number of V-H$^+$-ATPase-rich cells per 100 μm of lamella, per interlamellar region, and per 100 μm of lamella + interlamellar region ('sum').

Double-labeling of V-H$^+$-ATPase and Na$^+$/K$^+$-ATPase

To determine if V-H$^+$-ATPase and Na^+/K^+-ATPase are expressed in the same cells, we used a double-labeling technique modified from the method of Verlander et al. (Verlander et al., 1996). Tissue sections for double-labeling were deparaffinized, hydrated, blocked and stained for V-H$^+$-ATPase as described above. However, after being developed with the brown chromagen (DAB), the slides were rinsed in dH_2O for 10 min and reblocked with BPB for 20 min. A mouse anti-chicken Na^+/K^+-ATPase antibody (monoclonal antibody a5 culture supernatant diluted 1:100 in normal goat serum) was then applied to the sections overnight at 4 °C. The primary antibody, a5, developed by Dr Douglas Fambrough, was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. Rinsing and developing was performed as described above, except a blue chromagen was used (Vector SG). Double-labeled sections were then observed to determine if V-H$^+$-ATPase and Na^+/K^+-ATPase occurred in the same cells.

Statistical analyses

Differences in mean number of V-H$^+$-ATPase-rich cells were detected using a one-way analysis of variance (ANOVA), with a Student–Newman–Keuls *post-hoc* test. Differences in relative intensities of bands from immunoblots

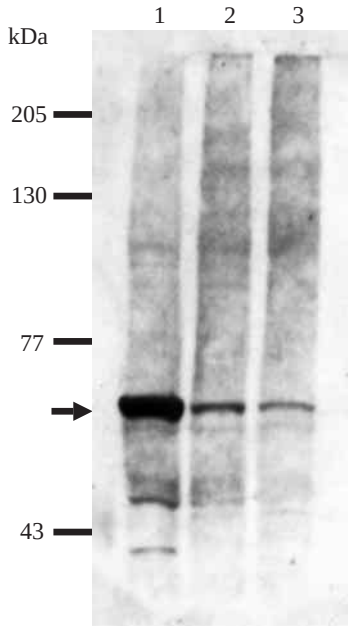

Fig. 1. Representative immunoblot for V-H$^+$-ATPase B-subunit in gill membrane enrichments from freshwater (lane 1), seawater-acclimated (lane 2) and seawater (lane 3) Atlantic stingrays. The positions of molecular mass markers (kDa) are shown. The antibody recognized a 60.5±1.08 kDa protein in gills (arrow). Note the reduction in band intensity from lanes 1 to 3.

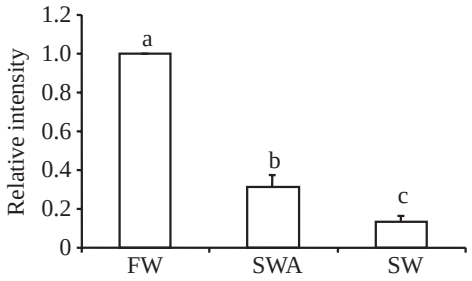

Fig. 2. Relative intensity of 60.5 kDa band representing the B subunit of V-H$^+$-ATPase in the gills of freshwater (FW), seawater-acclimated (SWA) and seawater (SW) stingrays. *N*=5 for all groups. Although statistical differences were detected with a non-parametric test (Kruskal–Wallis ANOVA), values are shown as means + 1 S.E.M. for clarity. Lower case letters indicate statistical categorization of groups as determined by a Kruskal–Wallis *post-hoc* test (P <0.05).

were detected using a Kruskal–Wallis non-parametric ANOVA, with a Kruskal–Wallis *post-hoc* test (Conover, 1980). All tests were 2-tailed and differences were considered significant if P<0.05.

Results

Immunoblotting of V-H$^+$-ATPase B-subunit

The antibody raised against the insect B-subunit detected a 60.5±1.08 kDa peptide in stingray gills (Fig. 1), which is close to the expected size of 58 kDa reported for the ubiquitous B2 isoform (Nelson and Harvey, 1999). Changes in relative abundance of the V-H$^+$-ATPase B-subunit associated with environmental salinity were examined using semi-quantitative immunoblotting. We found that branchial B-subunit relative abundance was highest in freshwater stingrays, followed by seawater-acclimated and seawater stingrays (Fig. 2).

Immunohistochemistry of V-H$^+$-ATPase B-subunit

We used immunohistochemistry to determine if environmental salinity affected the number, distribution and localization of V-H$^+$-ATPase-rich cells in the gill epithelium. We found that the 'sum' (lamellar + interlamellar) number of branchial V-H$^+$-ATPase-rich cells was different among the three groups, as mean 'sum' number of V-H$^+$-ATPase-rich cells was highest in freshwater, intermediate in seawater-acclimated, and lowest in seawater stingrays (Fig. 3, Fig. 4A). The distribution of V-H$^+$-ATPase-rich cells was also different among the three groups, as the mean number of V-H$^+$-ATPase-rich cells per 100 μm of lamellae was highest in freshwater, intermediate in seawater-acclimated and lowest in seawater stingrays (Fig. 3, Fig. 4B). Numbers of V-H$^+$-ATPase-rich cells on the interlamellar region were also reduced, but did not change as dramatically (Fig. 3, Fig. 4C).

Regardless of salinity, immunohistochemical staining for the V-H$^+$-ATPase B-subunit occurred in relatively large cells of the gill epithelium, and did not appear to associate with pavement or mucous cells (Fig. 5). Localization of the transporter within the V-H$^+$-ATPase-rich cells appeared to be qualitatively different among the three groups of stingrays. In freshwater stingrays, V-H$^+$-ATPase-rich cells were characterized by diffuse staining throughout the cytoplasm and discrete localization along the basolateral plasma membrane (Fig. 5A). In V-H$^+$-ATPase-rich cells of seawater-acclimated and seawater stingrays, staining appeared to be darker in the cytoplasm and less discrete along the basolateral membrane, relative to freshwater individuals (Fig. 5).

Double-labeling of V-H$^+$-ATPase and Na$^+$/K$^+$-ATPase

To determine if V-H$^+$-ATPase was expressed in the same cells as Na$^+$/K$^+$-ATPase we conducted a double-labeling technique, which resulted in cells stained brown (V-H$^+$-ATPase) and blue (Na$^+$/K$^+$-ATPase). Regardless of environmental salinity or location (lamellae *versus* interlamellar region), expression of V-H$^+$-ATPase and Na$^+$/K$^+$-ATPase occurred in separate cells (Fig. 6). It is interesting to note that V-H$^+$-ATPase-rich cells on gill lamellae of freshwater stingrays were usually located near the base of lamellae, whereas Na$^+$/K$^+$-ATPase-rich cells were usually found on the distal parts of lamellae (Fig. 6A). The functional implications of this distribution are not known. On the interlamellar regions, V-H$^+$-ATPase-rich and Na$^+$/K$^+$-ATPase-rich cells were interspersed among each other (Fig. 6).

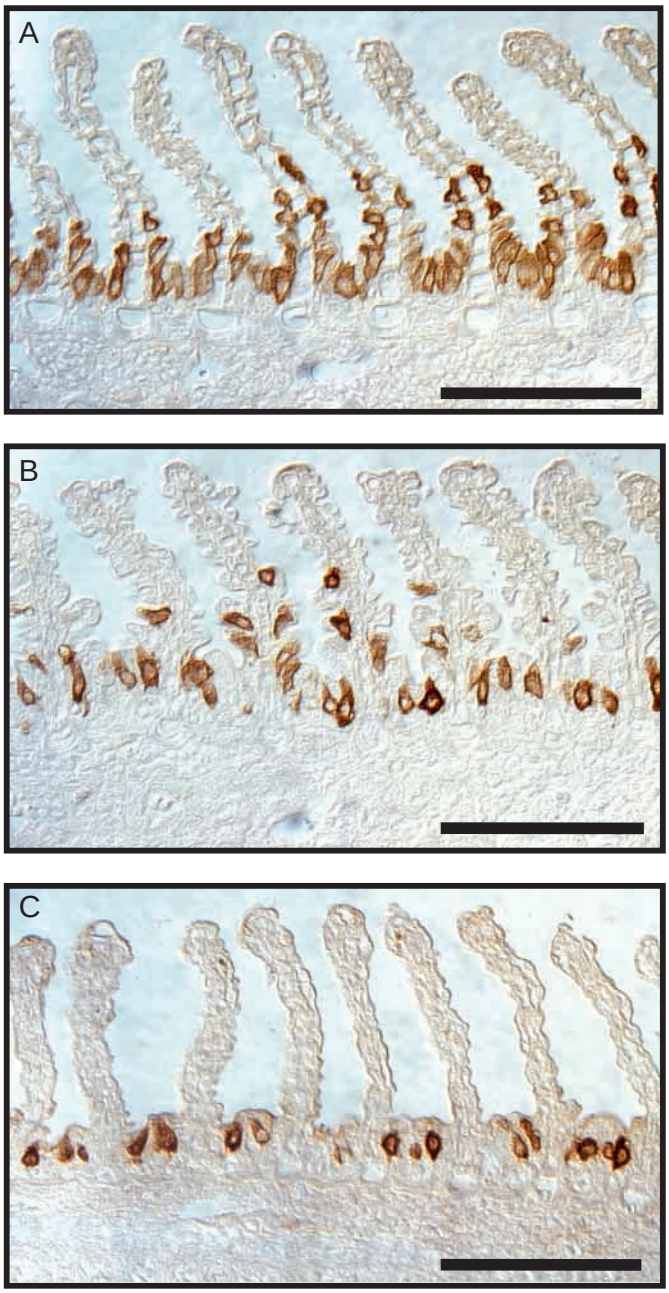

Fig. 3. Representative photomicrographs of immunostaining for V-H⁺-ATPase in longitudinal sections of gill filaments from freshwater (A), seawater-acclimated (B) and seawater (C) Atlantic stingrays (×400). Scale bars, 100 μm. V-H⁺-ATPase-rich cells occurred on lamellae (finger-like projections) and/or interlamellar regions (basal to and between lamellae). Note the differences in abundance and distribution (lamella *versus* interlamellar region) of V-H⁺-ATPase-rich cells among groups.

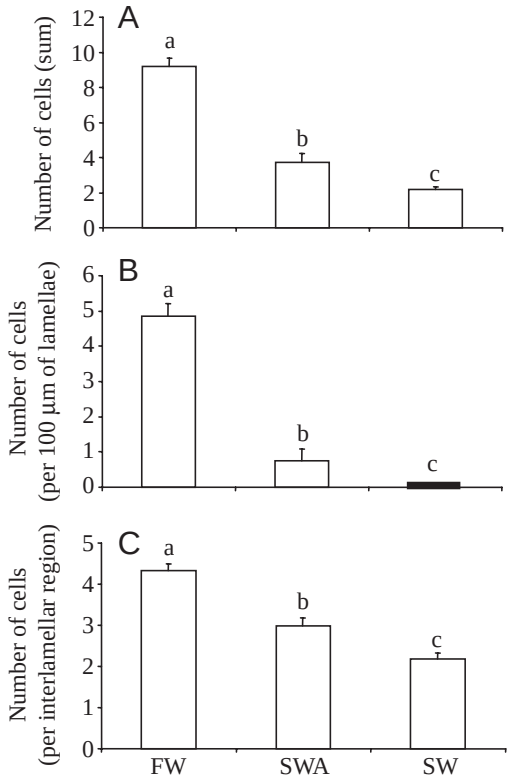

Fig. 4. V-H⁺-ATPase-rich cell numbers in the gills of freshwater (FW), seawater-acclimated (SWA) and seawater (SW) Atlantic stingrays. (A) 'Sum' number of V-H⁺-ATPase-rich cells (per 100 μm of lamella + per interlamellar region); (B) number of V-H⁺-ATPase-rich cells per 100 μm of lamella; (C) number of V-H⁺-ATPase-rich cells per interlamellar region. Values are means + 1 S.E.M. Lower case letters indicate statistical categorization of the means as determined by a Student–Newman–Keuls *post-hoc* test ($P<0.05$). Note that both mean and S.E.M. for the number of V-H⁺-ATPase-rich cells per 100 μm of lamella = 0 in SW stingrays.

Discussion

Results from the immunoblotting component of this study suggest that environmental salinity affects relative abundance of the V-H⁺-ATPase B-subunit in gills from the Atlantic stingray (Fig. 1, Fig. 2). We have previously reported a similar effect of environmental salinity on branchial Na^+/K^+-ATPase relative abundance and activity in Atlantic stingrays (Piermarini and Evans, 2000). These findings imply that the gill epithelium of freshwater stingrays has an overall greater active transport potential relative to seawater stingrays, possibly for the active uptake of NaCl and extrusion of H^+/HCO_3^-.

When freshwater stingrays were acclimated to sea water for 1 week, branchial V-H⁺-ATPase relative abundance decreased significantly (Fig. 2). This effect of salinity on branchial V-H⁺-ATPase expression has also been reported in a teleost (*Oncorhynchus mykiss*), where gill V-H⁺-ATPase activity and immunoreactivity decreased when freshwater trout were acclimated to sea water (Lin and Randall, 1993; Lin et al., 1994). A lower branchial V-H⁺-ATPase expression for the seawater-acclimated stingrays was expected, because active NaCl uptake would not be necessary in a seawater environment and passive Na^+/H^+ and Cl^-/HCO_3^- exchangers are thought to be responsible for acid–base extrusion in seawater fishes (Claiborne, 1998).

The immunohistochemical results of this study suggest that

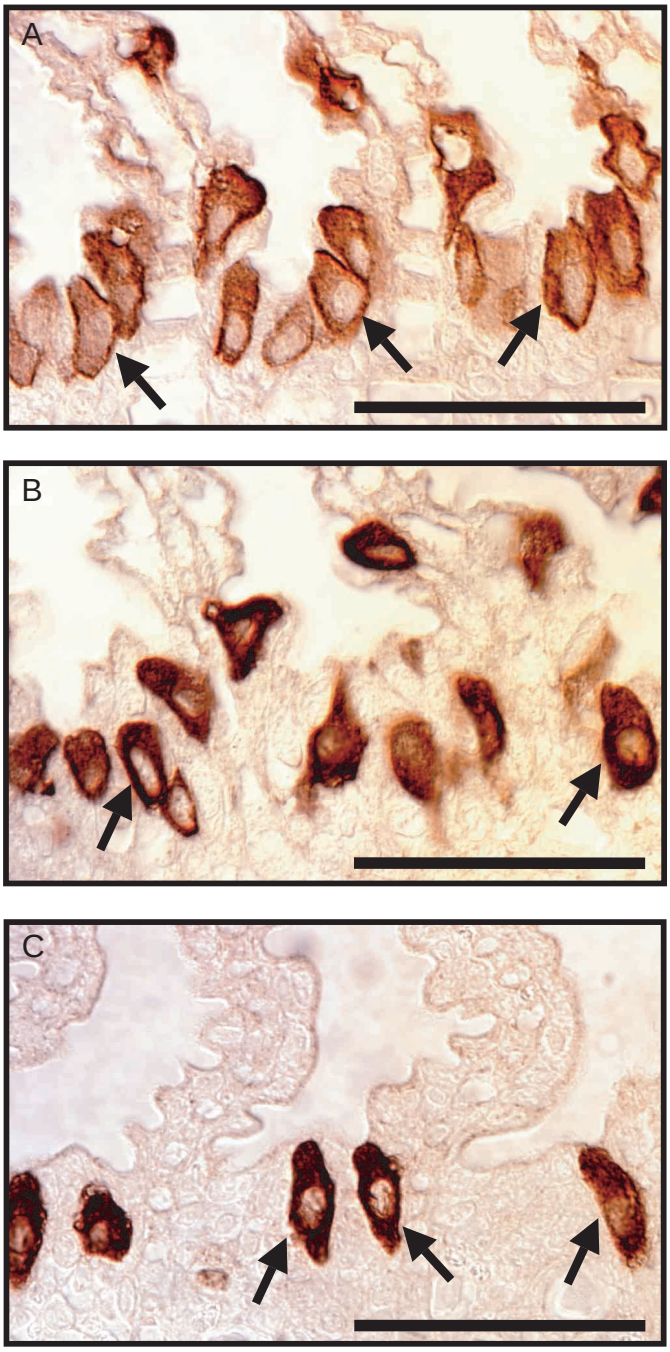

Fig. 5. Representative high-magnification (×1000) photomicrographs of V-H^+-ATPase-rich cells in gills from freshwater (A), seawater-acclimated (B), and seawater (C) Atlantic stingrays. Scale bars, 50 μm. Arrows indicate cells that best demonstrate the qualitative differences in staining observed among the groups. In freshwater stingrays, localization of V-H^+-ATPase was diffuse throughout the cytoplasm and discrete along the basolateral plasma membrane of relatively large cells. In seawater-acclimated and seawater stingrayss V-H^+-ATPase staining appeared to be stronger in the cytoplasm and less discrete along the basolateral membrane, relative to freshwater individuals. In all groups, no distinct staining was observed on the apical plasma membrane of V-H^+-ATPase-rich cells.

the higher overall abundance of V-H^+-ATPase in freshwater stingray gills (Fig. 2) can be attributed to a greater number of V-H^+-ATPase-rich cells found in the gill epithelium, especially on the lamellae (Fig. 3, Fig. 4). The dramatic decrease in the number of V-H^+-ATPase-rich cells found on the lamellae in the seawater-acclimated and seawater stingrays may suggest that those cells have a specialized function for freshwater NaCl and acid–base balance. We have previously reported a similar effect of environmental salinity on the number and distribution of Na^+/K^+-ATPase-rich cells in the gills of the Atlantic stingray (Piermarini and Evans, 2000).

In freshwater stingrays, localization of V-H^+-ATPase occurred diffusely throughout the cytoplasm and was associated with the basolateral membrane of relatively large cells (Fig. 5), presumably mitochondrion-rich (see Wilson et al., 1997). It was expected that the V-H^+-ATPase would be localized to the apical cell membrane of pavement cells and/or Na^+/K^+-ATPase-rich cells as it has been described in freshwater teleost species (Lin et al., 1994; Sullivan et al., 1995; Wilson et al., 2000). Basolateral localization of V-H^+-ATPase is relatively rare in vertebrates and to date has only been described in β-type intercalated cells of the mammalian collecting duct and turtle urinary bladder (Stetson and Steinmetz, 1985; Brown et al., 1988a; Brown et al., 1988b; Verlander et al., 1992; Brown and Breton, 1996). If the V-H^+-ATPase-rich cells of freshwater stingray gills are analogous in function to β-type intercalated cells then they would be involved with HCO_3^- excretion and Cl^- uptake *via* an apical Cl^-/HCO_3^- exchanger (Weiner and Hamm, 1990). This would be in contrast to freshwater teleost fishes in which Cl^-/HCO_3^- exchange is thought to occur in Na^+/K^+-ATPase-rich chloride cells (Sullivan et al., 1996; Wilson et al., 2000).

In the seawater-acclimated and seawater stingray gills, there appeared to be qualitative differences in the V-H^+-ATPase labeling, such as stronger staining in the cytoplasm and less distinct staining along the basolateral membrane, relative to freshwater individuals (Fig. 5). Although ultrastructural studies would be required to quantify these qualitative differences, our findings are consistent with V-H^+-ATPase regulation in other vertebrate tissues, in which recycling of the transporter between a cytoplasmic pool of vesicles and the plasma membrane has been demonstrated (Dixon et al., 1986; Stetson and Steinmetz, 1986; Verlander et al., 1992; Verlander et al., 1994; Brown and Breton, 2000). Therefore, the qualitative staining differences may indicate that seawater-acclimated and seawater stingrays have more V-H^+-ATPase stored in cytoplasmic vesicles, and less transporter on the basolateral membrane, relative to freshwater stingrays. If the V-H^+-ATPase-rich cells are functionally analogous to β-intercalated cells (see above), then this trend would be expected, because Cl^-/HCO_3^- exchange in marine stingrays could be driven by the favorable gradient for Cl^- to enter the cells from sea water, rather than the active generation of a gradient by a V-H^+-ATPase.

In seawater stingrays, V-H^+-ATPase-rich cells were only

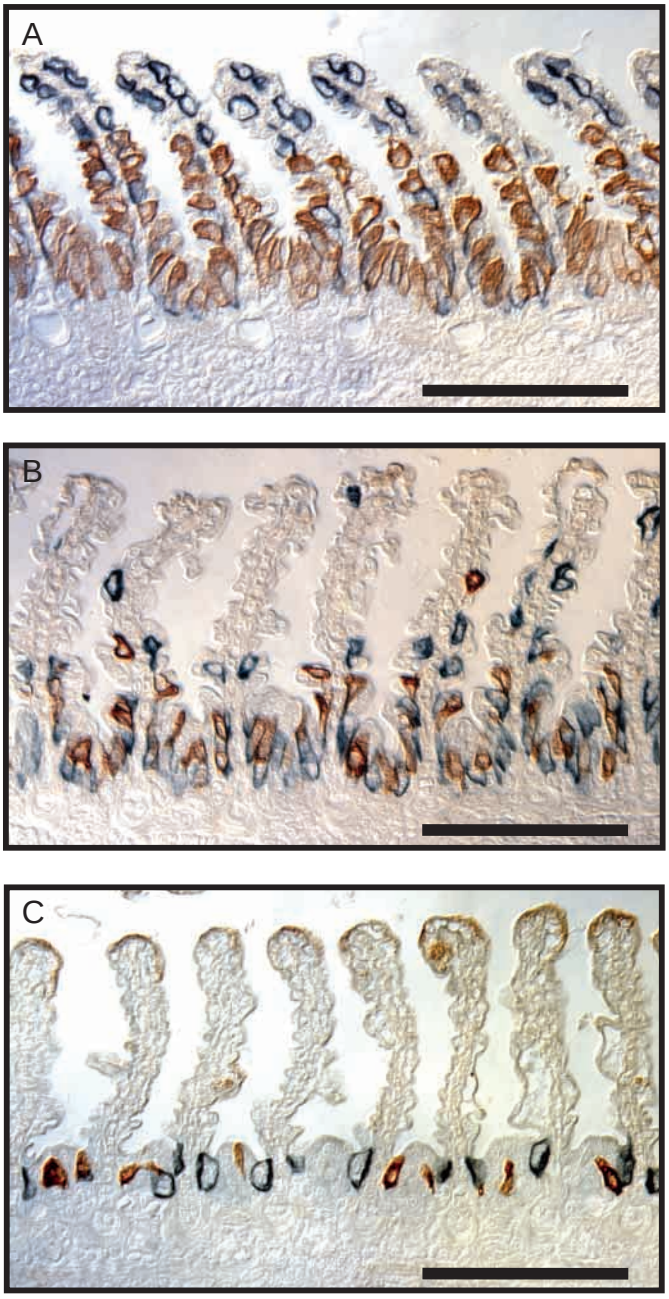

Fig. 6. Representative photomicrographs of double labeling for V-H$^+$-ATPase-rich (brown) and Na$^+$/K$^+$-ATPase-rich (blue) in longitudinal sections of gill filaments from freshwater (A), seawater-acclimated (B), and seawater (C) Atlantic stingrays (×400). Scale bars, 100 μm. Note that regardless of salinity there were separate brown and blue cells, indicating the two transporters occur in distinct cell types.

found on the interlamellar region of the gills, which corroborates the results of Wilson et al. (Wilson et al., 1997) who localized V-H$^+$-ATPase in the gills of a seawater elasmobranch (*Squalus acanthias*) using an antibody to the A-subunit. Wilson et al. (1997) reported a cytoplasmic localization for V-H$^+$-ATPase in mitochondrion-rich cells, and suggested it was stored in tubulovesicles that may be recruited to the apical membrane under conditions of acidosis (similar to α-intercalated cells). However, we hypothesize that these vesicles would be recruited to the basolateral membrane and function similar to a β-type intercalated cell. This would imply that another cell type and transporter are involved with acid excretion (see below).

Since branchial V-H$^+$-ATPase (this study) and Na$^+$/K$^+$-ATPase (Piermarini and Evans, 2000) abundance and distribution were affected similarly by salinity, we were interested in determining whether these two transporters are localized to the same cells. Results from double-labeling gills for V-H$^+$-ATPase and Na$^+$/K$^+$-ATPase demonstrated that these two transporters are in separate cells, regardless of environmental salinity (Fig. 6). This is important because it suggests there may be two types of ionocytes in the gill epithelium of elasmobranch fishes. Previous studies have suggested the elasmobranch gill epithelium contains two types of mitochondrion-rich cells, based on the appearance of two distinct apical cell membrane morphologies (Laurent and Dunel, 1980; Crespo, 1982). Our study provides the first immunohistochemical evidence for two mitochondrion-rich cell populations in the elasmobranch gill.

The finding of separate V-H$^+$-ATPase-rich and Na$^+$/K$^+$-ATPase-rich cells also has important functional implications. For example, this separation may indicate that Cl^- uptake/HCO_3^- excretion and Na^+ uptake/H^+ excretion occur in V-H$^+$-ATPase-rich and Na$^+$/K$^+$-ATPase-rich cells, respectively. Segregation of Cl^- and Na^+ uptake (and HCO_3^- and H^+ excretion) is known to occur in the mammalian collecting duct and turtle urinary bladder; β-type intercalated cells express basolateral V-H$^+$-ATPase, which helps drive HCO_3^- excretion and Cl^- uptake *via* an apical Cl^-/HCO_3^- exchanger (Stetson et al., 1985; Stetson and Steinmetz, 1985; Stetson and Steinmetz, 1986; Brown et al., 1988a; Verlander et al., 1992), α-type intercalated cells express apical V-H$^+$-ATPase that drives H^+ excretion and HCO_3^- reabsorption *via* a band-3 Cl^-/HCO_3^- exchanger (Stetson and Steinmetz, 1985; Stetson and Steinmetz, 1986; Brown et al., 1988a; Verlander et al., 1988), and principal cells express basolateral Na$^+$/K$^+$-ATPase that helps drive Na^+ uptake *via* an apical ENaC (Kashgarian et al., 1985; Alvarez de la Rosa et al., 2000). We propose that the V-H$^+$-ATPase-rich cells in the stingray gill are functionally analogous to β-type intercalated cells and are the putative sites of Cl^- uptake and HCO_3^- excretion. In contrast, we propose that the Na$^+$/K$^+$-ATPase-rich cells are a functional amalgam of α-type intercalated cells and principal cells. We hypothesize that Na$^+$/K$^+$-ATPase-rich cells excrete H^+ and absorb Na^+, but use different apical mechanisms than the mammalian collecting duct and turtle urinary bladder, such as an apical NHE isoform that has been colocalized to Na$^+$/K$^+$-ATPase-rich cells in the gills of marine elasmobranchs (Sue Edwards, Georgia Southern University, personal communication). Our hypothetical model of branchial NaCl and acid–base transport in the Atlantic stingray is presented in Fig. 7.

In conclusion, we found that branchial V-H$^+$-ATPase expression was different among Atlantic stingrays acclimated

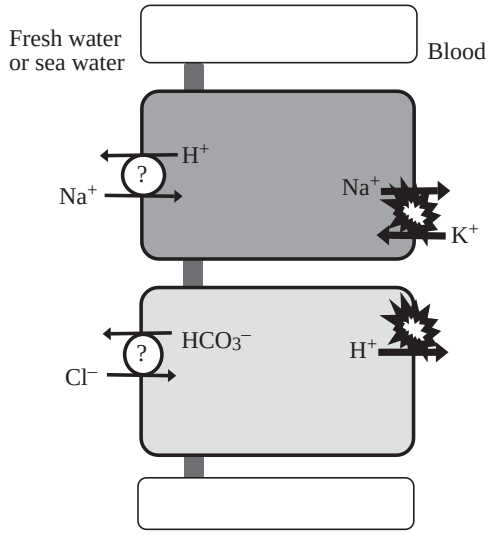

Fig. 7. Hypothetical model of NaCl and acid–base transport in the gills of the Atlantic stingray. Results from this and an earlier study (Piermarini and Evans, 2000) suggest that V-H^+-ATPase and Na^+/K^+-ATPase occur on the basolateral cell membrane of distinct mitochondrion-rich cell types. We hypothesize that the V-H^+-ATPase-rich cells act as base excreting cells *via* an apical Cl^-/HCO_3^- exchanger that would also result in Cl^- uptake. In contrast, we hypothesize that Na^+/K^+-ATPase-rich cells act as acid-excreting cells *via* an apical NHE that would also result in Na^+ uptake. This model can explain NaCl and acid–base transport in both freshwater and seawater stingrays. For example, in freshwater animals, the gradient for NaCl entry into the cells is unfavorable, therefore ATPases would be required to establish electrochemical gradients to drive Na^+/H^+ and Cl^-/HCO_3^- exchange, which is supported by the greater expression and number of ATPase-rich cells found in freshwater stingray gills. In seawater animals, the gradient for NaCl entry into the cells is favorable, therefore ATPases would not be as important for driving Na^+/H^+ and Cl^-/HCO_3^- exchange, which is supported by the lower overall expression and number of ATPase-rich cells found in seawater-acclimated and seawater stingrays. In addition, the qualitative differences in V-H^+-ATPase staining that we observed (see Fig. 5) may suggest that trafficking of cytoplasmic vesicles containing V-H^+-ATPase to the basolateral membrane is enhanced in freshwater stingrays, which would enhance active proton transport across this membrane. ? indicates that the presence of the transporter needs to be demonstrated in the Atlantic stingray.

to different environmental salinities. The basolateral localization of V-H^+-ATPase suggests that it may be involved with active HCO_3^- excretion and Cl^- uptake, which is in contrast to the transporter's proposed role of H^+ excretion and Na^+ uptake in freshwater teleost fishes. The lack of apical V-H^+-ATPase staining in the stingray gill suggests that another cell type may be involved with acid excretion, possibly Na^+/K^+-ATPase-rich cells. Results from this and an earlier study (Piermarini and Evans, 2000) have demonstrated that relatively high branchial expression of V-H^+-ATPase and Na^+/K^+-ATPase in the Atlantic stingray both appear to be important for life in fresh water. It is possible that these two transporters play a key role in the ability of euryhaline elasmobranchs to invade freshwater environments and both should be examined in other euryhaline species (e.g. *Carcharhinus leucas*) to test this hypothesis. Additionally, it would be interesting to study branchial V-H^+-ATPase and Na^+/K^+-ATPase expression in the stenohaline freshwater Potamotrygonid stingrays of the Amazon River to see if they have a pattern similar to that of freshwater Atlantic stingrays. This would determine if the relatively long evolution of Potamotrygonids in fresh water that has resulted in a similar body fluid osmotic composition to that of freshwater teleosts (e.g. lack of plasma urea, low plasma [NaCl]; Thorson et al., 1967) has also led to a pattern of V-H^+-ATPase and Na^+/K^+-ATPase expression that resembles the freshwater teleosts (Wilson et al., 2000). In any event, it is apparent that our understanding of the branchial mechanisms responsible for NaCl and acid–base regulation in fishes is far from complete and continues to be an area of exciting research.

We would like to thank the following people and organizations for their input and/or assistance with this study: Dr Lauren Chapman, Keith Choe, Dr Sarjeet Gill, Dr Louis Guillette, Jr, Dr William Harvey, Nicole Reid, Dr Colette St Mary, Marianne Thompson, Dr Jill Verlander-Reed, University of Florida Zoology Department, and the University of Florida Seahorse Key Marine Laboratory. This work was supported by an EPA-STAR Graduate Research Fellowship U-915419-01-0 (P.M.P.) and NSF Grant IBN-9604824 (D.H.E.).

References

Alvarez de la Rosa, D., Canessa, C. M., Fyfe, G. K. and Zhang, P. (2000). Structure and regulation of amiloride-sensitive sodium channels. *Annu. Rev. Physiol.* **62**, 573–594.

Brown, D. and Breton, S. (1996). Mitochondria-rich, proton-secreting epithelial cells. *J. Exp. Biol.* **199**, 2345–2358.

Brown, D. and Breton, S. (2000). H^+V-ATPase-dependent luminal acidification in the kidney collecting duct and the epididymis/vas deferens: vesicle recycling and transcytotic pathways. *J. Exp. Biol.* **203**, 137–145.

Brown, D., Hirsch, S. and Gluck, S. (1988a). An H^+-ATPase in opposite plasma membrane domains in kidney epithelial cell subpopulations. *Nature* **331**, 622–624.

Brown, D., Hirsch, S. and Gluck, S. (1988b). Localization of a proton-pumping ATPase in rat kidney. *J. Clin. Invest.* **82**, 2114–2126.

Brown, D., Smith, P. J. S. and Breton, S. (1997). Role of V-ATPase-rich cells in acidification of the male reproductive tract. *J. Exp. Biol.* **200**, 257–262.

Claiborne, J. B. (1998). Acid-Base Regulation. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 177–198. Boca Raton: CRC Press.

Claiborne, J. B., Blackston, C. R., Choe, K. P., Dawson, D. C., Harris, S. P., MacKenzie, L. A. and Morrison, S. A. I. (1999). A mechanism for branchial acid excretion in marine fish: Identification of multiple Na^+/H^+ antiporter (NHE) isoforms in gills of two seawater teleosts. *J. Exp. Biol.* **202**, 315–324.

Conover, W. J. (1980). *Practical Nonparametric Statistics*. New York: John Wiley & Sons.

Crespo, S. (1982). Surface morphology of dogfish (*Scyliorhinus canicula*) gill epithelium and surface morphological changes following treatment with zinc sulfate: A scanning electron microscope study. *Mar. Biol.* **67**, 159–166.

Dixon, T. E., Clausen, C., Coachman, D. and Lane, B. (1986). Proton transport and membrane shuttling in turtle bladder epithelium. *J. Membr. Biol.* **94**, 233–243.

Ehrenfeld, J. and Klein, U. (1997). The key role of the H^+ V-ATPase in acid–base balance and Na^+ transport processes in frog skin. *J. Exp. Biol.* **200**, 247–256.

Fenwick, J. C., Wendelaar Bonga, S. E. and Flik, G. (1999). In vivo bafilomycin-sensitive Na^+ uptake in young freshwater fish. *J. Exp. Biol.* **202**, 3659–3666.

Filippova, M., Ross, L. S. and Gill, S. S. (1998). Cloning of the V-ATPase B subunit cDNA from *Culex quinquefasciatus* and expression of the B and C subunits in mosquitoes. *Insect Mol. Biol.* **7**, 223–232.

Forster, R. P., Goldstein, L. and Rosen, J. K. (1972). Intrarenal control of urea reabsorption by renal tubules of the marine elasmobranch, *Squalus acanthias*. *Comp. Biochem. Physiol.* **42A**, 3–12.

Gill, S. S. and Ross, L. S. (1991). Molecular cloning and characterization of the B subunit of a vacuolar H^+-ATPase from the midgut and Malpighian tubules of *Helicoverpa virescens*. *Arch. Biochem. Biophys.* **291**, 92–99.

Harvey, B. J. (1992). Energization of sodium absorption by the H^+-ATPase pump in mitochondria-rich cells of frog skin. *J. Exp. Biol.* **172**, 289–309.

Kashgarian, M., Biemesderfer, D., Caplan, M. and Forbush, B. (1985). Monoclonal antibody to Na^+/K^+-ATPase: immunocytochemical localization along nephron segments. *Kidney Int.* **28**, 899–913.

Kormanik, G. A., Wilkins, T. and Banks, J. (1997). Proton-transporting ATPase activity in crude homogenates of gill tissue from the little skate, *Raja erinacea*. *Bull. Mt. Desert Isl. Biol. Lab.* **36**, 60–62.

Larsen, E. H., Christoffersen, B. C., Jensen, L. J., Sorensen, J. B. and Willumsen, N. J. (1996). Role of mitochondria-rich cells in epithelial chloride uptake. *Exp. Physiol.* **81**, 525–534.

Laurent, P. and Dunel, S. (1980). Morphology of gill epithelia in fish. *Am. J. Physiol.* **238**, R147–R159.

Lee, B. S., Holliday, L. S., Ojikutu, B., Krits, I. and Gluck, S. L. (1996). Osteoclasts express the B2 isoform of vacuolar H^+-ATPase intracellularly and on their plasma membranes. *Am. J. Physiol.* **270**, C382–C388.

Lin, H., Pfeiffer, D. C., Vogl, A. W., Pan, J. and Randall, D. J. (1994). Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. *J. Exp. Biol.* **195**, 169–183.

Lin, H. and Randall, D. J. (1993). Proton-ATPase activity in crude homogenates of fish gill tissue: Inhibitor sensitivity and environmental and hormonal regulation. *J. Exp. Biol.* **180**, 163–174.

Nelson, N. and Harvey, W. R. (1999). Vacuolar and plasma membrane proton-adenosine triphosphatases. *Physiol. Rev.* **79**, 361–385.

Nelson, R. D., Guo, X. L., Masood, K., Brown, D., Kalkbrenner, M. and Gluck, S. (1992). Selectively amplified expression of an isoform of the vacuolar H^+-ATPase 56-kilodalton subunit in renal intercalated cells. *Proc. Natl. Acad. Sci. USA* **89**, 3541–3545.

Niederstatter, H. and Pelster, B. (2000). Expression of two vacuolar-type ATPase B subunit isoforms in swimbladder gas gland cells of the European eel: nucleotide sequences and deduced amino acid sequences. *Biochim. Biophys. Acta* **1491**, 133–142.

Perry, S. F., Beyers, M. L. and Johnson, D. A. (2000). Cloning and molecular characterisation of the trout (*Oncorhynchus mykiss*) vacuolar H^+-ATPase B subunit. *J. Exp. Biol.* **203**, 459–470.

Piermarini, P. M. and Evans, D. H. (1998). Osmoregulation of the Atlantic stingray (*Dasyatis sabina*) from the freshwater Lake Jesup of the St John River, Florida. *Physiol. Zool.* **71**, 553–560.

Piermarini, P. M. and Evans, D. H. (2000). Effects of environmental salinity on Na^+/K^+-ATPase in the gills and rectal gland of a euryhaline elasmobranch (*Dasyatis sabina*). *J. Exp. Biol.* **203**, 2957–2966.

Stetson, D. L., Beauwens, R., Palmisano, J., Mitchell, P. P. and Steinmetz, P. R. (1985). A double-membrane model for urinary bicarbonate secretion. *Am. J. Physiol.* **249**, F546–F552.

Steinmetz, P. R., Kohn, O. F. and Hand, A. R. (1996). Scales of urine acidification: apical membrane-associated particles in turtle bladder. *Kidney Int.* **49**, 1655–1659.

Stetson, D. L. and Steinmetz, P. R. (1985). Alpha and beta types of carbonic anhydrase-rich cells in turtle bladder. *Am. J. Physiol.* **249**, F553–F565.

Stetson, D. L. and Steinmetz, P. R. (1986). Correlation between apical intramembrane particles and H^+ secretion rates during CO_2 stimulation in turtle bladder. *Pflugers Arch.* **407**, S80–S84.

Sudhof, T. C., Fried, V. A., Stone, D. K., Johnston, P. A. and Xie, X. S. (1989). Human endomembrane H^+ pump strongly resembles the ATP-synthetase of Archaebacteria. *Proc. Natl. Acad. Sci. USA* **86**, 6067–6071.

Sullivan, G., Fryer, J. and Perry, S. (1995). Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. *J. Exp. Biol.* **198**, 2619–2629.

Sullivan, G. V., Fryer, J. N. and Perry, S. F. (1996). Localization of mRNA for the proton pump (H^+-ATPase) and Cl^-/HCO_3^- exchanger in the rainbow trout gill. *Can. J. Zool.* **74**, 2095–2103.

Thorson, T. B., Cowan, M. C. and Watson, D. E. (1967). Potamotrygon spp.: Elasmobranchs with low urea content. *Science* **158**, 375–377.

Verlander, J. W., Madsen, K. M., Low, P. S., Allen, D. P. and Tisher, C. C. (1988). Immunocytochemical localization of band 3 protein in the rat collecting duct. *Am. J. Physiol.* **255**, F115–F125.

Verlander, J. W., Madsen, K. M., Galla, J. H., Luke, R. G. and Tisher, C. C. (1992). Response of intercalated cells to chloride depletion metabolic alkalosis. *Am. J. Physiol.* **262**, F309–F319.

Verlander, J. W., Madsen, K. M., Cannon, J. K. and Tisher, C. C. (1994). Activation of acid-secreting intercalated cells in rabbit collecting duct with ammonium chloride loading. *Am. J. Physiol.* **266**, F633–F645.

Verlander, J. W., Madsen, K. M. and Tisher, C. C. (1996). Axial distribution of band 3-positive intercalated cells in the collecting duct of control and ammonium chloride-loaded rabbits. *Kidney Int. Suppl.* **57**, S137–S147.

Weiner, I. D. and Hamm, L. L. (1990). Regulation of intracellular pH in the rabbit cortical collecting tubule. *J. Clin. Invest.* **85**, 274–281.

Wieczorek, H., Grber, G., Harvey, W. R., Huss, M., Merzendorfer, H. and Zeiske, W. (2000). Structure and regulation of insect plasma membrane H^+V-ATPase. *J. Exp. Biol.* **203**, 127–135.

Wilson, J. M., Laurent, P., Tufts, B. L., Benos, D. J., Donowitz, M., Vogl, A. W. and Randall, D. J. (2000). NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J. Exp. Biol.* **203**, 2279–2296.

Wilson, J. M., Randall, D. J., Vogl, A. W. and Iwama, G. K. (1997). Immunolocalization of proton-ATPase in the gills of the elasmobranch, *Squalus acanthias*. *J. Exp. Zool.* **278**, 78–86.