

# Pendrin immunoreactivity in the gill epithelium of a euryhaline elasmobranch

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**Piermarini, Peter M., Jill W. Verlander, Ines E. Royaux, and David H. Evans.** Pendrin immunoreactivity in the gill epithelium of a euryhaline elasmobranch. *Am J Physiol Regul Integr Comp Physiol* 283: R983–R992, 2002; 10.1152/ajpregu.00178.2002.—Pendrin is an anion exchanger in the cortical collecting duct of the mammalian nephron that appears to mediate apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange in bicarbonate-secreting intercalated cells. The goals of this study were to determine 1) if pendrin immunoreactivity was present in the gills of a euryhaline elasmobranch (Atlantic stingray, *Dasyatis sabina*), and 2) if branchial pendrin immunoreactivity was influenced by environmental salinity. Immunoblots detected pendrin immunoreactivity in Atlantic stingray gills; pendrin immunoreactivity was greatest in freshwater stingrays compared with freshwater stingrays acclimated to seawater (seawater acclimated) and marine stingrays. Using immunohistochemistry, pendrin-positive cells were detected on both gill lamellae and interlamellar regions of freshwater stingrays but were more restricted to interlamellar regions in seawater-acclimated and marine stingray gills. Pendrin immunolabeling in freshwater stingray gills was more apical, discrete, and intense compared with seawater-acclimated and marine stingrays. Regardless of salinity, pendrin immunoreactivity occurred on the apical region of cells rich with basolateral vacuolar-proton-ATPase, and not in  $\text{Na}^+\text{-K}^+\text{-ATPase}$ -rich cells. We suggest that a pendrin-like transporter may contribute to apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange in gills of Atlantic stingrays from both freshwater and marine environments.

anion exchanger; vacuolar-proton-adenosinetriphosphatase; sodium-potassium-adenosinetriphosphatase; sodium chloride regulation; acid/base regulation; *Dasyatis sabina*

PENDRIN is a recently discovered protein in mammals encoded by the Pendred disease syndrome (PDS) gene (9). Pendrin belongs to a diverse family of anion exchangers (SLC26) that includes such transporters as the sulfate-anion transporter (sat-1), diastrophic dysplasia sulfate transporter (DTDST), and congenital chloride diarrhea chloride-base exchanger (CLD) (10, 18, 30). This gene family is distinct from the SLC4 sodium-independent anion exchanger (AE) family that includes the well-studied AE-1 (band 3)  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (1, 18). Pendrin was first described in the

mammalian thyroid gland (9) and was discretely localized to the apical region of thyrocytes, where it is believed to function as a  $\text{Cl}^-/\text{iodide}$  exchanger (2, 15, 25). This transporter has  $\text{Cl}^-/\text{iodide}$ ,  $\text{Cl}^-/\text{HCO}_3^-$ ,  $\text{Cl}^-/\text{OH}^-$ , and  $\text{Cl}^-/\text{formate}$  exchange activity when expressed in *Xenopus* oocytes and HEK-293 cells (28, 29, 31).

Recent studies have detected both pendrin mRNA (26, 31) and protein (26) in the cortical collecting duct (CCD) of the mammalian nephron. Royaux et al. (26) localized pendrin to the apical region of vacuolar-proton-adenosinetriphosphatase (V-H-ATPase)-rich intercalated cells that did not have basolateral AE-1 immunoreactivity. Furthermore, Royaux et al. (26) demonstrated that isolated, perfused CCD tubules from bicarbonate-loaded pendrin-knockout mice had a decreased capacity for bicarbonate secretion compared with CCD tubules from wild-type mice. Taken together, these data not only suggested that intercalated cells with pendrin were bicarbonate-secreting cells but also that pendrin may represent an apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger in type B intercalated cells.

In cartilaginous fishes (elasmobranchs), the gills are the primary site of acid/base-related ion transport (13). However, no study has determined the identity or cellular location of a  $\text{Cl}^-/\text{HCO}_3^-$  exchanger in the elasmobranch gill, even though evidence suggests net bicarbonate excretion occurs across this epithelium (14, 33). In the gills of a euryhaline elasmobranch (Atlantic stingray, *Dasyatis sabina*), we have previously identified cells that are rich in basolateral V-H-ATPase, which may be functionally analogous to bicarbonate-secreting cells of the mammalian CCD (22). Therefore, V-H-ATPase-rich cells in the stingray gill may have mechanisms of apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange that are similar to the mammalian CCD, such as pendrin. To date, no studies of pendrin in the elasmobranch gill, or in transport epithelia from any lower vertebrate, have been reported.

The Atlantic stingray is unique because it is one of the few elasmobranch species with the physiological capability of living in both freshwater and marine environments. In previous studies, we have demon-

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strated that both  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and  $\text{V-H-ATPase}$  immunoreactivity are at relatively high levels in gills of freshwater Atlantic stingrays and subsequently decrease when the animals are acclimated to seawater (21, 22). Understanding how salinity influences expression of a branchial anion exchanger is of interest in the Atlantic stingray because it may reveal mechanisms this species uses for acid/base and  $\text{NaCl}$  regulation in freshwater and marine environments. For example, molecular, immunohistochemical, and pharmacological evidence in freshwater bony fish (teleost) gills suggests that an AE-1-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger contributes to bicarbonate secretion for acid/base balance and to chloride uptake for  $\text{NaCl}$  homeostasis (19, 20, 32, 36). In marine teleost gills, pharmacological evidence suggests an AE-1-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger contributes to bicarbonate secretion for acid/base regulation (5), but it could potentially complicate  $\text{NaCl}$  balance by leading to chloride accumulation (see Ref. 3). To our knowledge, the influence of environmental salinity on expression (mRNA or protein levels) of a branchial  $\text{Cl}^-/\text{HCO}_3^-$  exchanger has not been examined in any fish.

The goals of this study were to determine if pendrin immunoreactivity is present in the gills of the Atlantic stingray, to identify the specific cellular location of pendrin in the gill epithelium, and to determine if branchial pendrin immunoreactivity is influenced by environmental salinity. Our results present the first evidence of pendrin immunoreactivity in a lower vertebrate and suggest that pendrin or a pendrin-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger exists on the apical membrane of  $\text{V-H-ATPase}$ -rich cells in the Atlantic stingray gill epithelium. Furthermore, we find that branchial pendrin protein expression is decreased by increased environmental salinity.

## MATERIALS AND METHODS

**Animal collection and holding conditions.** During the spring of 1999, 10 freshwater Atlantic stingrays were captured from the St. Johns River in Florida (Lake Jesup or Lake George), using trotlines baited with shrimp (see Ref. 23). These stingrays were held in two 379-liter freshwater closed-system tanks [5 animals/tank; <1 parts per thousand (ppt) salinity]. In addition, five marine Atlantic stingrays were captured via hook and line from Cedar Key, FL, transported to Gainesville, FL, and held in a 379-liter seawater closed-system tank (32 ppt salinity).

Five of the freshwater stingrays were left in freshwater (referred to as freshwater stingrays), while the other five were gradually acclimated to seawater as follows (referred to as seawater-acclimated stingrays). After 1 wk in freshwater, the salinity was raised to 16 ppt over 2 days (8 ppt per day). After 2 days in 16 ppt, the salinity was raised to 32 ppt seawater over 3 days. The animals remained in 32-ppt seawater for 1 wk before tissue samples were taken. The stingrays from Cedar Key, FL, remained in 32-ppt seawater for the entire period (referred to as marine stingrays). All animals were fed live grass shrimp (*Palaemonetes* sp.) every other day and were starved 48 h before tissue collection.

All holding tanks were held in the same room, which contained a thermostat set at 25°C, with a 12:12-h light-dark photoperiod. Water temperature of the tanks ranged from 23

to 27°C during the experiment, and pH was adjusted as necessary to 8.2 using Malawi/Victoria Buffer (Seachem) for freshwater and Marine Buffer (Seachem) for seawater. The tanks were also equipped with biological filtration, which maintained ambient  $\text{NH}_3$  and  $\text{NO}_2$  levels below 1 part per million. Ion composition of the freshwater and seawater has been reported in a previous study (23).

**Collection of gill tissue.** Animals were anesthetized in 4 liters of a 0.01% 3-aminobenzoic acid ethyl ester (MS-222, Sigma) solution made with tank water. Malawi/Victoria Buffer was added to the anesthetic of freshwater stingrays to offset acidification caused by the MS-222. Once anesthetized, animals were placed ventral side up in a slanted water bath with their gills immersed in the anesthetic.

To clear the gills of red blood cells, the animal was perfused with a 4°C marine elasmobranch Ringer solution (12). However, for freshwater stingrays,  $\text{NaCl}$ , urea, and trimethylamine-oxide concentrations in the Ringer were reduced to 200, 200, and 41 mM, respectively. The skin ventral to the heart and pericardium was removed, and 0.5–1.0 ml of blood was removed from the ventricle with a heparinized 25-gauge needle attached to a 1-ml syringe. An equal volume of heparinized Ringer solution was then injected into the ventricle and allowed to circulate for a few minutes. A cannula, connected to a perfusion bottle (1 m above animal), was inserted into the conus arteriosus and held by forceps. Once the perfusion was started, the sinus venosus was cut to relieve backpressure. The perfusion was continued until the gills appeared bleached and the fluid exiting the pericardial cavity was clear of blood (usually 3–5 min).

Immediately after the perfusion, the animal was pithed, and the second left and right gill arches were removed and placed in an elasmobranch Ringer solution on ice. For immunohistochemistry, gill filaments were trimmed off the arches and placed in fixative (3% paraformaldehyde, 0.05% glutaraldehyde, and 0.05% picric acid in 10 mM PBS, pH 7.3) for 24 h at 4°C, then transferred to two changes of 75% ethanol for removal of fixative. Tissues were left in the second change of 75% ethanol until embedded. Additional filaments were snap-frozen in liquid nitrogen for immunoblot analysis and stored at –80°C until analyzed.

**Antibodies.** The antibody used to detect pendrin was developed by Royaux et al. (25) and is an affinity-purified rabbit polyclonal antibody raised against amino acids 630–643 of human pendrin. This sequence of amino acids is near the carboxy terminus of pendrin and is completely conserved among humans, rats, and mice (see Ref. 11). The antibody has been used to immunolabel pendrin in HEK-293 cells transfected with pendrin cDNA and in rat thyroid tissue (25). To detect  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , a mouse anti-chicken  $\text{Na}^+\text{-K}^+\text{-ATPase}$   $\alpha$ -subunit monoclonal antibody, a5, developed by Dr. D. Fambrough was obtained from the Developmental Studies Hybridoma Bank 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). To detect  $\text{V-H-ATPase}$ , a rabbit anti-insect  $\text{V-H-ATPase}$  B-subunit polyclonal antibody was kindly provided by Dr. W. Harvey, Whitney Laboratory, University of Florida, with permission from Dr. S. Gill, University of California at Riverside. In previous studies (21, 22), we have successfully used the latter two antibodies to detect  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and  $\text{V-H-ATPase}$  in gills of the Atlantic stingray.

**Immunoblot analysis of pendrin immunoreactivity.** Immunoblots were performed on polyvinylidene difluoride membranes (PVDF; Bio-Rad) containing 20  $\mu\text{g}$  of total gill membrane protein per lane that were prepared for a previous

study (21). Preparation of tissue for immunoblots was similar to Claiborne et al. (4), with modifications. On a single day, gill filaments from a freshwater, seawater-acclimated, and marine stingray were prepared. First, the tissue was placed in ice-cold homogenization buffer (250 mM sucrose, 1 mM Na-EDTA, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, 100  $\mu$ g/ml PMSF, and 30 mM Tris) and was homogenized with a motorized Tissue-tearor (Biospec Products) in a 4°C cold room on ice. Homogenates were filtered through cheesecloth and centrifuged (3,000 *g*) for 5 min at 4°C to remove nuclei and debris. The supernatant was then filtered through cheesecloth and centrifuged (50,000 *g*) for 30 min at 4°C to pellet membrane fractions. The pellet was resuspended with a minimal volume of ice-cold homogenization buffer, and then an equal volume of a modified Laemmli sample buffer (16), without bromophenol blue and  $\beta$ -mercaptoethanol, was added to solubilize the proteins.

The resulting protein samples were centrifuged for 5–10 s at 16,000 *g* to pellet any undissolved material. The total protein content of the supernatant was determined with a detergent-compatible assay (Bio-Rad), after which bromophenol blue and  $\beta$ -mercaptoethanol were added to final concentrations of 0.01 and 2%, respectively.

A 20- $\mu$ g sample of total gill membrane protein for one stingray in each condition was loaded in triplicate and run on a 7.5% Tris-HCl precast polyacrylamide gel (Bio-Rad) for 1 h at 125 V. Proteins were then transferred to a PVDF membrane using a wet (20% methanol, Tris-glycine) transfer unit for 2.5 h at 90 V in a 4°C cold room with stirring. The protein preparation, electrophoresis, and blotting were repeated for the remaining gill samples, which resulted in a total of five PVDF membranes with each containing, in triplicate, gill membrane protein from a single freshwater, seawater-acclimated, and marine stingray.

Because these PVDF membranes were previously immunostained (see Refs. 21, 22), it was necessary to remove antibodies that were bound to the membrane with a strip buffer (62 mM Tris base, 2% sodium lauryl sulfate, 0.6%  $\beta$ -mercaptoethanol, pH 6.7). Each PVDF membrane was first soaked in 100% methanol for 15 min and then placed in the strip buffer for 30 min at 60°C. The PVDF was then placed in three washes of deionized H<sub>2</sub>O (5 min each) to remove any residual  $\beta$ -mercaptoethanol. The PVDF was blocked with blocking buffer (Tris-buffered saline with 5% nonfat dry milk, 0.1% Tween-20, and 0.02% sodium azide, pH 7.4) for 1.5 h at 25°C and then incubated with the rabbit anti-human pendrin polyclonal antibody (diluted 1:5,000 in blocking buffer) overnight at 4°C.

The PVDF was washed three times (15 min each) with Tris-buffered saline containing 0.1% Tween-20 (TTBS, pH 7.4) and then incubated with an alkaline-phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad; diluted 1:3,000 in blocking buffer) 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) was applied to the PVDF for 5 min at 25°C to generate a luminescent signal. Binding of antibody was detected by exposing Hyperfilm-ECL imaging film (Amersham) to the PVDF membrane. Negatives were digitized into TIFF files using a UMAX flatbed scanner with transparency adapter. As a control, we incubated stripped membranes with normal rabbit serum (Biogenex) because preimmune serum from the rabbits that generated the anti-human pendrin antibody was not available.

To quantify the relative abundance of pendrin immunoreactivity, we measured the optical density (uncalibrated OD) of the immunopositive band in each animal using

Scion Image version 4.02 (Scion). On a given blot, optical density values were measured for each animal and then standardized to the freshwater condition to calculate relative abundance. Therefore, all relative abundance measurements of freshwater gills were 1.0, and those of seawater-acclimated and marine gills were a fraction of the freshwater value.

*Immunohistochemical localization of pendrin immunoreactivity.* The fixed gill tissue (stored in 75% ethanol) was dehydrated in an ethanol series and embedded into paraffin wax. Serial sections of gill tissue, parallel to the long axis of the filament, were cut at 6  $\mu$ m and placed on poly-L-lysine-coated slides (3 sections per slide). Longitudinal sections of gill filaments provide cross-sectional orientations of gill lamellae, which appear as fingerlike projections, and interlamellar regions, which are basal to and between lamellae (see Fig. 3). Sections were deparaffinized in Hemo-De (Fisher Scientific), hydrated in an ethanol series, and washed in 10 mM PBS. A hydrophobic PAP-Pen (Electron Microscopy Sciences) was used to draw circles around the tissue sections, and then 3% H<sub>2</sub>O<sub>2</sub> 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<sub>3</sub>, and 0.1% Tween-20) for 20 min. The polyclonal rabbit anti-human pendrin antibody (diluted 1:1,500 in BPB) was then incubated on the sections overnight at 4°C.

The antibody was rinsed off with PBS, and then the sections were soaked in PBS for 5 min. The sections were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Biogenex) and a horseradish peroxidase-labeled streptavidin solution (Biogenex) for 20 min each at 25°C (with a 5-min PBS wash after each incubation). Antibody binding was visualized by applying the chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Biogenex) to the sections for 5 min at 25°C. In each experiment, one section was exposed to normal rabbit serum or BPB in place of the anti-human pendrin antibody as a negative control, because preimmune serum from the rabbits that generated the anti-human pendrin antibody was not available.

*Colocalization of pendrin immunoreactivity with V-H-ATPase and Na<sup>+</sup>-K<sup>+</sup>-ATPase.* To determine if pendrin immunoreactivity was expressed in V-H-ATPase-rich and/or Na<sup>+</sup>-K<sup>+</sup>-ATPase-rich cells, we used a double-labeling technique consisting of sequential immunolocalization procedures using two different chromagens. Tissue sections for double labeling were first deparaffinized, hydrated, blocked, and stained for pendrin as described above (except a 1:1,000 dilution of the anti-pendrin antibody was used when double labeling with V-H-ATPase). After treatment with the brown chromagen substrate (DAB), the slides were rinsed in deionized H<sub>2</sub>O for 10 min and blocked with BPB for 20 min. An antibody to Na<sup>+</sup>-K<sup>+</sup>-ATPase (monoclonal antibody a5 culture supernatant diluted 1:100 in BPB) or V-H-ATPase (polyclonal antibody serum diluted 1:5,000 in BPB) was then applied to the sections overnight at 4°C. Rinsing and developing were performed as described above, except a blue chromagen was used (Vector SG, Vector Laboratories).

*Statistical analyses.* Differences in pendrin immunoreactivity relative abundance measurements were detected using a Kruskal-Wallis nonparametric ANOVA, with a Kruskal-Wallis multiple comparisons test (6). All tests were two tailed, and differences were considered significant at  $P < 0.05$ .

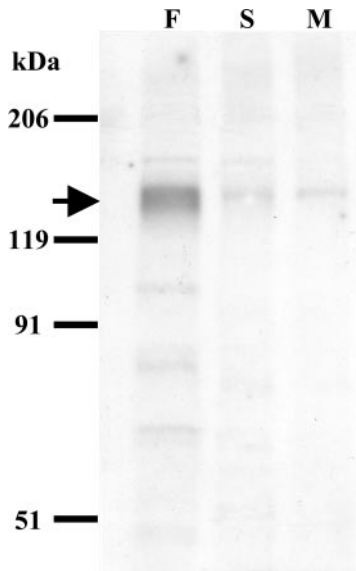


Fig. 1. Representative immunoblot for pendrin immunoreactivity in gill membrane enrichments from freshwater (F), seawater-acclimated (S), and marine (M) Atlantic stingrays. Black lines represent migration of molecular mass markers (kDa). The anti-human pendrin antibody recognized an ~144-kDa protein (arrow). Note the 144-kDa band appears darker in gill membrane protein of freshwater stingrays relative to seawater-acclimated and marine stingrays.

## RESULTS

**Immunoblot analysis of pendrin immunoreactivity.** In immunoblots of membrane proteins isolated from stingray gills, under all salinities tested, the anti-human pendrin antibody bound to a protein of ~144 kDa (Fig. 1). No detectable signal was present when PVDF membranes were incubated with normal rabbit serum instead of the primary antibody (data not shown). Semiquantitative immunoblotting revealed that the relative abundance of pendrin immunoreactivity was highest in gill membrane protein from freshwater stingray gills (Fig. 2). In gill membrane protein

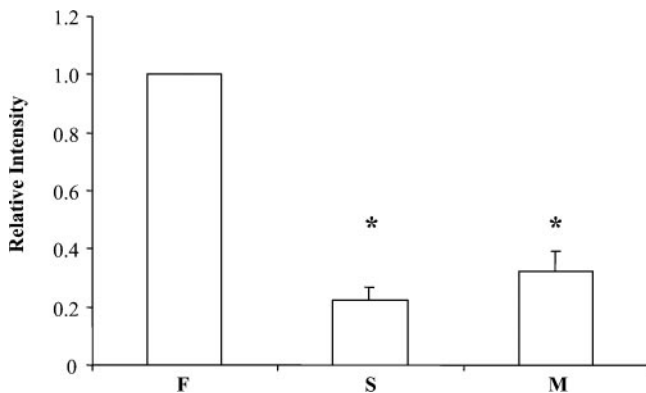


Fig. 2. Histogram demonstrating relative abundance of pendrin immunoreactivity, based on optical density measurements of the 144-kDa band in the gill membrane protein of freshwater, seawater-acclimated, and marine Atlantic stingrays;  $n = 5$  for all groups. Values are presented as means + SE. Note that no error bar is present in freshwater stingray because of the standardization procedure (see MATERIALS AND METHODS). \*Statistical difference from freshwater ( $P < 0.05$ ).

from seawater-acclimated stingrays, pendrin immunoreactivity was diminished compared with freshwater gill protein and was comparable to that from marine stingrays (Fig. 2).

**Immunohistochemistry of pendrin in stingray gills.** In freshwater stingrays, numerous pendrin-positive cells were found on both gill lamellae and interlamellar regions (Fig. 3A). In seawater-acclimated stingray

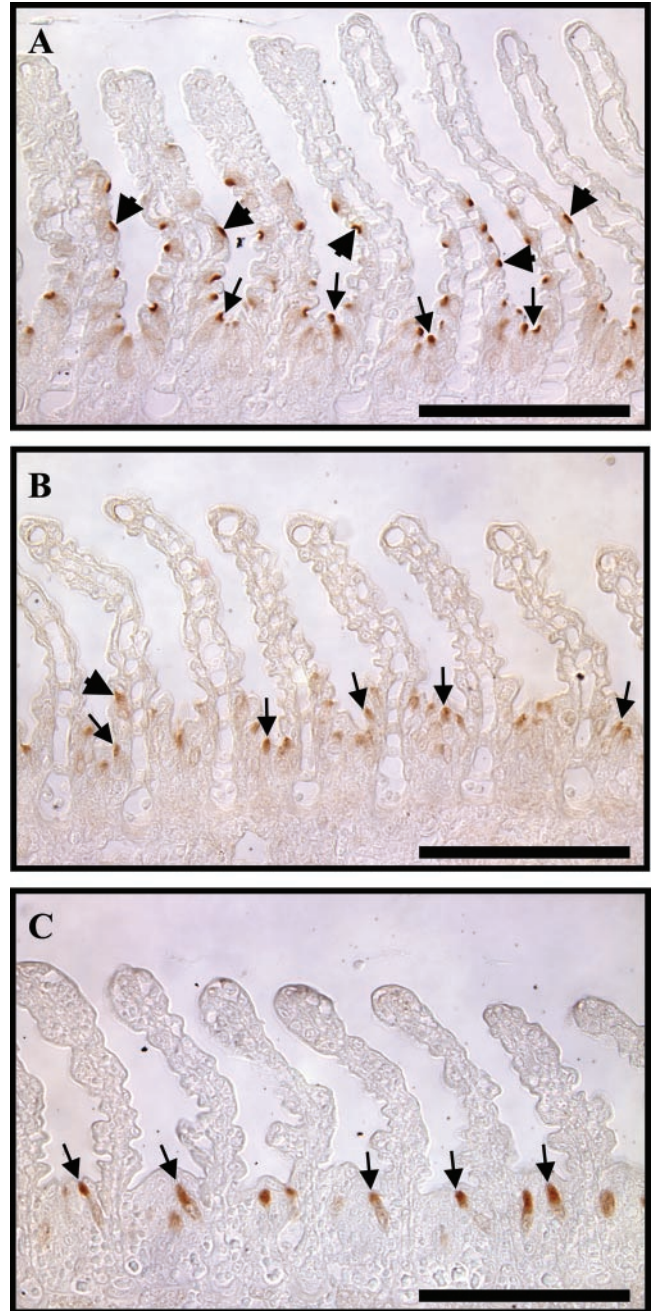


Fig. 3. Representative photomicrographs of pendrin immunolabeling in longitudinal sections of gill filaments from freshwater (A), seawater-acclimated (B), and marine (C) Atlantic stingrays ( $\times 400$ ). Pendrin-positive cells stain brown and occur on lamellae (wide arrows) and/or interlamellar regions (thin arrows). Note differences in pendrin-positive cell distribution (lamellae vs. interlamellar region) among groups. Scale bar, 100  $\mu\text{m}$ .

gills, pendrin-positive cells were primarily detected on interlamellar regions and were infrequently found on lamellae (Fig. 3B). In marine stingrays, pendrin-positive cells were found exclusively on interlamellar regions, with no detectable immunoreactivity on lamellae (Fig. 3C). No staining was detected when normal rabbit serum or BPB was used instead of primary antibody (data not shown).

Higher-magnification images of pendrin-positive cells revealed that pendrin immunoreactivity was localized to the apical region (Fig. 4), regardless of salinity. However, there were qualitative differences in immunostaining among the three stingray groups. In freshwater stingray gills, localization of pendrin immunoreactivity was most apical, discrete, and intense (Fig. 4A). In contrast, pendrin immunostaining in seawater-acclimated and marine stingray gills was diffuse throughout the subapical cytoplasm and did not have discrete apical pendrin immunoreactivity (Fig. 4, B and C, respectively).

*Colocalization of pendrin immunoreactivity with V-H-ATPase and  $\text{Na}^+\text{-K}^+\text{-ATPase}$ .* Double labeling of gills for pendrin and V-H-ATPase immunoreactivity revealed that the two transporters occurred in the same cells (Fig. 5). Regardless of environmental salinity, all pendrin immunolabeling was in the apical region of cells that stained for V-H-ATPase (Fig. 6). Double labeling of gills for pendrin and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  immunoreactivity demonstrated that the two transporters occurred in separate cells, regardless of environmental salinity (Figs. 7 and 8).

## DISCUSSION

Our findings present the first evidence of a pendrin-like transporter in an ion-transporting tissue from any lower vertebrate. Immunoblotting with an anti-human pendrin antibody demonstrated the presence of an ~144-kDa protein in gill membrane enrichments from Atlantic stingrays (Fig. 1), a molecular mass that is slightly greater than the reported size of 95–115 kDa for pendrin in mammals (24, 25, 27, 31). The difference in size may indicate that the pendrin-like protein in elasmobranchs is composed of more amino acids and/or that the protein is heavily glycosylated in elasmobranchs compared with mammals. Pendrin has two potential glycosylation sites in mammals (9), but the protein has not been sequenced in elasmobranchs; therefore, the number of amino acids and potential glycosylation sites in the elasmobranch protein are unknown.

Semiquantitative immunoblotting revealed that pendrin immunoreactivity was most abundant in gill tissue from freshwater stingrays compared with seawater-acclimated and marine stingrays (Fig. 2). Our immunohistochemical findings demonstrated that the distribution of cells with detectable pendrin immunoreactivity was also influenced by environmental salinity. In freshwater stingray gills, pendrin-positive cells were found on both gill lamellae and interlamellar regions, whereas pendrin-positive cells were primarily

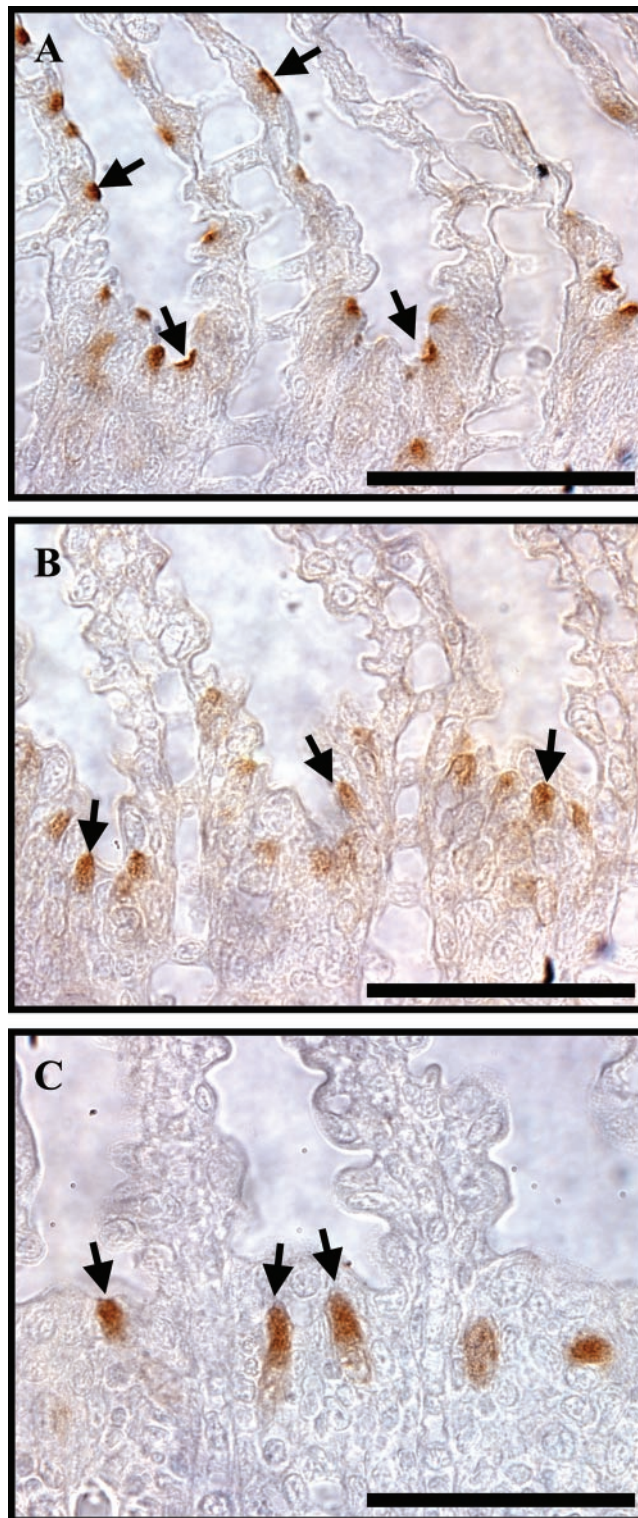


Fig. 4. Higher-magnification photomicrographs of pendrin immunolabeling in longitudinal sections of gill filaments from freshwater (A), seawater-acclimated (B), and marine (C) Atlantic stingrays ( $\times 1,000$ ). Note that staining in pendrin-positive cells occurs in the apical region and that localization is more discrete, intense, and apical in freshwater stingray gills relative to seawater-acclimated and marine stingrays. Arrows indicate apical regions of cells that best demonstrate the qualitative differences described in text. Scale bar, 50  $\mu\text{m}$ .

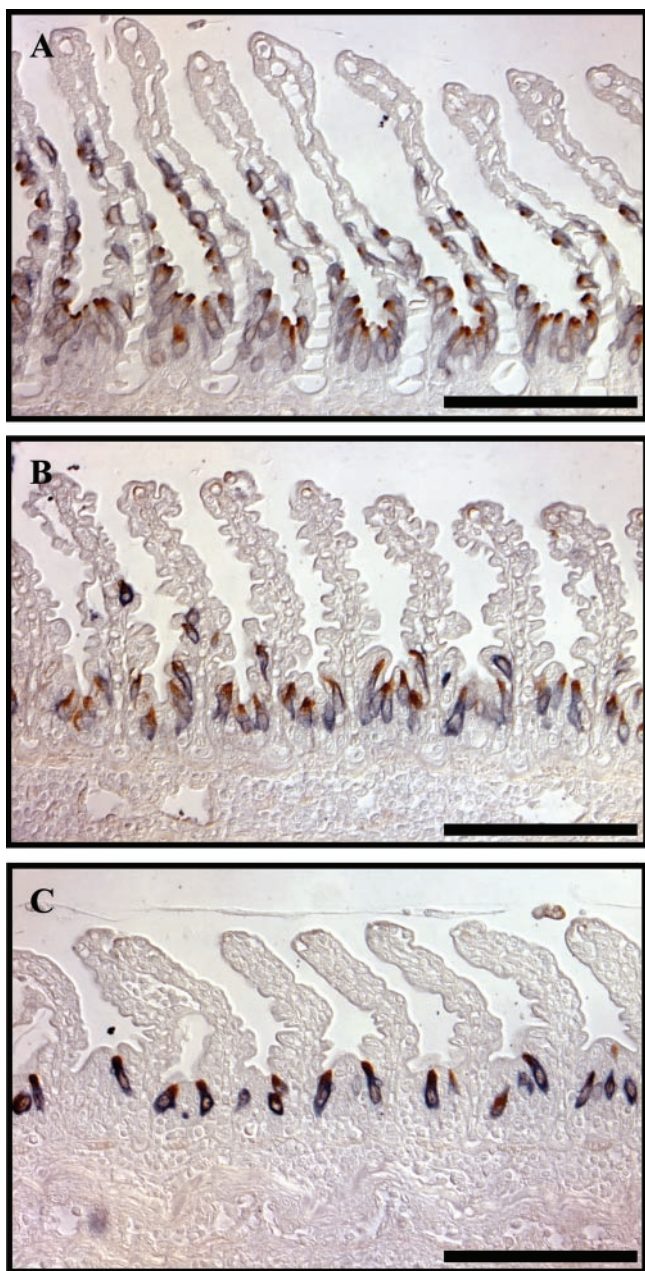


Fig. 5. Representative photomicrographs of pendrin immunolabeling (brown) colocalized with vacuolar-proton-adenosinetriphosphatase (V-H-ATPase; blue) in longitudinal sections of gill filaments from freshwater (A), seawater-acclimated (B), and marine (C) Atlantic stingrays ( $\times 400$ ). Note that pendrin and V-H-ATPase immunoreactivity are in the same cells. Scale bar, 100  $\mu\text{m}$ .

found in the interlamellar regions of seawater-acclimated and marine stingray gills (Fig. 3). Furthermore, the intensity of the immunolabeling appeared to be stronger in pendrin-positive cells of freshwater stingray gills relative to seawater-acclimated and marine stingrays (Fig. 4). These salinity-related differences in cellular distribution and immunostaining intensity are consistent with the greater pendrin immunoreactivity detected in freshwater stingray gills by immunoblotting compared with gills of seawater-acclimated and marine stingrays (Fig. 2).

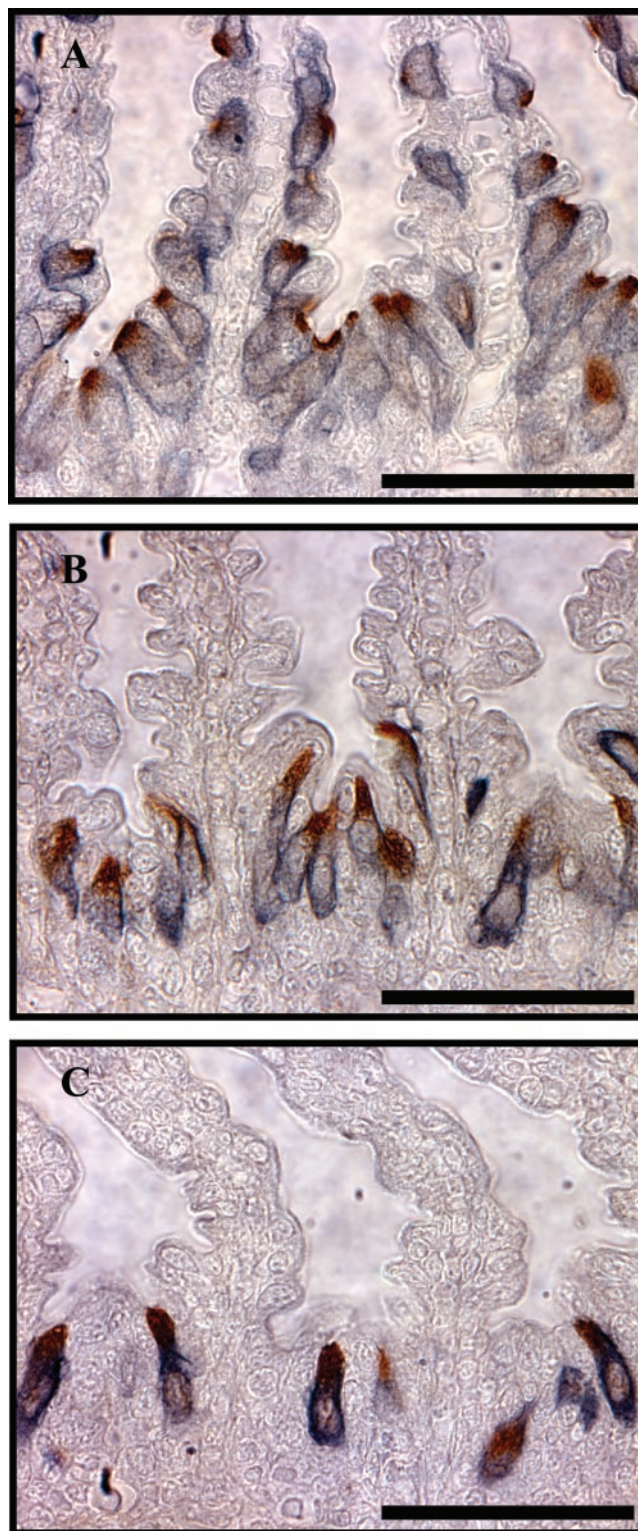


Fig. 6. Higher magnification photomicrographs of pendrin immunolabeling (brown) colocalized with V-H-ATPase (blue) in longitudinal sections of gill filaments from freshwater (A), seawater-acclimated (B), and marine (C) Atlantic stingrays ( $\times 1,000$ ). Note that pendrin immunostaining occurs in the apical region of V-H-ATPase-rich cells, regardless of salinity. Scale bar, 50  $\mu\text{m}$ .

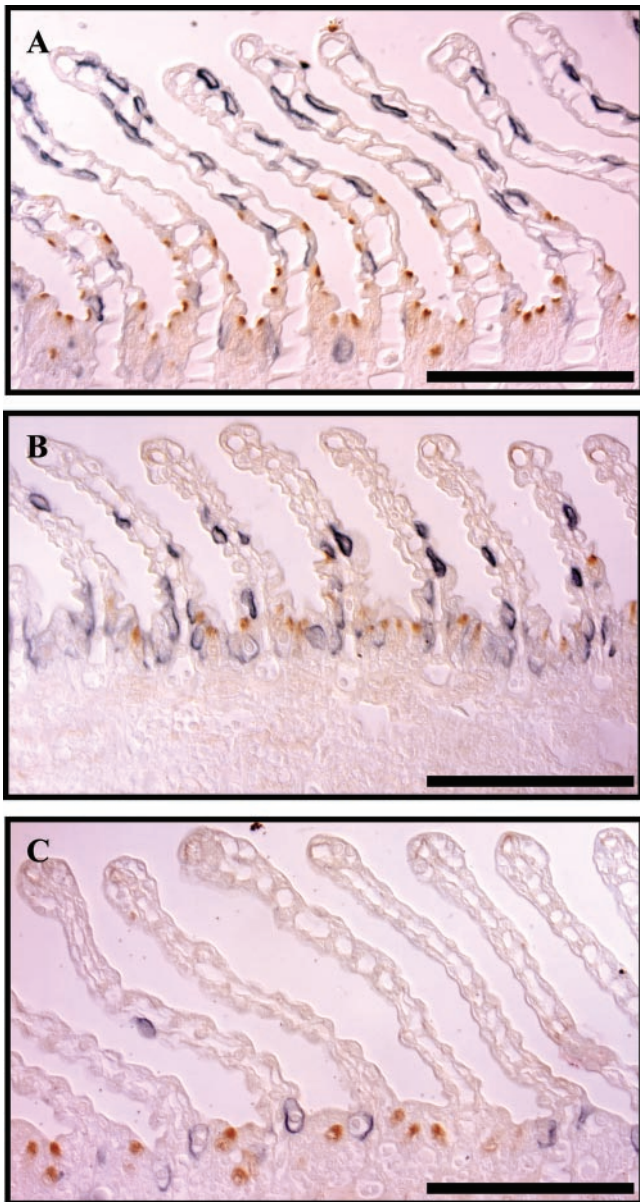


Fig. 7. Representative photomicrographs of pendrin immunolabeling (brown) colocalized with  $\text{Na}^+\text{-K}^+\text{-ATPase}$  (blue) in longitudinal sections of gill filaments from freshwater (A), seawater-acclimated (B), and marine (C) Atlantic stingrays ( $\times 400$ ). Note that pendrin and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  immunoreactivity occur in different cells, regardless of salinity. Scale bar,  $100\ \mu\text{m}$ .

Immunohistochemistry also demonstrated that localization of pendrin immunoreactivity within pendrin-positive cells occurred in the apical region and that this subcellular localization was influenced by environmental salinity. In freshwater stingray gills, pendrin-positive cells had discrete, intense apical localization (Fig. 4A). In seawater-acclimated and marine stingray gills, pendrin-positive cells lacked discrete apical staining and instead exhibited weaker, diffuse staining throughout the subapical cytoplasm (Fig. 4, B and C, respectively). These observations are similar to findings reported for transporters in other ion-secreting epithelia that are trafficked between a cytoplasmic

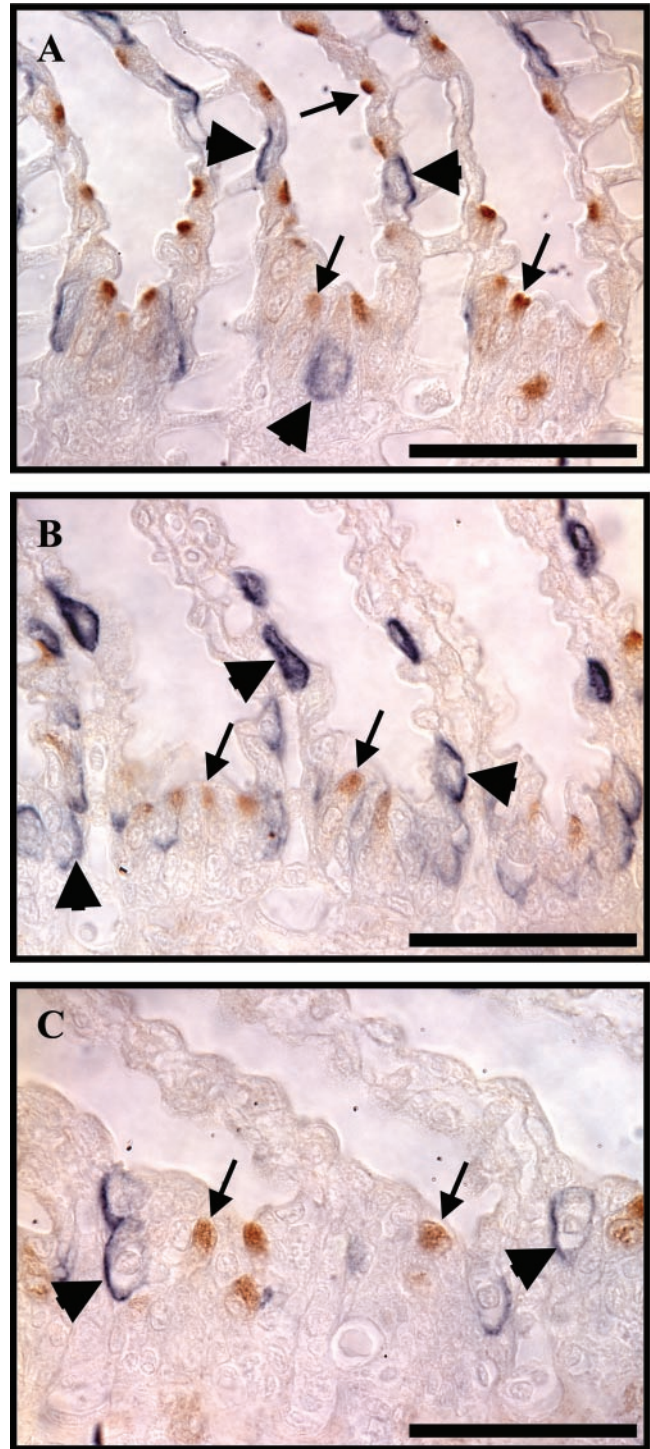


Fig. 8. Higher-magnification photomicrographs of pendrin immunolabeling (brown) colocalized with  $\text{Na}^+\text{-K}^+\text{-ATPase}$  (blue) in longitudinal sections of gill filaments from freshwater (A), seawater-acclimated (B), and marine (C) Atlantic stingrays ( $\times 1,000$ ). Thin arrows indicate apical region of pendrin-positive cells, and wide arrows indicate basolateral region of  $\text{Na}^+\text{-K}^+\text{-ATPase}$ -rich cells. Note that pendrin immunoreactivity does not occur in the apical region of cells with basolateral  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , regardless of salinity. Scale bar,  $50\ \mu\text{m}$ .

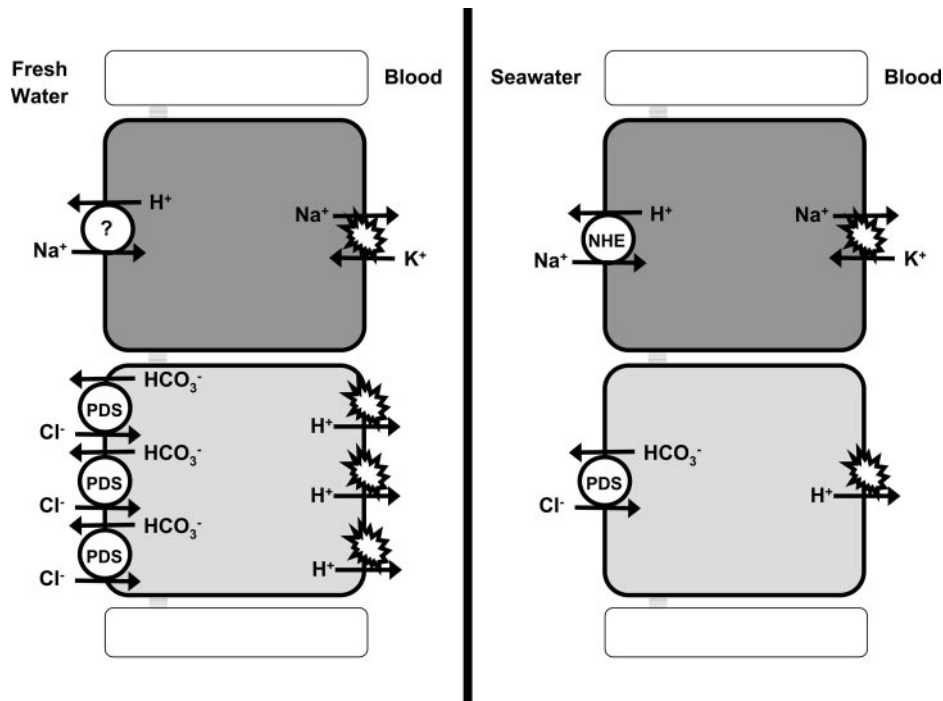


Fig. 9. Hypothetical model of NaCl and acid/base regulation in freshwater (*left*) and seawater-acclimated/marine (*right*) Atlantic stingray gills, based on results from this study, Piermarini and Evans (21, 22), and Edwards et al. (8). In freshwater Atlantic stingrays, we propose that V-H-ATPase-rich cells are a site of chloride uptake and bicarbonate secretion via an apical pendrin-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. Basolateral V-H-ATPase would actively pump protons across the basolateral membrane, which would create an intracellular bicarbonate concentration that is favorable for apical bicarbonate secretion and chloride uptake via a pendrin-like transporter. To balance the absorption of protons and chloride by the V-H-ATPase-rich cells, we hypothesize that  $\text{Na}^+/\text{H}^+$  exchange in freshwater elasmobranchs has not been identified to date. In seawater-acclimated/marine Atlantic stingrays, we propose that V-H-ATPase-rich cells function similarly to those in gills of freshwater elasmobranchs, but with less plasma membrane insertion of an apical pendrin-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger and basolateral V-H-ATPase. We hypothesize that the  $\text{Na}^+/\text{H}^+$  exchange in seawater-acclimated/marine elasmobranchs is a site of  $\text{Na}^+/\text{H}^+$  exchange via apical  $\text{Na}^+/\text{H}^+$  exchangers that have been localized to  $\text{Na}^+/\text{H}^+$  exchanger-rich cells in other marine elasmobranchs by Edwards et al. (8). NHE,  $\text{Na}^+/\text{H}^+$  exchanger; PDS, pendrin-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. "?" Indicates that presence of the transporter has not been demonstrated. Note that depicted differences in plasma membrane insertion of pendrin-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger and V-H-ATPase between freshwater and seawater-acclimated/marine elasmobranchs are based on qualitative immunohistochemical observations from this study and Piermarini and Evans (22) and do not reflect exact quantitative differences.

pool of vesicles and the plasma membrane (7, 17, 34, 35). Therefore, in addition to the greater relative abundance of pendrin immunoreactivity and number of pendrin-positive cells (see above), freshwater stingray gills may also have more pendrin-like transporters inserted into the apical plasma membrane of pendrin-positive cells than do seawater-acclimated and marine stingrays. This suggests the gill epithelium of freshwater stingrays has a relatively high potential for pendrin-like anion exchange activity, such as  $\text{Cl}^-/\text{base}$  exchange.

Greater protein expression and membrane insertion of a pendrin-like exchanger in freshwater stingray gills are consistent with the physiological need for enhanced chloride uptake in freshwater environments to counteract the large diffusional and urinary losses of chloride to the environment. Chloride uptake via a pendrin-like transporter would also provide a route for base secretion that could contribute to acid/base regulation. In seawater-acclimated and marine stingrays,

lesser expression and apical membrane insertion of a pendrin-like transporter may be adequate, because  $\text{Cl}^-/\text{base}$  exchange would only be required for acid/base balance; chloride uptake is no longer physiologically necessary in seawater environments and would actually be opposite to the needs of NaCl homeostasis.

Double-labeling experiments clearly demonstrated that pendrin immunoreactivity was exclusively found in the apical region of V-H-ATPase-rich cells and not  $\text{Na}^+/\text{K}^+$ -ATPase-rich cells (Figs. 5–8). These results are similar to pendrin localization reported in the mammalian CCD, where pendrin immunolabeling occurred in the apical region of bicarbonate-secreting V-H-ATPase-rich intercalated cells that were not immunoreactive for AE-1 (26). Because the pendrin immunolabeling in the Atlantic stingray gill occurs in the apical region of cells that are rich with basolateral V-H-ATPase, we suggest that the V-H-ATPase-rich cells are analogous in function to type B intercalated cells of the mammalian CCD and are a site of pendrin-



mediated apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange. In particular, we propose that proton extrusion by a basolateral V-H-ATPase would result in intracellular bicarbonate accumulation and create a favorable bicarbonate gradient for apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange via the pendrin-like transporter.

It is important to note that our results do not rule out the possibility that other anion exchangers exist in the elasmobranch gill epithelium, such as an AE-1-like  $\text{Cl}^-/\text{HCO}_3^-$  exchanger that has been detected in teleost gills (19, 20, 32, 36). It would be interesting for future studies to determine if other anion exchangers are present in the elasmobranch gill, what cells they are expressed in, and if their expression is influenced by environmental salinity.

Overall, the salinity-related differences in pendrin and V-H-ATPase (see Ref. 22) immunoreactivity of the Atlantic stingray gill are consistent with the model of branchial  $\text{NaCl}$  and acid/base transport mechanisms we proposed in an earlier study (22). We have incorporated our current results and those from a recent study on  $\text{Na}^+/\text{H}^+$  exchangers in elasmobranch gills (8) to further develop our hypothetical model of ion transport in the Atlantic stingray gill epithelium (Fig. 9).

### Perspectives

In summary, we have demonstrated that pendrin immunoreactivity is present in the gills of the Atlantic stingray, which is the first evidence of a pendrin-like transporter in any tissue from a lower vertebrate. Pendrin immunoreactivity is most abundant and most apical in the gills of freshwater stingrays compared with seawater-acclimated and marine stingrays and only occurs in the apical region of V-H-ATPase-rich cells, regardless of salinity. In conclusion, our findings suggest that a pendrin-like transporter may contribute to apical  $\text{Cl}^-/\text{HCO}_3^-$  exchange in the gill epithelium of the Atlantic stingray and may play an important role in the mechanisms of  $\text{NaCl}$  and acid/base homeostasis that allow this euryhaline species to inhabit both freshwater and marine environments.

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