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Immunolocalization of Na^+/K^+-ATPase, Carbonic Anhydrase II, and Vacuolar H^+-ATPase in the Gills of Freshwater Adult Lampreys, *Geotria australis*

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ABSTRACT As adults, anadromous lampreys migrate from seawater into freshwater rivers, where they require branchial ion (NaCl) absorption for osmoregulation. In teleosts and elasmobranchs, pharmological, immunohistochemical, and molecular data support roles for Na^+/K^+-ATPase (NPPase), carbonic anhydrase II (CAII), and vacuolar H^+-ATPase (V-ATPase) in two different models of branchial ion absorption. To our knowledge, these transport-related proteins have not been studied in adult freshwater lampreys, and therefore it is not known if they are expressed, or have similar functions, in lampreys. The purpose of this study was to localize NPPase, CAII, and V-ATPase in the gills of adult freshwater lampreys and determine if any of these transport-related proteins are expressed in the same cells. Heterologous antibodies were used to localize the three proteins in gill tissue from pouched lamprey (*Geotria australis*). Immunoreactivity (IR) for all three proteins occurred between, and at the base of, lamellae in cells that match previous descriptions of mitochondrion-rich-cells (MRCs). NPPase-IR was always on the basolateral side of cells that did not stain for CAII or V-ATPase. In contrast, CAII-IR was always on the apical side of cells that also contained diffuse V-ATPase-IR. Therefore, we have identified two types of MRC in adult freshwater lamprey gills based on immunohistochemical staining for three transport proteins. A model of ion transport, based on our results, is proposed for adult freshwater lampreys. *J. Exp. Zool. 301A:654–665, 2004.*

INTRODUCTION

The pouched lamprey (*Geotria australis*), is an anadromous fish with a four-stage life cycle. The first is an ammocoete larval stage that is spent in fresh water environments (Potter et al., '80). The ammocoete then undergoes a metamorphosis into a young adult that migrates downstream to seawater (Potter et al., '82). Following a period of growth at sea, adult lampreys stop feeding and re-enter river systems where they migrate upstream to shallow waters for reproduction (Hardisty and Potter, '72). As the adults re-enter fresh water from seawater, they must switch from a hyposmotic strategy requiring ion secretion by the gills to a hyperosmotic strategy requiring ion absorption by the gills (Morris, '72). Like the gill epithelium of freshwater teleosts and elasmobranchs, the gill epithelium of these upstream migrating lampreys is thought to absorb Na^+ and Cl^- ions from the environmental water in exchange for secretion of H^+ and HCO_3^-, respectively (Bartels et al., '98).

Mechanisms of branchial Na^+-absorption/H^+-secretion and Cl^--absorption/HCO_3^--secretion

Abbreviations
BPB Biogenex's protein block
α5 NPPase antibody
CAII CAII antibody
E11 V-ATPase antibody

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have been studied extensively in freshwater teleosts such as salmonids and tilapia (reviewed by Claiborne, '98; Claiborne et al., 2002; Marshall, 2002). Most of these studies support a model where H^+ secretion is driven by vacuolar H^+-ATPase (V-ATPase), which secondarily drives Na^+ uptake through an epithelial Na^+ channel (ENaC) (Lin et al., '94; Sullivan et al., '95; Perry et al., 2000; Wilson et al., 2000b, 2002b). Although evidence is limited, Cl^--absorption/HCO_3^- secretion is thought to be via a band III-like Cl^-/HCO_3^- exchanger (Sullivan et al., '96; Wilson et al., 2000b, 2002b). Carbonic anhydrase has been localized in the gills of teleosts, and is thought to support ion transport by providing H^+ and HCO_3^- to V-ATPase and Cl^-/HCO_3^- exchangers, respectively (Dimberg and Hoglund, '87; Rahim et al., '88; Kültz et al., '92). The specific location of these proteins is more controversial, and probably varies with water characteristics and the species of teleost. However, V-ATPase is generally believed to be in the apical membranes of pavement cells and Cl^-/HCO_3^- exchangers are thought to be in the apical membranes of freshwater chloride cells (Wilson et al., 2000b). These freshwater chloride cells are morphological equivalents of Na^+ and Cl^- secreting chloride cells of seawater teleosts, which contain numerous mitochondria and express high levels of basolateral Na^+/K^+-ATPase (NPPase) (Perry, '97; Marshall, 2002).

Studies of branchial Na^+-absorption/H^+-secretion and Cl^--absorption/HCO_3^--secretion mechanisms in freshwater elasmobranchs are limited to the euryhaline Atlantic stingray (*Dasyatis sabina*) and the stenohaline Potamotrygonids. Atlantic stingrays (*D. sabina*) have two types of mitochondrion-rich-cells (MRCs) in their branchial epithelium, one expressing high levels of NPPase in the basolateral membrane, and the other expressing high levels of V-ATPase in the basolateral membrane (Piermarini and Evans, 2000, 2001a). The expression of both transporters was shown to be markedly higher in fresh water than in seawater, and therefore both MRC types are thought to absorb ions (Piermarini and Evans, 2001a). Specifically, the stingray NPPase-rich cells are thought to absorb Na^+ via an apical Na^+ transporter that is driven by an intracellular $[Na^+]$ made low by NPPase. Although evidence is lacking for Atlantic stingrays, this apical Na^+ transporter has been predicted to be a Na^+/H^+ exchanger (NHE), which has been localized to the apical membrane of marine elasmobranch gills (Edwards et al., 2002). V-ATPase-rich cells are thought to absorb Cl^- and secrete HCO_3^- via an apical anion exchanger similar to the recently discovered pendrin transporter of mammalian thyroid glands and B type collecting duct intercalated cells (Piermarini et al., 2002). Carbonic anhydrase has not been studied in a freshwater elasmobranch, but is known to be expressed in the gills of at least one seawater elasmobranch (*Squalus acanthias*) (Swenson and Claiborne, '85; Wilson et al., 2000c). Although a kinetic and pharmological study by Wood et al. (2002) was unable to determine what specific types of transporters are responsible for branchial Na^+-absorption/H^+-secretion and Cl^--absorption/HCO_3^--secretion in Potamotrygonid rays of the Amazon River, they are thought to be similar to those of euryhaline elasmobranchs.

Based on ultrastructural morphology studies, at least two epithelial cell types thought to be responsible for ion transport have been identified in adult lamprey gills (Morris, '57, '75; Nakao, '77; Peek and Youson, '79a; Bartels, '89; Bartels et al., '96, '98). The best characterized are called chloride cells, which first develop in the interlamellar area of metamorphosizing young adults, and persist in feeding adults at sea (Morris, '57). Because of several morphological traits and their association with seawater environments (Peek and Youson, '79a; Peek and Youson, '79b), lamprey chloride cells are thought to secrete NaCl in seawater via a mechanism identical to chloride cells of marine teleost gills (reviewed by Karnaky, '98). However, unlike in teleosts where chloride cells persist in fresh water (Perry, '97), lamprey chloride cells degenerate when adults reenter fresh water to begin their up-stream spawning migration (Morris, '57; Nakao, '77), and therefore cannot be involved in Na^+-absorption/H^+-secretion or Cl^--absorption/HCO_3^--secretion in fresh water. The putative ion-transporting cell types thought to have this role have been given several names, including chloride uptake cells (Morris, '57), type 3 cells (Nakao, '77), and MRCs (Bartels et al., '98). They occur in ammocoetes, degenerate in seawater adults, and then regenerate as adults reenter fresh water (Morris, '57; Bartels et al., '98). The apical membrane of these putative ion-transporting cells includes either well-developed microvilli or microfolds, and as one of their names indicates, they contain numerous mitochondria (Nakao, '77; Bartels et al., '98). Lastly, Bartels et al. ('98) used freeze-fracture replicas to show that there may be two subtypes of these MRCs, one subtype with high-densities of globular and

rod-shaped particles in the apical membrane and the other subtype with these particles in the basolateral membranes.

The presence of two subtypes of MRCs in freshwater adult lamprey gills suggests that mechanisms of ion absorption in lampreys resemble those of elasmobranch gills rather than those of teleost gills (see above). However, no studies have identified or localized ion transporters or transport-related enzymes in the gills of freshwater adult lampreys using immunological or molecular techniques. Therefore, this study used immunohistochemical techniques to identify and localize NPPase, V-ATPase, and CAII –immunoreactive branchial epithelial cells in the gills of upstream migrating freshwater adult lampreys. These proteins have defined roles in teleost and elasmobranch models of ion absorption, and therefore locating them is an important step in developing a detailed model of branchial ion transport mechanisms in freshwater lampreys.

MATERIALS AND METHODS

Animals and holding conditions

Adult lampreys on their upstream migration were captured in eel traps from the Derwent River, Tasmania and shipped directly to Deakin University, Geelong, Victoria. The lampreys were maintained in recirculating, filtered fresh water at 10°C and a 12 h dark/light cycle for three to five days.

Tissue collection and preparation

Lampreys were anaesthetized in ethyl-m-amino benzoate (MS–222) (1:1000) diluted in tank water. Gills were removed from each animal, and either snap frozen in liquid nitrogen for immunoblots or placed into a modified Zambonies fixative (4% paraformaldehyde, 10% picric acid in a 0.1 M phosphate buffer pH 7.4) for immunohistochemistry.

Antibodies

The mouse monoclonal antibody for NPPase (α5) was developed by Dr. Douglas Fambrough, and was obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development of the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. It was made against the alpha subunit of *Gallus domesticus* (chicken) NPPase and binds to all isoforms. This antibody recognizes fish NPPase, and is now used widely for studies on fish branchial cells (Choe et al., '99, 2002; Piermarini and Evans, 2000, 2001a; Wilson et al., 2000b; Edwards et al., 2002).

The rabbit polyclonal antibody for CAII (CAII) was a gift from Paul Linser at the Whitney Laboratory and Department of Anatomy and Cell Biology, University of Florida. It was made against chicken retinal CAII, and has been used to localize CAII in fish tissues (Linser, '91; Wilson et al., 2000a, 2002a).

The monoclonal antibody for V-ATPase (E11) was a gift from Stephen Glück at the Department of Medicine, University of Florida. It was made against a peptide that represents the carboxy-terminal 10 residues of the bovine kidney 31 kDa E subunit of V-ATPase, and has been used to localize V-ATPase (e.g. Yurko and Glück, '87; Bastani and Haragsim, '96; Bastani and Glück, '97).

Immunoblotting

Frozen gill tissue was placed into ice cold homogenization buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, 100μg/ml Phenylmethylsulfonyl Fluoride, and 5 mg/ml protease inhibitor cocktail [Sigma, St. Louis, MO]). The tissue was homogenized, and then centrifuged to remove cell debris (1,200 $\times$ g for 15 min). The supernatant was then centrifuged (20,700 $\times$ g for 25 min) and the resulting pellet was suspended in a minimal volume of 50% homogenizing buffer and 50% modified Laemmli sample buffer (1.21 M Tris, 10% glycerol, 0.4% SDS). The protein concentration was measured by BCA protein assay reagent (Pierce Endogen, Rockford, IL) according to manufacturer's protocol. Twenty-five μg of total protein was resolved in a 12% polyacrylamide gel using a mini protean II electrophoresis cell (90V for 5 h at 4°C) (Bio-Rad, Hercules, CA). Proteins were then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes.

The PVDF membranes were blocked in Blotto (5% non-fat milk in Tris-buffered saline) for 2 h at about 24°C, and then incubated in Blotto with either CAII (1:40,000) or E11 (1:10), overnight on ice. Membranes were then washed with four changes (15 min each) of Tris-buffered saline with 0.1% Tween–20 (TBST), and incubated in either goat anti-rabbit or goat anti-mouse IgG secondary antibody (Bio-Rad, alkaline-phosphatase conjugated) diluted 1:3000 in Blotto for 1–2 h at about 24°C. After four more washes in TBST, the

membranes were incubated in Bio-Rad's Immun-Star ECL substrate for 5 min at 24°C. Luminescent bands were then detected with Amersham-Pharmacia's Hyperfilm-ECL, according to the manufacturer's protocol. Lastly, immunoblot results were digitized with a Cannon flatbed scanner.

Immunohistochemical localization

Immunohistochemistry was carried out on paraffin embedded sections as described previously (Piermarini and Evans, 2000, 2001a), with minor modifications. Fixed gill tissues were dehydrated in an ethanol series, and embedded in paraffin wax. Sections were cut at 6 μm, and dried onto poly-L-lysine-coated slides. The tissue sections were then deparaffinized in Citrisolv (Fisher Scientific, Pittsburgh, PA) and rehydrated in an ethanol series ending with phosphate-buffered saline (PBS). A hydrophobic barrier was created around each section with a PAP-pen (Electron Microscopy Suppliers, Fort Washington, PA), and endogenous peroxidase activity was inhibited by incubating with 3% H_2O_2 for 25 min at about 24°C. Non-specific binding sites on the tissues were blocked by incubating with protein block (BPB: normal goat serum with 1% bovine serum albumin, 0.09% NaN_3, and 0.1% Tween–20) (Biogenex, San Ramon, CA) for 20 min. Sections were incubated with one of three primary antibodies, diluted in BPB: α5 (1/100), CAII (1/40,000), or E11 (1/25) overnight at 4°C, in a humidified chamber. Alternatively, negative control sections were incubated with BPB lacking antibodies. Unbound primary antibodies were removed with a 5 min rinse in PBS. Sections were then incubated with multilink solution (biotinylated goat anti-mouse, rabbit, guinea pig, and rat antibodies diluted in BPB), followed with horseradish-peroxidase streptavidin solution for 20 min at 24°C each (all from Biogenex). After another wash in PBS for 5 min, antibody binding was visualized by incubating with either 3,3'–diaminobenzadine tetrahydrochloride (DAB) or Vector SG (Biogenex) for five min at 24°C. Sections were then rinsed with running tap water for 5 minutes, dehydrated in an ethanol-Citrosolv series, and mounted permanently with a coverslip using permount (Fisher Scientific). For the high magnification micrographs of E11 staining, sections were counterstained with hematoxylin to help determine what region(s) of the cells stained.

Double-labeling experiments

A double labeling technique, described previously (Piermarini and Evans, 2001a), was used to compare α5, CAII, and V-ATPase staining. Gill sections were deparaffinized, hydrated, and stained with either antibody CAII or E11 as described above. However, after rinsing with water, the sections were re-blocked with BPB for 20 min, and incubated with either antibody α5 or CAII as above. Concentrations of antibodies during the second staining were 1/10 or 1/50 for α5 and 1/20,000 for CAII. The first antibody was detected with DAB and the second antibody was detected with Vector SG. Lastly, sections were permanently mounted as described above.

RESULTS

Immunoblots

The polyclonal antibody for CAII bound to a single, 32 kDa band on immunoblots of lamprey gill (Fig. 1A). The monoclonal antibody E11 bound to a single, 29 kDa band (Fig. 1B). None of the secondary antibodies bound to bands on immunoblots of gill tissue (not shown).

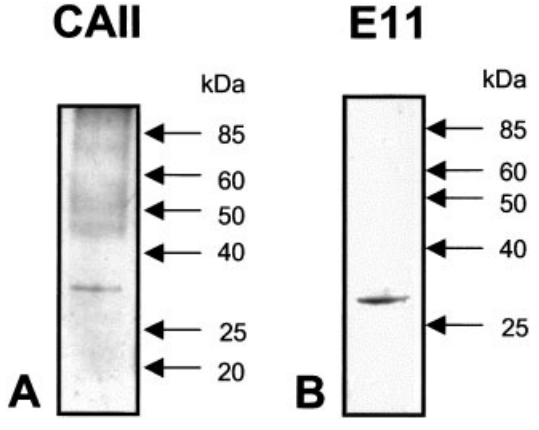

Fig. 1. Representative immunoblots of antibodies CAII (**A**) and E11 (**B**) with proteins from lamprey gills. The CAII antibody recognized a single protein band of approximately 32 kDa (A) and the E11 antibody recognized a single protein band of approximately 29 kDa (B). There was also some weak association of the CAII antibody with proteins above 40 kDa. E11 = V-ATPase antibody. The locations of molecular weight markers are indicated to the right of each blot.

Immunohistochemistry

Antibodies α5, CAII, and E11 all displayed strong immunoreactivity (IR) with epithelial cells of lamprey gills (Figs. 2 and 3). As seen in low magnification micrographs, the strongest IR for all three antibodies occurred in epithelial cells between, and at the bases of the lamellae (Fig. 2). At concentrations of the three antibodies needed to observe specific labeling in epithelial cells, no staining was observed in vascular and structural tissues that lie underneath the epithelium. At four-fold greater CAII antibody concentrations, diffuse staining was observed in presumed erythrocytes, identified as ''free cells'' between pillar cells of the gill lamellae (not shown). No staining was observed in sections that were incubated with BPB instead of primary antibody.

As seen in high magnification micrographs, the interlamellar α5-IR was limited to the basolateral regions of cuboidal epithelial cells that did not appear to be directly adjacent to each other (Fig. 3A). Lighter α5-IR also occurred on the basolateral side of squamous cells on the lamellae, with diminishing intensity toward the distal tips of lamellae (Fig. 2B). Intense interlamellar CAII-IR was limited to the apical most areas of cuboidal epithelial cells that did not appear to be directly adjacent to each other (Fig. 3B). This apical staining was most concentrated in microvilli that sometimes formed a convex surface extending out from the epithelium (Fig. 3B). Some CAII staining also occurred in squamous cells of the lamellae, but unlike α5, this lamellar staining was strongest on the most distal tips (Fig. 2C). The strongest, and most common, E11-IR was throughout the cytoplasm of large, cuboidal epithelial cells of the intermellar area (Figs. 2D and 3C). In a few cells ($<2\%$), this E11-IR was in the apical regions, above the nucleus, but never extended into microvilli (Fig. 3D). Unlike α5 and CAII, E11 showed no evidence of immunoreactivity in squamous cells of the lamellae (Fig. 2D).

Double-labeling experiments

Because all three antibodies demonstrated IR within the same regions (interlamellar region) of the gill, we conducted a series of double-labeling experiments to determine if there was an overlap of staining for any of the proteins in the same cells. For all of these experiments, the first antibody was stained brown (DAB) and the second was stained dark-blue (Vector SG). Strong CAII-IR and α5-IR did not occur in the same cells, and sometimes appeared to alternate from one cell to the next (Fig. 4A). Similarly, E11-IR and NPPase-IR were almost never observed in the same cells, and sometimes appeared to alternate from one cell to the next (Fig. 4B). Conversely, CAII and E11 showed strong IR in the same cells, with CAII-IR always in the outermost, apical region, and E11-IR usually throughout the cytoplasm (Fig. 4C). Occasionally, E11–IR occurred near CAII-IR in the apical region, but while CAII-IR extended into the microvilli, E11-IR was absent from microvilli (Fig. 4C).

DISCUSSION

Immunoblots

Because none of the three transport-related proteins (NPPase, CAII, and V-ATPase) have been sequenced from lampreys, and no lamprey antibodies exist for these proteins, we used heterologous antibodies. The antibody for NPPase (α5), is commonly used in fish gills, and its specificity is widely accepted (Choe et al., '99, 2002; Piermarini and Evans, 2000, 2001a; Wilson et al., 2000b; Edwards et al., 2002). To evaluate the specificity of antibodies CAII and E11, we used immunoblots of lamprey gill proteins. The 32 kDa protein detected with antibody CAII is virtually the same size as CAII in spiny dogfish, *Squalus acanthias* (33 kDa), skates, *Raja sp.* (34 kDa), and carp, *Cyprinus carpio* (30 kDa) (Rahim et al., '88; Linser, '91; Wilson et al., 2000c), and therefore is probably a lamprey homologue of CAII. Similarly, the 29 kDa band detected with antibody E11 is virtually the same size as the E subunit in mammals (31 kDa), and therefore is probably a lamprey homologue of V-ATPase E subunit (Brown et al., '88).

Na^+/K^+-ATPase

The strong interlamellar NPPase-IR that we observed in freshwater lampreys was in a location consistent with chloride cells of seawater lampreys, which are predicted to express high levels of NPPase (Beamish et al., '78; Karnaky, '98). However the morphology, sub-cellular staining pattern, and timing (existence in fresh water) of the NPPase-rich cells in this study were all more consistent with presumed ion-absorptive MRCs, than with chloride cells. For example, like MRCs, the interlamellar NPPase-rich cells that we observed were cuboidal, were 10-15 μm in diameter, appeared to occur separately, and only occupied the surface epithelium (Fig. 3A) (Morris,

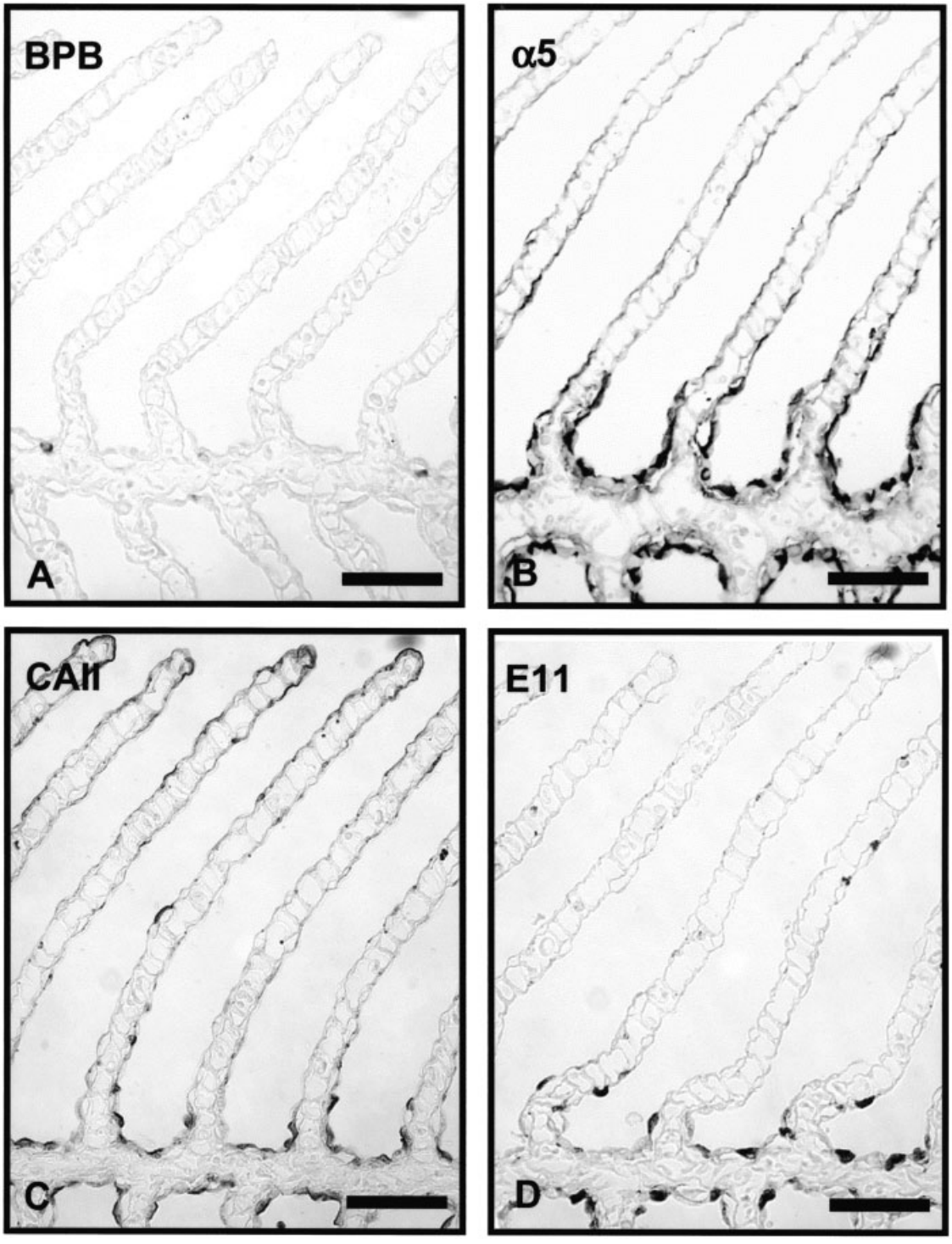

Fig. 2. Representative low-magnification, light micrographs of lamprey gill sections incubated with BPB (**A**), antibody α5 (**B**), antibody CAII (**C**), or antibody E11 (**D**). No immunoreactivity occurred in negative control sections that were incubated with BPB, in place of primary antibody (A). However, strong immunoreactivity was obtained in epithelial cells lining the inter-lamellar region with antibodies α5 (B), CAII (C), and E11 (D). Lighter, and more sparse immunoreactivity was observed on the lamellae with antibodies α5 and CAII (B and C). Scale bars = 100 μm.

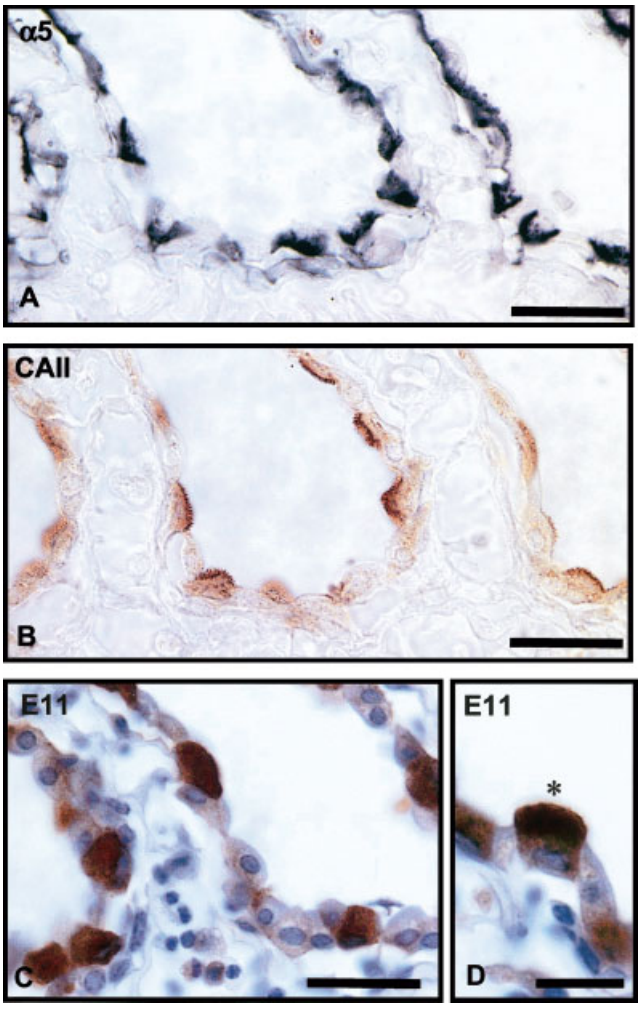

Fig. 3. Representative high-magnification light micrographs of lamprey gill sections incubated with antibody α5 (**A**), antibody CAII (**B**), or antibody E11 (**C** and **D**). Strong immunoreactivity with α5 was concentrated in the basolateral regions of cuboidal cells (A). Alternatively, CAII immunoreactivity was concentrated in the apical regions of cuboidal cells, including microvilli (B). E11 immunoreactivity (counterstained with hematoxylin) usually occurred throughout the cytoplasm (C) and occasionally in the apical region (D) of cuboidal cells. * = supranuclear E11 immunoreactivity. Scale bars = 20 μm.

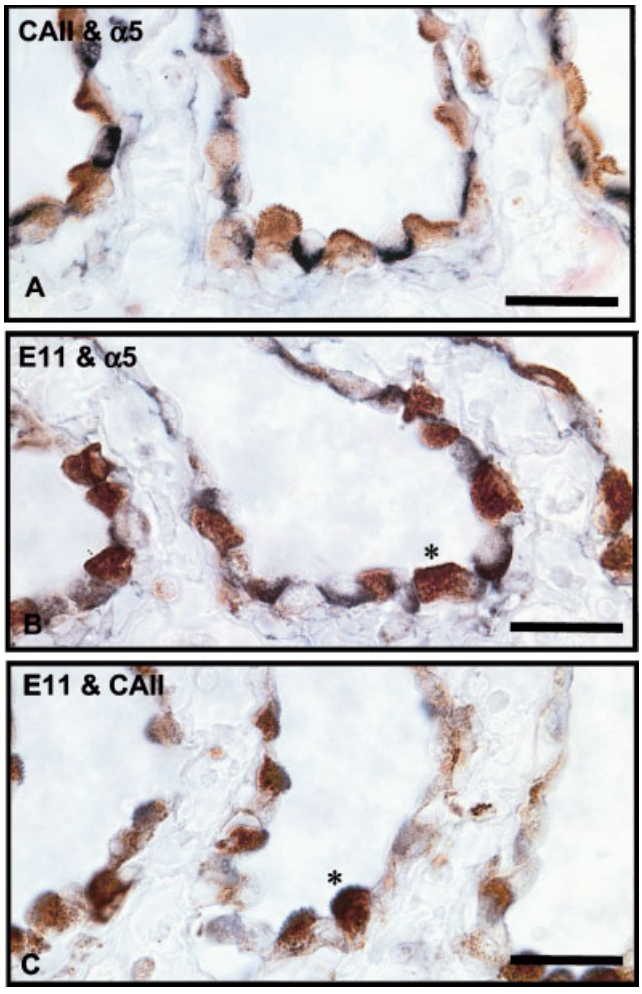

Fig. 4. Representative high-magnification light micrographs of lamprey gill sections incubated with antibodies CAII and α5 (**A**), E11 and α5 (**B**), or E11 and CAII (**C**). The α5 immunoreactivity (always dark-blue) was not in the same cells as CAII (brown, A) or E11 (brown, B) immunoreactivity. Alternatively, E11 (brown) and CAII (dark-blue) immunoreactivity occurred in the same cells (C). The CAII immunoreactivity was concentrated in the apical regions, and E11 immunoreactivity was usually diffuse, and occasionally in the apical region. * = supranuclear E11 staining. Scale bars = 20 μm.

'57; Nakao, '77; Bartels et al., '98). In contrast, the chloride cells of seawater lampreys are usually columnar or flask shaped, are 20–23 μm in length, occur in groups, and extend into basal layers of cells below the surface epithelium (Morris, '57; Peek and Youson, '79a, '79b; Bartels et al., '93).

The sub-cellular distribution of NPPase-IR was also inconsistent with ion-secreting chloride cells. In seawater teleost chloride cells, a tubule system fills most of the cytoplasm and contains high levels of NPPase so that the entire cells appear to stain when labeled for NPPase (Witters et al., '95; Shikano and Fujio, '98a, '98b; Wilson et al., 2002b). Lamprey chloride cells also have this tubule system, suggesting that they should show a staining pattern similar to teleost chloride cells (Peek and Youson, '79a, '79b). However, the NPPase-IR in freshwater gills that we observed was limited to the basolateral regions, suggesting that the NPPase-rich cells of freshwater lamprey

gills are not chloride cells (Fig. 3A). Lastly, previous studies have shown that chloride cells degenerate when adult lampreys re-enter fresh water for their spawning migration, and are replaced by MRCs (Morris, '57; Nakao, '77). Therefore, we conclude that the NPPase-rich cells of this study are not chloride cells that remained from when the lampreys were in seawater, but are instead one of the freshwater MRC types that have been described by Bartels et al. ('98). We cannot conclude which freshwater MRC type from our results.

CA II

Like NPPase-IR, the strongest CAII-IR occurred between, and at the base of, lamellae in epithelial cells with a diameter of 10–15 μm (Fig. 2D), suggesting that CAII is also expressed in freshwater MRCs. CAII-IR also occurred on the tips of lamellae in squamous cells that are presumed to be pavement (respiratory) cells. This region of the lamprey gill does not contain MRCs, and is considered to be primarily involved in gas exchange (Morris, '57). Similar diffuse, lamellar staining has been observed for CAII in elasmobranch (Wilson et al., 2000c) and teleost (Rahim et al., '88) gills, where the enzyme is thought to function in respiratory CO_2 secretion. Therefore, the CAII-staining pavement cells at the tips of lamellae in this study may function for CO_2 secretion. A four-fold higher concentration of the CAII antibody was required to obtain staining in presumed erythrocytes within the gill. This observation is consistent with enzymatic studies of lamprey CA, which suggested that lamprey erythrocytes have low levels of CA activity ($1/16^{th}$ of teleost activities) and that lamprey erythrocytes express a type I CA isozyme (Henry et al., '93).

The strong association of CAII-IR with the apical side of interlamellar cells (Fig. 3B) is consistent with teleost branchial epithelial cells. For example, studies using the Hanson histochemical technique detected carbonic anhydrase activity at the apical side of seawater killifish (*Fundulus heteroclitus*) chloride cells (Lacy, '83), and immunological studies have detected CAII at the apical side of branchial epithelial cells in rainbow trout (*Onchorynchus mykiss*), carp (*Cyprinus carpio*), and Osorezan dace (*Tribolodon hakonensis*) (Rahim et al., '88; Hirata et al., 2003). Therefore, CAII may be at the apical side of branchial cells in several fish species, including lampreys.

A possible explanation for the apical location of this water soluble enzyme is that it might be bound to a membrane protein. For example, CAII has recently been shown to bind to anion exchanger 1 (AE1), the Cl^-/ HCO_3^- exchanger of mammalian erythrocytes and mammalian renal A type intercalated cells (Reinhart and Reithmeier, 2001). This association has been termed a transport metabolon, where carbonic anhydrase is attached to the exchanger, so that HCO_3^- is delivered directly to the catalytic transport site. This increases transport rates by limiting the diffusive loss of HCO_3^- catalyzed by CAII, and therefore concentrates HCO_3^- at the transport site (Reinhart and Reithmeier, 2001). For example, the CAII-binding site of AE1 has been identified, and preventing CAII binding by mutating this site reduces HCO_3^- transport by 90% (Sterling et al., 2001). This functional association may be widespread for HCO_3^- transporters, because AE2 and 3 are also dependent on CAII binding for maximal transport rates, and other anion exchangers (e.g., pendrin) have stretches of amino acids that are similar to the CAII-binding site of AE1 (Sterling et al., 2001). Therefore, it is possible that the apical CAII that we observed in freshwater lamprey gills is bound to an apical Cl^-/HCO_3^- exchanger. Future studies should be conducted to determine what Cl^-/ HCO_3^- exchanger is expressed in lamprey gills, and if they are expressed in CAII-rich cells.

V-ATPase

Like NPPase-IR and CAII-IR, V-ATPase-IR occurred between, and at the base of, lamellae (Fig. 2D), consistent with freshwater MRCs (Morris, '57). The double-labeling experiments showed the presence of V-ATPase-IR and CAII-IR in the same population of cells. The CAII-IR always appeared apical while V-ATPase-IR usually appeared throughout the entire cell, excluding microvilli. NPPase-IR was seen in a separate population of cells (Fig. 4). With a few exceptions, V-ATPase-IR was diffuse and did not appear to be confined to a specific membrane (apical or basolateral) (Figs. 3C, 3D, 4B, and 4D). Even within the few cells that the IR was sub-apical, it did not extend into microvilli like CAII (Fig. 3B), and it is uncertain if the transporter was in contact with the environmental water. Therefore, it is possible that most of the V-ATPase is in pools of vesicles associated with, or connected to, one side of the cell (apical or basolateral). This leaves the possi-

bility that V-ATPase can be either in the apical or basolateral membranes in freshwater lamprey MRCs, similar to intercalated cells of mammalian renal collecting ducts (Glück et al., '96).

Diffuse V-ATPase-IR, similar to that observed in freshwater lampreys (present study), has been observed in branchial epithelial cells of two elasmobranchs, spiny dogfish (Wilson et al., '97) and Atlantic stingrays (Piermarini and Evans, 2001a). Immunogold transmission electron microscopy and confocal microscopy suggest that most of the V-ATPase is associated with the basolateral membranes in these elasmobranchs (Piermarini and Evans, 2001b; Evans et al., 2004). The apical regions of these V-ATPase-rich cells were shown later to stain for an apical Cl^-/HCO_3^- exchanger (pendrin) (Piermarini et al., 2002). Based on these immunological results, the V-ATPase-rich cells of elasmobranchs have been called B type MRCs (Piermarini et al., 2002), which are thought to absorb Cl^- and secrete HCO_3^- like B type intercalated cells of mammalian renal collecting ducts (Royaux et al., 2001). The CAII and V-ATPase-rich cells observed in freshwater lampreys (present study) may have the same functions if they express a Cl^-/HCO_3^- exchanger in their apical membranes.

Model of ion absorption

Immunoreactivity for all three transport-related proteins was present in cells with a size (10–15 μm), shape (cuboidal), and distribution (interlamellar) consistent with adult freshwater MRCs (Bartels et al., '98). Double-labeling experiments showed that α5 stained one population of presumed MRCs, and CAII and E11 stained another (Fig. 4), with no apparent overlap. Therefore, for the first time, we have identified two independent MRCs in the gills of freshwater lamprey on the basis of immunological staining for transport related proteins. These findings agree with the conclusions of Bartels et al. ('98), who characterized two subtypes of freshwater MRCs, based on the location of globular and rod-shaped particles viewed with freeze fracture electron microscopy. Based on morphological analogies to mammalian renal collecting ducts, they speculated that one cell type may be responsible for Na^+-absorption/H^+-secretion similar to A type intercalated cells, and the other may be responsible for Cl^--absorption/HCO_3^--secretion similar to B type intercalated cells (Bartels et al., '98).

Although our results support Bartel et al.'s ('98) prediction of two separate types of MRCs in freshwater lampreys, we hypothesize a different mechanism of Na^+ absorption and H^+ secretion. For example, A type intercalated cells use V-ATPase for H^+ secretion at the apical membrane, and basolateral AE1 for HCO_3^- transport into the blood (Glück et al., '96). Alternatively, we hypothesize that Na^+-absorption/H^+-secretion occur in the NPPase-rich cells of freshwater lampreys, as has been predicted for elasmobranch gills (Piermarini and Evans, 2001a). In this scenario, NPPase in the basolateral membrane would create a low intracellular [Na^+] that would provide a gradient for Na^+ to enter through the apical membrane via a Na^+ transporter (possibly an NHE (Fig. 5)). This mechanism is similar to mammalian renal proximal tubule cells (Aronson, 2002), and would satisfy the freshwater lamprey's needs for Na^+ absorption and H^+ secretion.

In our model, the mechanism for Cl^--absorption/HCO_3^--secretion in freshwater lampreys is similar to B type MRCs of elasmobranch gills and intercalated cells of mammalian renal collecting ducts (Fig. 5). In this scenario, CAII would be providing HCO_3^- to a Cl^-/HCO_3^- exchanger in the apical membrane via hydration of CO_2. Most of the V-ATPase would be in the basolateral membrane removing protons that are created when CO_2 is hydrated by CAII. This would satisfy the freshwater lamprey's needs for Cl^- absorption and HCO_3^- secretion. It is also possible that some of the V-ATPase may be in the apical membrane secreting H^+ into the environmental water. Apical H^+ secretion (via V-ATPase) and Cl^-/HCO_3^- exchange would still satisfy the lampreys need for Cl^- absorption, but would have no net effect on acid-base transport.

Our model of ion absorption for freshwater lampreys is similar to that of elasmobranchs, which separates Na^+-absorption/H^+-secretion and Cl^--absorption/HCO_3^--secretion into two types of MRC (A and B, respectively). Our model for lampreys is different from that of freshwater teleosts, where pavement cells function for Na^+-absorption and H^+-secretion and MRCs (chloride cell) function for Cl^--absorption/HCO_3^--secretion. However, the presence and location of NHEs and anion exchangers is speculative and based on analogies to other transport tissues. Therefore, future studies should test and further develop this model by determining if and where NHEs and anion exchangers are expressed in freshwater lamprey gills.

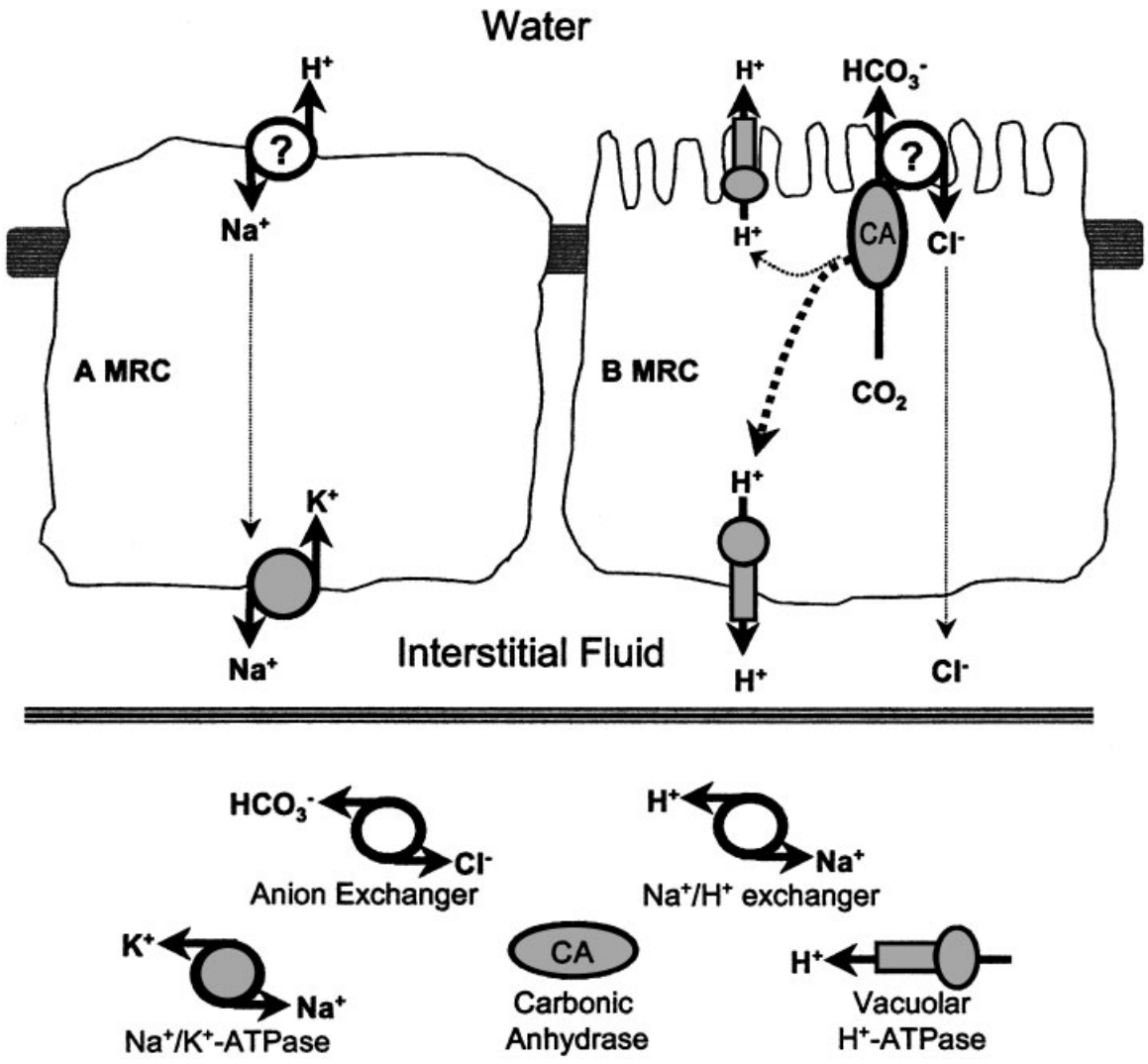

Fig. 5. Model of potential ion and acid-base exchange mechanisms in freshwater adult lamprey gills based on previous morphological studies (Morris, '57; Nakao, '77; Bartels et al., '98) and similarities of our immunocytochemical results with those of elasmobranchs (Piermarini and Evans, 2000; Piermarini and Evans, 2001a). Our results show that there are two types of MRC, and we predict that one is responsible for Na^+ absorption and H^+ secretion, and the other for Cl^- absorption and HCO_3^- secretion. In the first, high amounts of NPPase are expressed in the basolateral membranes, which would create a low intracellular [Na^+]. This could then drive the entry of Na^+ from the environmental water through an apical Na^+ transporter (possibly an NHE). In the second MRC type, high amounts of CAII are expressed in the apical membrane along with high levels of V-ATPase in the basolateral and/or apical membranes. CAII may be providing HCO_3^- to an apical anion exchanger as part of a metabolon secreting HCO_3^- and absorbing Cl^-. V-ATPase can also support this by removing H^+ that is produced when CO_2 is hydrated by CAII. Shading indicates proteins that were located in this study, and "?" indicates proteins that have not yet been studied in lampreys.

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