

A putative H⁺-K⁺-ATPase in the Atlantic stingray, *Dasyatis sabina*: primary sequence and expression in gills

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Submitted 4 September 2003; accepted in final form 21 June 2004

Choe, Keith P., Jill W. Verlander, Charles S. Wingo, and David H. Evans. A putative H⁺-K⁺-ATPase in the Atlantic stingray, *Dasyatis sabina*: primary sequence and expression in gills. *Am J Physiol Regul Integr Comp Physiol* 287: R981–R991, 2004. First published June 24, 2004; 10.1152/ajpregu.00513.2003.—In mammals, the gastric H⁺-K⁺-ATPase (HK α 1) mediates acid secretion in the stomach and kidneys. Like mammals, elasmobranchs also secrete acid from their stomachs, but unlike mammals they primarily use their gills for systemic acid excretion instead of their kidneys. The purpose of this study was to determine if an HK α 1 orthologue exists in an elasmobranch (Atlantic stingray, *Dasyatis sabina*), to determine if it is expressed in gills and, if so, to localize its expression and determine if its expression is regulated during hypercapnia or freshwater acclimation. A polyclonal antibody made against an HK α 1 peptide detected HK α 1 immunoreactivity in protein isolates and tissue sections of stingray stomachs and gills. Immunohistochemistry demonstrated that HK α 1 immunoreactivity was present in a subpopulation of epithelial cells in both organs. Double-labeling experiments in the gills showed that HK α 1 immunoreactivity occurred in Na⁺-K⁺-ATPase-rich cells and not in V-type H⁺-ATPase-rich cells. RT-PCRs were used to deduce the primary sequence of a putative H⁺-K⁺-ATPase from the stomach of Atlantic stingrays. The 3,421-base pair cDNA includes a coding region for a 1,025-amino acid protein that is over 80% identical to HK α 1 of mammals. RT-PCRs were then used to demonstrate that this transcript is also expressed in the gills. To our knowledge, this is the first H⁺-K⁺-ATPase sequence reported for any elasmobranch and the first full-length sequence for any fish. We also provide the first evidence for its expression in the gills of any fish and demonstrate that its expression increased during freshwater acclimation but not exposure to hypercapnia.

elasmobranch; proton pump; gastric; stomach

H⁺-K⁺-ATPASES consist of an α -subunit that catalyzes ATP hydrolysis and ion translocation and a β -subunit that is thought to be involved in stabilizing and cellular targeting of the transporter. The α -subunit belongs to the large P-type ATPase (P-ATPase) superfamily of membrane proteins, which actively transport charged substrates across membranes (33). Other P-ATPases include sarcoplasmic reticulum Ca²⁺-ATPases (SERCA), plasma membrane Ca²⁺-ATPases, and Na⁺-K⁺-ATPases (21). A highly homologous subgroup of this family (type IIc) includes the gastric H⁺-K⁺-ATPases (HK α 1), non-gastric/colonic H⁺-K⁺-ATPases (HK α 2), and the Na⁺-K⁺-ATPases (NaK α 1, 2, and 3) (21).

Gastric H⁺-K⁺-ATPase activity was first measured as K⁺-stimulated ATPase in microsomes prepared from bullfrog (*Rana catesbeiana*) stomach (15) and was later characterized

as a nonelectrogenic H⁺-K⁺-ATPase in vesicular membranes prepared from pig stomachs (39). It functions as a heterodimeric complex of an α - or catalytic subunit (HK α 1) and a β -subunit (HK β) in tubulovesicular and canalicular membranes of mammalian gastric parietal cells, where it exchanges luminal K⁺ for cytoplasmic H⁺ (42). Chloride channels in the apical plasma membrane facilitate Cl⁻ loss, and the net result is HCl secretion. In mammalian stomachs, this mechanism can create acidic secretions with a pH as low as 0.8 and work against up to 3 million-fold [H⁺] gradients (19). H⁺-K⁺-ATPase has also been predicted to function in secretion of acid in fish stomachs, but the evidence is sparse. For example, although teleosts and elasmobranchs are known to secrete acid from their stomachs (23), the only fish sequence data in GenBank is an HK α 1-like cDNA fragment from the stomach of a teleost fish, *Pleuronectes americanus* (AW013156) (7, 18). The only evidence in an elasmobranch is a study that detected immunoreactivity for HK α 1 in gastric oxyntic cells of the Atlantic stingray (*Dasyatis sabina*) (43).

HK α 1 protein and mRNA expression have also been demonstrated in mammalian kidneys, where it is thought to function in systemic acid-base and ion regulation. For example, cDNA identical to HK α 1 has been sequenced from mammalian kidneys (26), and pharmacological, immunohistochemical, and in situ hybridization studies suggest that HK α 1 is expressed in the connecting segment and the collecting duct where it secretes H⁺ and reabsorbs K⁺ (25, 48–50). In elasmobranchs, gills have many of the functions of kidneys in mammals. For example, the gills of elasmobranchs are responsible for 90–100% of systemic net-acid excretion during acidosis (2, 5) and are the site of active K⁺ absorption in freshwater (8, 16, 17). Appropriately, parallels between epithelial cells in the gills of an elasmobranch, Atlantic stingray (*Dasyatis sabina*), and renal tubule cells of mammals have been discovered. For example, in one cell type of stingray gills thought to secrete base, apical immunoreactivity for a Cl⁻/HCO₃⁻ exchanger (pendrin) and basolateral immunoreactivity for vacuolar H⁺-ATPase (V H⁺-ATPase) occur similar to type B intercalated cells in the cortical collecting duct of mammalian kidneys (37). High levels of basolateral NaK α immunoreactivity were observed in another gill cell type thought to secrete acid and absorb electrolytes (34), but the mechanisms of acid and electrolyte transport in this cell type are not known.

The discovery that HK α 1 participates in systemic ion and acid-base regulation in mammalian kidneys and the reported similarities between mammalian intercalated cells and elasmobranch gills suggest that HK α 1 may have evolved from a common ancestor.

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branch epithelial cells led us to hypothesize that HK α 1 may be expressed in the gills of elasmobranchs. Therefore, the first goals of this study were to determine if HK α 1 immunoreactivity exists in the gills of the Atlantic stingray and, if so, to determine if it colocalizes with NaK or V H⁺-ATPase. Furthermore, we also sought to determine if HK α 1-like mRNA exists in Atlantic stingrays and, if so, to obtain its primary sequence, verify whether it is expressed in the gills, and determine if its expression is altered during hypercapnia or acclimation to freshwater. Here we report the first cDNA sequence for an H⁺-K⁺-ATPase in an elasmobranch, provide the first evidence for expression of an HK α 1-like protein in the gills of any fish, and demonstrate that HK α 1 mRNA expression increases during acclimation to freshwater.

MATERIALS AND METHODS

Animals and holding conditions. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Atlantic stingrays (*Dasyatis sabina*, ~0.5–1.0 kg) were captured from waters surrounding Sea Horse Key, FL, in the Gulf of Mexico using a cast net. They were transported to the University of Florida in Gainesville, FL, where they were held in a 380-liter Rubbermaid tank containing 100% seawater. The water pH was maintained between 7.8 and 8.2 with a commercial aquarium carbonate buffer (Seachem, Stone Mountain, GA), and NH₃ and NO₃⁻ were maintained below 1 ppm with a biological filter. The room that housed the stingrays was on a 12:12-h light/dark cycle, and the stingrays were fed shrimp to satiation three times a week, up to 2 days before they were killed.

Tissue collection for Western blots and immunohistochemistry. Three stingrays were anesthetized by an initial immersion in 150 mg/l MS-222 diluted in tank water. The anesthetic was diluted to 75 mg/l by addition of tank water, and stingrays were perfused through the conus arteriosus with cold (~4°C) elasmobranch Ringer (14) to remove blood. The stingrays were then pithed, and the stomachs were either placed in fixative (3% paraformaldehyde, 0.05% glutaraldehyde, 0.05% picric acid in 10 mmol/l phosphate-buffered saline, pH 7.3) for 24 h at 4°C or scraped with a glass slide and snap frozen in liquid nitrogen. Similarly, gill filaments were trimmed from the arches and either fixed for immunohistochemistry or frozen for Western blots. The rectal gland from one animal was cut into pieces (no dimension longer than 3 mm) and placed in fixative. After the 24 h of fixation, the tissues were rinsed and stored in two changes of 75% ethanol.

Antibodies. The mouse monoclonal antibody for NaK α (α 5) was developed by Dr. D. 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. It was made against avian NaK α and binds to all isoforms. This antibody recognizes fish NaK α and is now used widely for studies on fish branchial cells (3, 4, 9, 34, 35, 47).

The rabbit polyclonal antibody for V H⁺-ATPase was developed by Filippova et al. (13) and was a gift from Dr. W. Harvey at the Whitney Laboratory, University of Florida (with permission from Dr. S. Gill, University of California at Riverside). It was made against a 279-amino acid peptide that matches residues 79–357 of *Culex quinquefasciatus* B subunit. This antibody has been used to localize V H⁺-ATPase in Atlantic stingrays (35).

The rabbit polyclonal antibody for HK α 1 (C2) was a gift of A. Smolka in the Department of Medicine and Cell Biology and Anatomy, Medical University of South Carolina. It was made against a synthetic peptide based on the 16 carboxyl-terminal amino acids of the pig HK α 1 (43). It stains the apical cytoplasm of pig and rat gastric parietal cells and labels a single protein band of 94 kDa on Western blots of pig gastric microsomes.

Western blotting. Immunoblots were prepared from stomach scrapings and gill tissue using a procedure modified from Piermarini and Evans (34). Briefly, tissues were minced, and homogenized in buffer [250 mmol/l sucrose, 30 mmol/l Tris, 1 mmol/l Na₂EDTA, 5 μ l/ml protease inhibitor cocktail (Sigma P8340), 100 μ g/ml phenylmethylsulfonyl fluoride, pH 7.8] with a mechanical homogenizer for 30 s at maximum speed on ice. Homogenates were first centrifuged at 300 g for 5 min at 4°C to remove debris and whole nuclei. This supernatant was then centrifuged at 10,000 g for 10 min at 4°C to remove mitochondria. Finally, the supernatant from the second spin was centrifuged at 100,000 g for 60 min at 4°C to form a pellet of microsomes.

This microsomal pellet was resuspended in homogenization buffer and an equal volume of 2 \times Laemmli sample buffer (28) that lacked bromophenol blue and β -mercaptoethanol. The resulting protein samples were centrifuged at 16,000 g for 5–10 s to remove any undissolved material. The total protein concentration was determined with the Pierce BCA assay (Rockford, IL), and bromophenol blue and β -mercaptoethanol were added to final concentrations of 0.01 and 2%, respectively. Thirty micrograms of protein samples was loaded and run in 7.5% Tris-HCl precast polyacrylamide gels (Bio-Rad, Hercules, CA) and then transferred onto PVDF membranes.

The PVDF membranes were blocked in Blotto (5% nonfat milk in Tris-buffered saline) for 2 h at ~24°C and then incubated in Blotto with either monoclonal antibody α 5 (1:1,000) or polyclonal antibody C2 (1:10,000) overnight at 4°C. Membranes were then washed with four changes (15 min each) of Tris-buffered saline with 0.1% Tween-20 (TBST) and incubated in goat anti-mouse (Bio-Rad) or goat-anti rabbit (Bio-Rad) IgG secondary antibody (alkaline-phosphatase conjugated) diluted 1:3,000 in Blotto for 2 h at ~24°C. After four more washes in TBST, the membranes were incubated in ImmunoStar ECL substrate (Bio-Rad) for 5 min at 24°C. Luminescent bands were then detected with Amersham-Pharmacia's Hyperfilm-ECL according to manufacturer's protocol. Lastly, Western blot results were digitized with a Canon D660U flatbed scanner and Adobe Photoshop 5.0 software.

Immunohistochemical localization. Immunohistochemistry was completed on paraffin-embedded sections as described previously (34, 35), with minor modifications. Fixed tissues were dehydrated in an ethanol series and embedded in paraffin wax. Sections were cut at 6 μ m and dried onto poly-L-lysine-coated slides. For gills, sections from the trailing half of the filaments were selected for immunohistochemical staining, because they contain the highest density of ion transport cells (PM Piermarini, unpublished observation). Tissue sections were deparaffinized in Citrisolv (Fisher Scientific, Pittsburgh, PA) and rehydrated in an ethanol series followed by PBS. Endogenous peroxidase activity was inhibited by incubating with 3% H₂O₂ for 25 min at 24°C. Nonspecific binding sites on the tissues were blocked by incubating with Biogenex's protein block (San Ramon, CA; BPB; normal goat serum with 1% bovine serum albumin, 0.09% NaN₃, and 0.1% Tween-20) for 20 min.

Sections were incubated with antibody C2 (1/2,000 to 1/3,500 in BPB) overnight at 4°C in a humidified chamber. 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 Biogenex's multilink solution (biotinylated goat anti-mouse, rabbit, guinea pig, and rat antibodies diluted in BPB) followed with Biogenex's horseradish-peroxidase streptavidin solution for 20 min at 24°C each. After another wash in PBS for 5 min, antibody binding was visualized by incubating with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 min at 24°C. Sections were then rinsed with running tap water for 5 min, dehydrated in an ethanol-Citrosolv series, and mounted with a coverslip using Permount (Fisher Scientific).

Labeling of HK α 1 and NaK α or V H⁺-ATPase in the same sections. A double-labeling technique described previously (35) was used to compare the location of HK α 1 immunoreactivity to NaK α and

V H⁺-ATPase immunoreactivity in gills from seawater stingrays. Gill sections were deparaffinized, hydrated, and stained for HK α 1 with antibody C2 as described above. However, after treatment with DAB and rinsing with water, the sections were again blocked with BPB for 20 min and incubated with either the primary antibody α 5 (NaK α) or anti-V H⁺-ATPase as above. Concentrations were 1/10 or 1/50 for α 5 and 1/10,000 for V H⁺-ATPase. Detection of bound antibody was done as described above, except Vector SG, which produced a blue reaction product, was used instead of DAB, which produces a brown reaction product. Lastly, sections were mounted as described above.

RT-PCR, cloning, and sequencing. A seawater stingray was perfused with Ringer as described above, and stomach mucosa was removed under sterile conditions. Total RNA was isolated with TRI-reagent (Sigma) and reverse transcribed with a Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA), using oligo(dT) as a primer, and stored at -20°C .

The first set of primers (F1 deg and R2 deg, Table 1) were designed for amino acid sequences that are conserved between *Xenopus laevis* (GenBank accession number AAA76601) and *Pleuronectes americanus* (GenBank accession number AAD56285) HK α 1 proteins. Each PCR was performed on 1/20 of a reverse transcriptase reaction with a FastStart Taq DNA Polymerase kit (Roche Applied Science, Indianapolis, IN) in a PCR Express thermocycler (ThermoHybaid, Franklin, MA) with the following parameters: initial denaturing for 5 min at 94°C , followed by 35 cycles of 1 min at 94°C , 30 s at 52°C , and 1 min 20 s at 72°C . The final cycle was followed by 7 min at 72°C , and the reaction was held at 4°C . A 1,047-base pair (bp) product was visualized by ethidium bromide staining in a 1% agarose gel. This product was ligated into pCR 4-TOPO vectors and transformed into TOP10 chemically competent cells using a TOPO TA Cloning kit for sequencing (Invitrogen). Plasmid DNA was then sequenced in both directions at the Marine DNA Sequencing Facility at the Mount Desert Island Biological Laboratory (Salisbury Cove, ME).

OLIGO 6.7 software (Cascade, CO) was then used with this 1,008-bp fragment (excluding primer sequence) to design a nondegenerate antisense primer (R1742, Table 1) for amplification toward the 5'-end of the stomach transcript. A sense degenerate primer (F318 deg, Table 1) was designed to match amino acids that are conserved in all the HK α 1 sequences within GenBank. RT-PCR was completed as above, except that the PCR annealing temperature was increased to 58.7°C and the elongation steps were extended to 1 min 45 s. A 1,442-bp product was visualized, cloned, and sequenced as above.

5'/3'-Rapid amplification of cDNA ends. 5'/3'-Rapid amplification of cDNA ends (5'- and 3'-RACE) cDNA was prepared with a GeneRacer kit (Invitrogen) according to the manufacturer's protocols. The 2,300-bp consensus alignment of the two original sequences obtained with RT-PCR and degenerate primers was used to design nondegenerate antisense (R471 and R387, Table 1) and sense (F2602,

Table 1) primers for 5'- and 3'-RACE, respectively. PCR reactions for 5'- and 3'-RACE were completed as described above, except for the use of touchdown cycle parameters and nested PCR to increase specificity (see below).

For 5'-RACE, the first PCR reaction used primer R471 and a kit primer that anneals to the 5'-end of the GeneRacer oligonucleotide with the following denaturing, annealing, and elongation parameters: 5 cycles of 1 min at 94°C and 1 min at 72°C ; 5 cycles of 1 min at 94°C and 1 min at 70°C ; and 25 cycles of 1 min at 94°C , 1 min at 65°C , and 1 min at 72°C . Nested PCR was performed at two annealing temperatures, each with primer R387 and a kit primer that anneals to the 3'-end of the 5' GeneRacer oligonucleotide with the following denaturing, annealing, and elongation parameters: 25 cycles of 1 min at 94°C , 1 min at 61°C or 70°C , and 1 min at 72°C . An ~ 470 -bp product was visualized with both annealing temperatures. They were both cloned, and eight colonies were sequenced.

For 3'-RACE, the first PCR reaction used primer F2602 and a kit primer that anneals to the 3'-end of the 3' GeneRacer oligo(dT) primer with the denaturing, annealing, and elongation parameters identical to the first 5'-RACE PCR reaction, except elongation steps were increased to 2 min each. Nested PCR was performed on this product with primer F2602 and a kit primer that anneals to the 5'-end of the 3' GeneRacer oligo(dT) primer with the following denaturing, annealing, and elongation parameters: 25 cycles of 1 min at 94°C , 1 min at 70°C , and 1 min 30 s at 72°C . An ~ 800 -bp product was visualized and cloned as described above, and six colonies were sequenced as described above.

Sequence analysis. Sequences were assembled with GeneTools software (BioTools, Edmonton, Alberta), and the assembled nucleotide sequence was searched for open reading frames. The predicted amino acid sequence was aligned with type IIc P-type ATPases of rat (*Rattus norvegicus*) using the ClustalV method of MegaAlign software (InforMax, Frederick, MD). An unrooted distance tree of the same sequences was generated with Prodist, Fetch, and Neighbor-Joining software of the PHYLIP program package (12). The predicted location of the conserved phosphorylation site and of transmembrane regions were taken from Sweadner and Donnet (44) and Okamura et al. (33). A rooted tree of HK α 1 sequences was generated with MegaAlign software.

Multiple tissue relative quantitative PCR (RQ RT-PCR) and verification of gill expression. To determine if other acid-base and/or ion transport tissues express the putative HK α 1 sequenced from stomach, relative quantitative RT-PCR was performed on stomach, rectal gland, gill, and kidney total RNA. A seawater stingray was perfused with Ringer as described above, and an ~ 100 -mg sample of each tissue was removed with an independent set of sterile scissors and forceps. Each tissue sample was then placed in a sterile microfuge tube, and homogenized with a sterile disposable pestle in TRI-reagent (Sigma).

Table 1. Primers used in PCR

Name	Orientation	Nucleotide Sequence (5' to 3')	Amino Acids
F1 deg	sense	GAY GAR CAR TGG AAR GAR GC	DEQWKE
R2 deg	antisense	GGR AAC CAN CCY TCY TGN GCC	AQEGWF
R1742	antisense	GTG GGG AAG TTC ATC TCG T	NA
F318 deg	sense	N CTN CAR TGY CTN ATG TGG GT	LQCLMWA
R471	antisense	CAC CAC CAC CAC TGC GAT GAG	NA
R387	antisense	GCG ATG AAG CAG ATG ACG GA	NA
F2602	sense	GCA GCT CGC TGT CTA CTC CTA TTT C	NA
F196	sense	CGT GAT GAA ATG CCG TAC C	NA
R646	antisense	ACG ATG GAG GCG AAG TTG TCA	NA
F3c	sense	ACT TCG CCT CCA TCG TCA	NA
R3c	antisense	TGT TCT TCG TCA GCG TGT ATG C	NA
L8F1	sense	AAG AAG GCT CAG TTG AAC ATT GGA	NA
L8R2	antisense	TGT ACT TGT GAT AAG CCC GAC CAG	NA

Amino acids are only given for the degenerate primers. NA, not applicable.

Total RNA and cDNA were produced as described above, but random primers were used so that ribosomal and messenger RNA would be reverse transcribed.

Nondegenerate primers (F196 and R646, Table 1) were designed to the stomach sequence with OLIGO 6.7 to amplify a 471-bp PCR product that spans three intron locations that are conserved between HK α 1 and NaK α (32). These primers were successfully tested on stomach and gill cDNA as described above with the following denaturing, annealing, and elongation parameters: 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C.

A QuantumRNA 18S internal standard primer kit (Ambion, Austin, TX) was used to control for variability in RNA quality and quantity between the different tissues tested. This kit was chosen for two reasons: 1) the amount of 18S ribosome has been shown to be constant between different cells types and stages of the cell cycle, and 2) competitors are included, which reduce the efficiency of 18S amplification so that it approximates that of the product of interest. Multiplex PCR with 18S and putative HK α 1 primer sets was then optimized as suggested by the manufacturer. First, the range of cycle numbers where the amount of putative HK α 1 product was linearly proportional to the cycle number was determined for gill cDNA. Gill was used instead of stomach, because stomach was expected to express far more putative HK α 1 than any other tissue. Second, a series of multiplex PCR reactions were performed to determine a ratio of 18S primers to competitors that would make the kinetics of 18S amplification approximate to those of the putative HK α 1. Finally, the optimized multiplex PCR (37 cycles with an 18S primers to competitors ratio of 1:9) was performed on cDNA from all the tissues with the following denaturing, annealing, and elongation parameters: 37 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The products were visualized as above and photographed with Polaroid 667 film.

To verify expression of the putative HK α 1 in gills, more RT-PCR experiments were conducted on gill total RNA from three more stingrays. PCR was conducted as described above and included reactions lacking template and with RNA that was not reverse transcribed. The results were photographed as above and products were sequenced as above.

Quantitative real-time PCR. For the hypercapnia experiments, 20 stingrays were captured and held in 100% seawater as indicated above, except they were never fed. These stingrays were then exposed to normocapnic (air) or hypercapnic (1% CO₂ in air) water, as described previously (2). Briefly, stingrays were transferred into a 10.5-liter closed-recirculating chamber (one/chamber) fitted with a countercurrent gas/water exchange column. All stingrays were first exposed to a pretreatment period of 4–5 h, when the exchange column received air from a gas mixer. After this pretreatment period, the exchange column received air (control) or 1% CO₂ in air (hypercapnia). Total RNA was then isolated from the gills of stingrays after 2–4 h for quantitative real-time PCR (qRT-PCR) as described above.

For the salinity experiments, eight stingrays were captured and held in 100% seawater as indicated above, except they were never fed. The stingrays were then divided into two groups; one group remained in 100% seawater (approximate concentrations in mmol/l: 517.36 Na⁺, 8.66 Ca²⁺, 11.54 K⁺, 485.60 Cl⁻), and the other was transferred to a separate 380-liter tank where they were gradually exposed to freshwater (buffered Gainesville tap water, approximate concentrations in mmol/l: 3.50 Na⁺, 1.16 Ca²⁺, 0.03 K⁺, 0.40 Cl⁻; 1 day in 75% seawater, 2 days in 50% seawater, and 1 day in 25% seawater) (2). Commercial carbonate salt buffers (Seachem Laboratories, Covington, GA) were added to make the pH and [HCO₃⁻] stable and approximately equal in the two salinities (2). Stingrays remained in either seawater or freshwater for an additional 7 days, and total RNA was isolated for quantitative real-time PCR analysis as described above.

To measure relative expression levels, poly A RNA was reverse transcribed as described above and the resulting cDNA was subjected

to PCR in the presence of SYBR Green (Molecular Probes, Eugene, OR) binding dye in a real-time thermal cycler (1). Nondegenerate primers for stingray HK α 1 (F3c and R3c, Table 1) and ribosomal protein L8 (L8F1 and L8R2) were designed to amplify amplicons with high efficiency according to the specifications of Applied Biosystems SYBR Green master mix protocol (Foster City, CA). To minimize the chance of amplifying contaminating genomic DNA, primer pairs were designed to include an intron-exon boundary that is conserved between vertebrate homologues. qRT-PCR reactions with gill RNA samples that were not reverse transcribed verified that no products were amplified from contaminating genomic DNA.

All qRT-PCR reactions were run in triplicate and included 0.2 μ l of cDNA (2.0 μ l of a 1/10 dilution of original cDNA), 7.4 pmol of each primer, and SYBR Green Master Mix (Applied Biosystems) in a total volume of 25 μ l. All qRT-PCR reactions were run in an ABI 5700 sequence detection thermal cycler (Applied Biosystems) with the following cycling parameters: initial denaturing for 10 min at 95°C, followed by 40 cycles of 35 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The final cycle was followed by a melting curve analysis to verify the amplification of a single product in each well. Relative gene expression was calculated from a relative standard curve that used tenfold serial dilutions of purified plasmid DNA as the template, and all results were normalized to ribosomal protein L8, a highly conserved gene for which expression in the gills remains constant during salinity and acid-base changes (unpublished observation). Unpaired Student's *t*-tests were used to compare seawater with freshwater acclimated and control to hypercapnic expression levels. Alpha was set at 0.05.

RESULTS

Immunoblots. The anti-HK α 1 polyclonal antibody C2 bound to a single 109-kDa band on immunoblots of the microsomal fraction of stomach mucosa (Fig. 1A). It also bound to a single 93-kDa band on immunoblots of the microsomal fraction of gill tissue (Fig. 1A). The anti-NaK α monoclonal antibody α 5 bound to a band of ~112 kDa on immunoblots of the microsomal fraction of stomach and gill tissue (Fig. 1B). No bands were observed in negative controls experiments that omitted primary antibodies (not shown).

Immunohistochemistry. The anti-HK α 1 antibody C2 reacted strongly with epithelial cells in stingray gastric glands (Fig. 2A). No specific immunostaining was observed when BPB

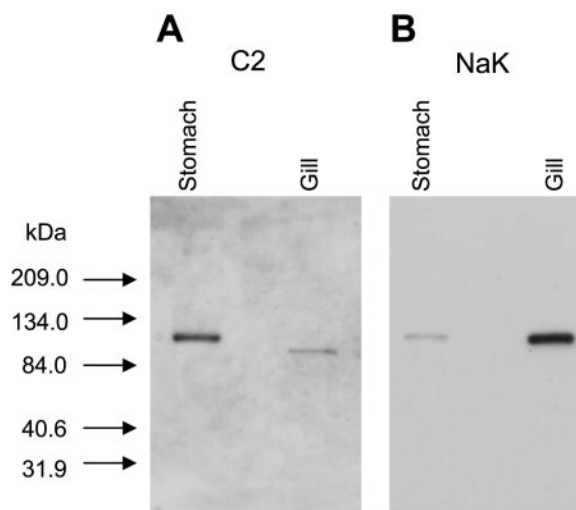


Fig. 1. Representative immunoblots of microsomal proteins from stomach and gill with antibody C2 (A) and antibody α 5 (B).

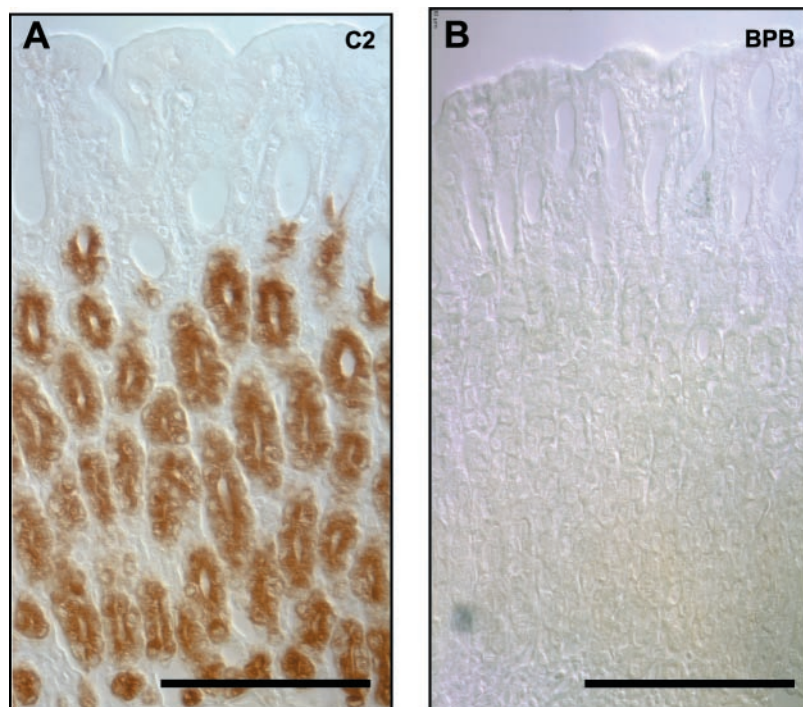


Fig. 2. Representative light micrographs of Atlantic stingray stomach gastric gland sections incubated with antibody C2 (A) or BioGenex's protein block (BPB) (B). No immunolabeling was observed in negative control sections that were incubated with BPB in place of primary antibody followed by multilink (anti-mouse, rabbit, and donkey) (B) secondary antibodies. Strong immunolabeling was obtained in cells lining the gastric glands with antibody C2 (A). Scale bars 100 μ m.

alone was used in place of primary antibody (Fig. 2B) or when C2 was incubated with stingray rectal gland sections (not shown).

Antibody C2 also reacted strongly with a subpopulation of epithelial cells in stingray gills (Fig. 3, A and C). Staining was limited to the cytoplasm of large, cuboidal epithelial cells in the filament and at the base of lamellae and did not stain thin cells on the lamellae (i.e., pavement cells) (Fig. 3C). No staining was observed when BPB alone was used in place of primary antibody (Fig. 3B).

Gill double labeling of HK α 1 and NaK α 1 or V H⁺-ATPase. We stained for HK α 1 and NaK α 1 or V H⁺-ATPase in the same gill sections to determine if HK α 1 immunoreactivity colocalized with either of the latter ATPase ion pumps. HK α 1 immunoreactivity was in the same cells as NaK α immunoreactivity (Fig. 4A). As with the single labeling of HK α 1, these cells were usually on the filament (Fig. 4A). Within these double-labeled cells, NaK α staining was confined to the basolateral region and HK α 1 staining was distributed throughout the cytoplasm. HK α 1 immunoreactivity was not found in cells that stained for V H⁺-ATPase (Figs. 4B).

Molecular identification of a putative stingray HK α 1. In initial PCR reactions, oligonucleotide primers F1 deg and R2 deg supported the amplification of a 1,047-bp product from stingray stomach cDNA. This product matched the size expected from vertebrate HK α 1 nucleotide alignments and was 75% identical to the homologous region of *X. laevis* HK α 1 (GenBank accession number U17249). Primers F318 deg and R1742 were then used to amplify and sequence a 1,442-bp product that overlapped 109 bp with the first product and was 74.3% identical to *X. laevis* HK α 1. Lastly, nondegenerate primers based on an assembly of these first two PCR products were used with RACE to amplify and sequence the remaining 5'- and 3'-ends of the putative stingray HK α 1 transcript.

The complete putative stingray HK α 1 cDNA (accession number AY283439) contains 3,421 nucleotides with a 3,071-nucleotide open reading frame. The likely start codon (ATG) is 96 nucleotides downstream from the 5'-end of the cDNA obtained by RACE, and the likely in-frame stop codon (TAA) is 250 nucleotides upstream from the 3'-end of the cDNA. A transcript cleavage sequence (AATAAA) is 33 nucleotides upstream from a poly-A tail.

Distribution of putative HK α 1 expression. Multiplex RT-PCR with cDNA from gill, rectal gland, and kidney was conducted to determine if the putative stingray HK α 1 mRNA was expressed in transport tissues other than stomach mucosa. After 37 cycles of PCR, the 471-bp product expected from the putative stingray HK α 1 specific primers (F196 and R636) was only observed for stomach and gill, with stomach yielding markedly more product (Fig. 5A). Alternatively, the 315-bp product expected from the 18S internal control primers was observed in all tissues at roughly equivalent levels.

More RT-PCR experiments were conducted with RNA from gills of three separate stingrays to verify the expression of the putative HK α 1 mRNA in gills. Primers F196 and R646 amplified 471-bp products (see Fig. 5B for representative results) from all three that, after cloning and sequencing, were identical to the corresponding region of the stomach sequence. These products were not amplified from PCR reactions lacking template or with total RNA that had not been reverse transcribed (Fig. 5B).

qRT-PCR. There was no difference in expression of HK α 1 in the gills from normocapnic and hypercapnic stingrays after either 2 or 4 h of exposure (Fig. 6A). Alternatively, the expression of HK α 1 in the gills from freshwater-acclimated stingrays was 2.39-fold greater than in gills from seawater stingrays (Fig. 6B).

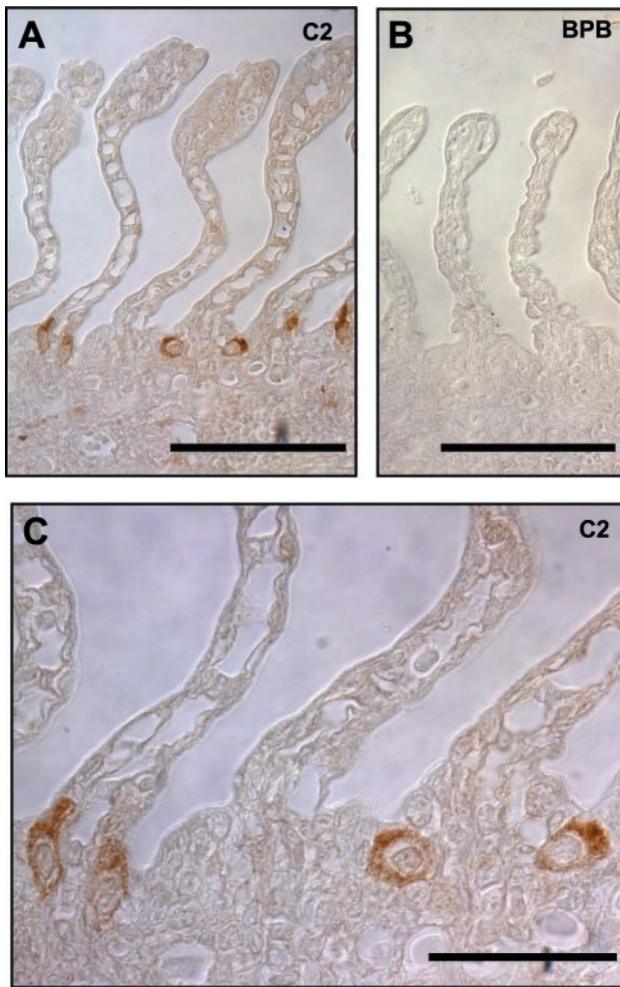


Fig. 3. Representative light micrographs of gill sections from Atlantic stingrays incubated with antibody C2 (A and C) or BPB (B). No immunolabeling was observed in negative control sections that were incubated with BPB in place of primary antibody followed by multilink (anti-mouse, rabbit, and donkey) (B) secondary antibodies. However, strong immunolabeling occurred in epithelial cells with antibody C2 (A and C). Most of these cells occurred on the filament, and within these cells, immunolabeling occurred throughout the cytoplasm (C). Scale bars 100 μm (A and B) or 50 μm (C).

DISCUSSION

Our results are the first to demonstrate that an HK α 1, similar to that of mammalian gastric parietal cells and renal intercalated cells, is present not only in the gastric glands of an elasmobranch but also in NaK-rich gill epithelial cells where it may function in K⁺ absorption. These conclusions are based on our demonstration of HK α 1 immunoreactivity in stingray stomachs and gills, amplification of HK α 1-like cDNA from stingray stomachs and gills, and a greater expression of HK α 1 in the gills of freshwater-acclimated stingrays than in seawater stingrays.

HK α 1 immunoreactivity. The band detected on immunoblots of stingray stomach mucosa is slightly larger than the band detected on immunoblots of stingray gills, suggesting different posttranslational modifications, posttranscriptional modifications, and/or expression of different isoforms in the two tissues. Both bands are close to the calculated molecular weight of the putative stingray HK α protein (114.6 kDa, sum of amino acids).

Elasmobranch stomachs secrete acid (23), and stingray stomachs were shown previously to be immunoreactive with antibody C2 (43). Our results confirm this immunoreactivity and suggest that C2 recognizes a stingray gastric proton pump. Elasmobranch rectal glands excrete a hyperionic fluid to maintain ion balance in seawater (11, 22) via a secondarily active mechanism that depends on high levels of Na⁺-K⁺-ATPase activity (10, 40). Therefore, the lack of specific immunoreactivity between rectal gland and C2 suggests that this antibody does not crossreact with stingray NaK α .

Our results indicate that, within gills, HK α 1 immunoreactivity occurs exclusively within NaK-rich epithelial cells. This cell type contains abundant NaK in its basolateral membrane and is thought to drive acid secretion and electrolyte absorption as mechanisms of systemic acid-base and ion regulation (9, 34, 35). Therefore, if apical, HK α 1 may be a mechanism of acid secretion and/or K⁺ absorption in this putative ion-transporting cell type. The apparent cytosolic distribution of staining with C2 suggests a vesicular location, similar to HK α 1 immunolocalization in resting mammalian gastric parietal cells and renal collecting duct intercalated cells (25, 49). Stimulation of acid

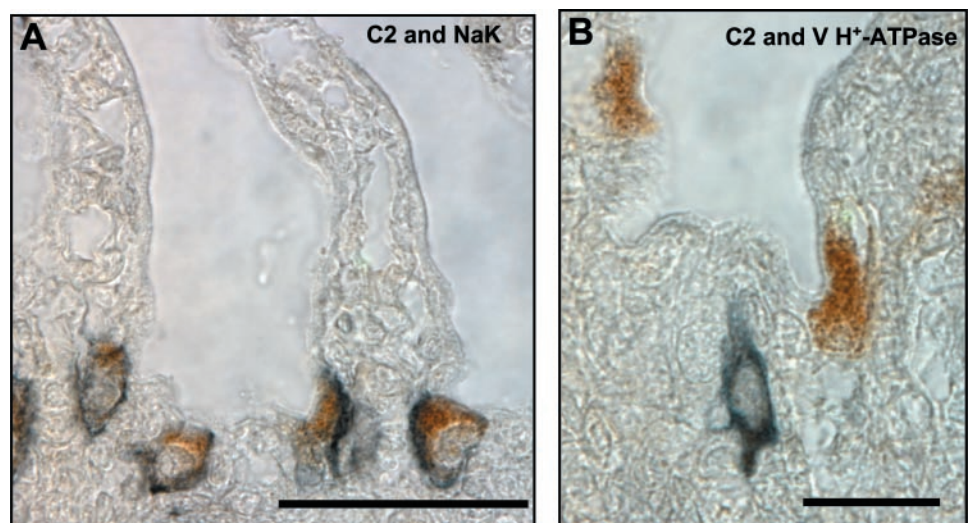


Fig. 4. Representative light micrographs of Atlantic stingray gill sections incubated with antibodies C2 (brown) and α 5 (blue) (A) or C2 (brown) and the antibody for V H⁺-ATPase (blue) (B). C2 and α 5 labeled the same cells with C2 throughout the cytoplasm and α 5 restricted to the basolateral region (A). Most of the immunopositive cells occurred on the filament. Scale bar 50 μm . C2 and V H⁺-ATPase labeling occurred in different cells (B). Scale bar 20 μm .

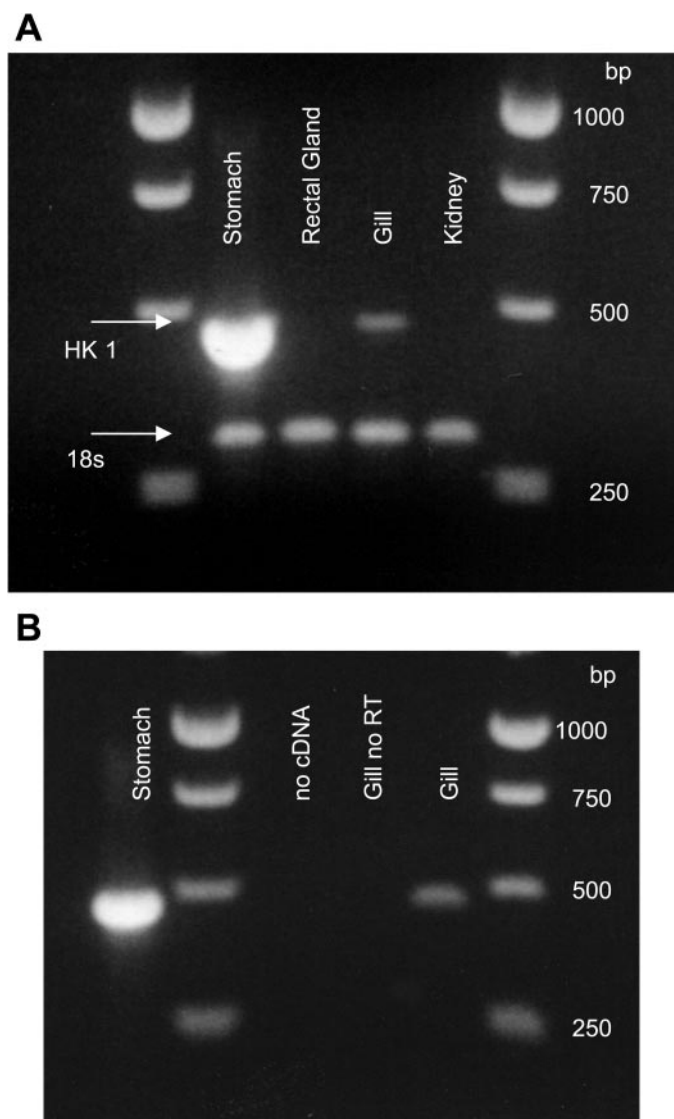


Fig. 5. Representative results from multiple tissue relative quantitative PCR (A) and verification of gill expression (B). Multiplex PCR with primer pairs for a 471-bp fragment of the putative stingray HK α 1 (F196/R646) and a 315-bp fragment of the 18S ribosome was conducted with randomly primed stingray cDNA as a template. Although 18S was amplified approximately equally from cDNA of all tissues, the putative HK α 1 fragment was only amplified from stomach and gill (A). Expression of the putative HK α 1 in gills was further verified by conducting PCR with 2 negative controls: 1) no template and 2) RNA that was not reverse transcribed as a template (B). Size of molecular weight markers are noted to the right of DNA ladders.

secretion in mammalian stomachs is known to cause HK α 1 containing vesicles to fuse with a canaliculus that is continuous with the apical membrane of parietal cells (38). This HK α 1 cycling has been proposed for collecting duct intercalated cells (25) and might also exist in stingray branchial cells. Unfortunately, we have yet to observe cells with unambiguous apical staining, even in stingrays made acidotic by hypercapnia or NH $_4$ Cl injections or in stingrays acclimated to freshwater (unpublished results). A complex apical membrane structure and/or limitations of immunocytochemistry and light microscopy to resolve specific localization within cells might explain the diffuse staining pattern.

Molecular identification of a putative stingray HK α 1. Although our immunoreactivity results suggest the expression of an HK α 1-like protein in stingray stomachs and gills, we could not make any definitive conclusions about the identity of these putative HK α 1-like proteins based on these results alone. Before this study, the most ancestral organism for which HK α 1 sequence data existed was a teleost (*Pleuronectes americanus*) (18), and it was not known if an orthologue of HK α 1 existed in elasmobranchs. In addition, although the HK α 1 antibodies did not appear to react with NaK α on Western blots or in rectal glands of stingrays, the possibility of crossreactivity with other type IIc P-ATPases could not be eliminated because all the type IIc P-ATPases share homology. For example, the hog antigen for antibody C2 shares 9/16 and 8/16 homologous amino acids with HK α 2 and NaK α of rat, respectively, leaving the possibility of cross-reactions to these other isoforms. Therefore, we sought to obtain more definitive results supporting the existence of an HK α 1 in stingrays and its expression in gills with molecular techniques.

The open reading frame of the putative HK α 1 from stomach encodes for a 1,025-amino acid protein (114.6 kDa, sum of amino acids) that appears to be a type IIc P-ATPase (Fig. 7). For example, an alignment with rat type IIc P-ATPases from GenBank shows that the predicted stingray protein has many regions conserved with P-ATPases, including the invariant phosphorylation site that is characteristic of all P-ATPases and the predicted transmembrane regions of type IIc P-ATPases (33) (Fig. 7). Further comparisons of the stingray protein to the type IIc P-ATPases of rat suggest that it is an HK α 1 isoform. For example, the stingray sequence is 81.4, 65.6, 62.7, and 62.8% identical to rat HK α 1, HK α 2, NaK α 1, and NaK α 2 proteins, respectively. In addition, the unrooted distance tree based on this alignment confirms that it is closest to HK α 1, and is of roughly equal distance from HK α 2, NaK α 1, and NaK α 2

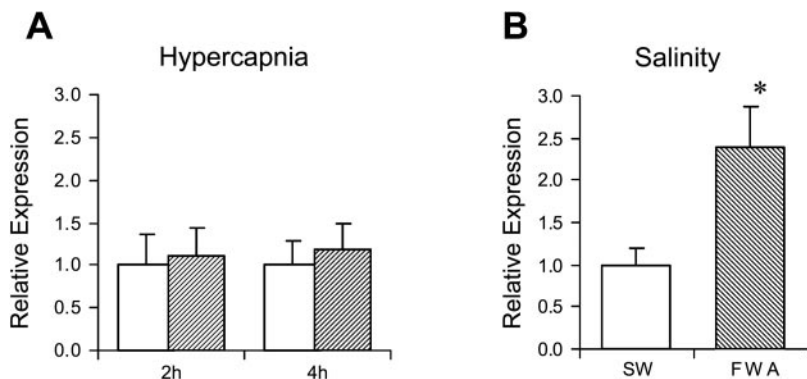


Fig. 6. Expression of putative stingray HK α 1 in gills during hypercapnia (A) and freshwater acclimation (B). White bars are means for control animals (normocapnia in A and salt water in B) and hatched bars are means for treated animals [hypercapnia in A and freshwater acclimated (FWA) in B]. Means + SE; $n = 5$ for A and $n = 4$ for B. * $P < 0.05$.

(Fig. 8). Importantly, the stingray sequence shares 14/16 homologous amino acids in the antigen site for antibody C2, supporting our conclusion that it does recognize the putative stingray HK α 1. Lastly, the stingray protein has a glutamic acid

in transmembrane domain six (Fig. 7), which has been shown to bind to the gastric proton pump inhibitor, SCH28080 [2-methyl-8-(phenylmethoxy)-imidazo-(1,2a)-pyridine-3-acetonitrile], a characteristic of only HK α 1 (33).

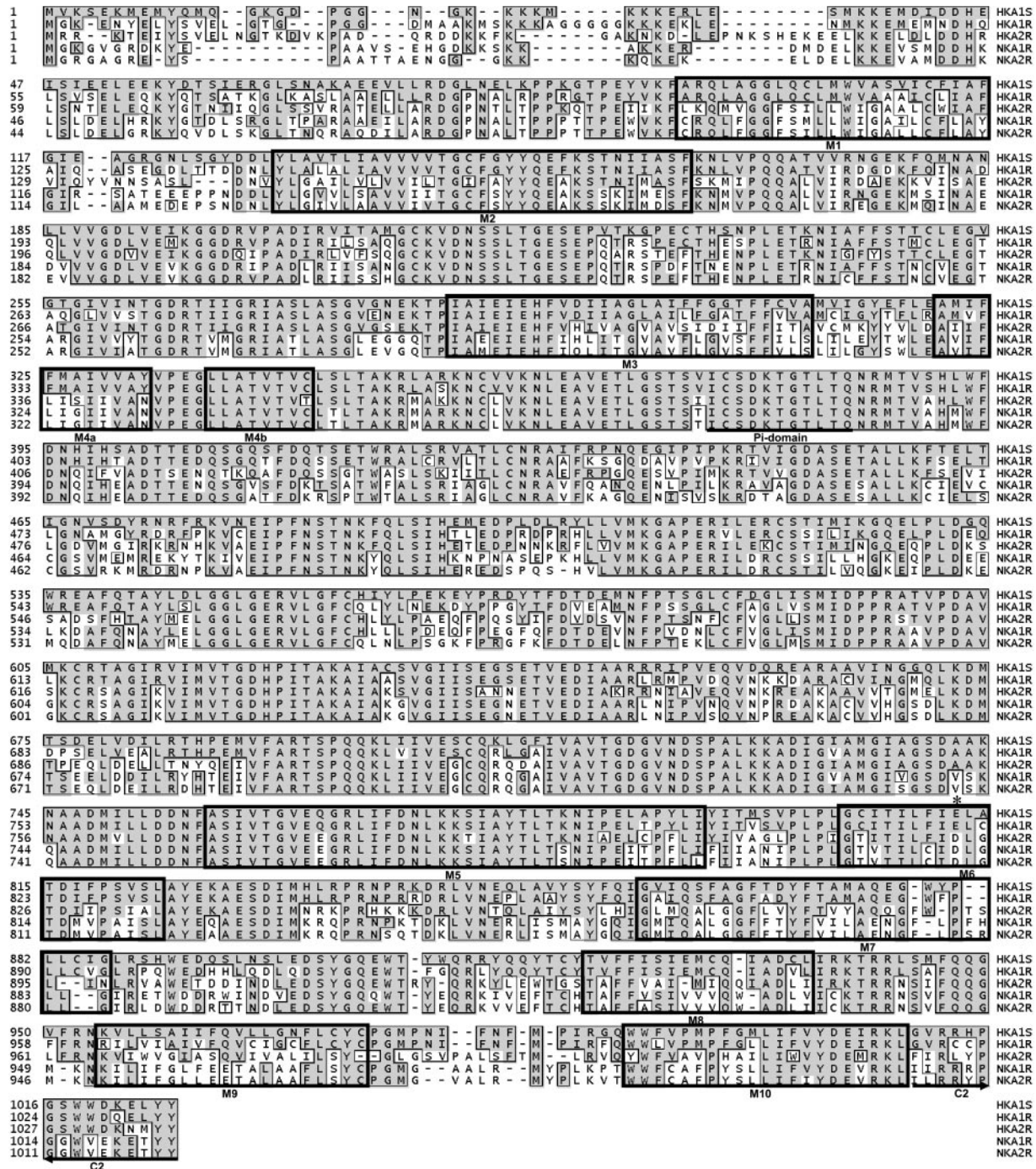


Fig. 7. Amino acid alignment of the putative stingray HK α 1 with the type IIc P-type ATPase α -subunits of rat. Gaps (dashes) were introduced to maintain alignment. Amino acids that are similar (within 4 PAM100 distance units) to those of the putative stingray HK α 1 are enclosed within light boxes and those that are identical are shaded. Putative transmembrane domains are enclosed in heavy boxes, the highly conserved phosphorylation site is underlined (Pi-domain, 374–384), and the location of a glutamic acid that binds SCH28080 in HK α 1 is marked by an asterisk (812). The region homologous to the antigen of antibody C2 is underlined (1010–1025). HKA, H⁺-K⁺-ATPase α -subunit; NKA, Na⁺-K⁺-ATPase α -subunit; S, Atlantic stingray (*Dasyatis sabina*); R, Norway rat (*Rattus norvegicus*). GenBank accession numbers are: HKA1S (AY283439), HKA1R (P09626), HKA2R (NP598201), NKA1R (NP036636), and NKA2R (NP036637).

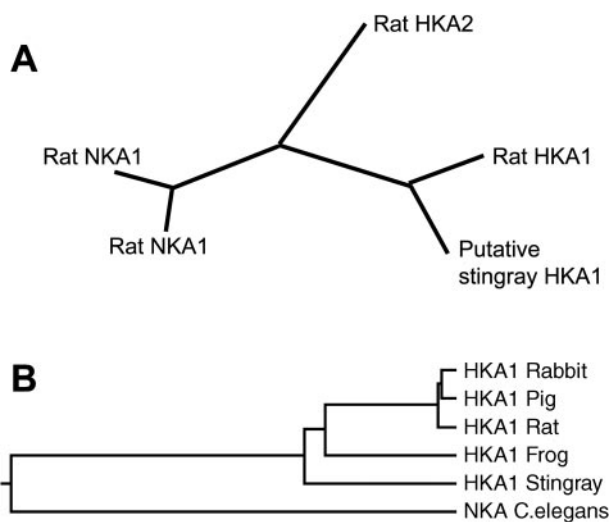


Fig. 8. Unrooted distance tree of the protein sequences from Fig. 7 produced with Prodist, Fitch, and Neighbor-joining software of Phylip (A). Branch lengths are proportional to distances between the sequences. Stingray sequence is closest to HK α 1 and of roughly equal distance from the other type IIc P-ATPases. Rooted phylogenetic tree based on a ClustalV alignment of HK α 1 sequences (B). Stingray sequence is closest to frog and is the earliest HK α 1. *C. elegans*, *Cestrum elegans*; stingrays, *Dasyatis sabina*; frog, *Xenopus laevis*; rat, *Rattus norvegicus*; pig, *Sus scrofa*; rabbit, *Oryctolagus cuniculus*.

A phylogenetic analysis of our sequence with available full-length HK α 1 sequences, demonstrates that it is most similar to frog HK α 1 and is the earliest known H⁺-K⁺-ATPase (Fig. 8B). A recent analysis by Okamura et al. (33) showed that H⁺-K⁺-ATPases only evolved in the vertebrate lineage, with no equivalent in invertebrate genomes. They are thought to have originated by gene duplication of an ancestral Na⁺-K⁺-ATPase and then to have later split into HK α 1 and 2. Our putative stingray HK α 1 dates the divergence of H⁺-K⁺-ATPases from Na⁺-K⁺-ATPases to before the evolution of cartilaginous fish in the Devonian era ~400 million years ago (6). In fact, the earliest phylogenetic appearance of acid secretion by the stomach is in elasmobranchs (23), suggesting that they may be the oldest living organisms to have an HK α 1 orthologue. However, although the jawless, more ancestral fishes (hagfishes and lampreys) lack a stomach, future studies should be conducted to determine if they express H⁺-K⁺-ATPases in their gills.

Distribution of putative HK α 1 expression. The high expression of the putative stingray HK α 1 in the stomach was expected because of this organ's unique requirement of a highly acidic luminal pH. Alternatively, the inability to detect expression in kidneys was not expected, because HK α 1 is expressed in the kidneys of mammals (41) and two previous studies provided immunological and pharmacological evidence for an H⁺-K⁺-ATPase in the kidneys of two sharks, *Scyliorhinus caniculus* and *Squalus acanthias* (20, 45). However, marine elasmobranch renal tubules have four loops and at least 16 morphologically distinguishable segments (27). Therefore, HK α 1 expression could be confined to specific segments, so that the amount of transcript is below detectable limits in whole tissue RNA preparations. It is also possible that the kidney expresses a different isoform of H⁺-K⁺-ATPase that is not detectable with the nondegenerate primers we used to evaluate tissue distribution. Our demonstration of expression in the gills

supports the immunological evidence of HK α 1 expression in this acid-base regulatory organ. Unfortunately, because of a low expression level in the gills, we have been unable to sequence the ends of the transcript from the gills. Therefore we are unable to determine if the stomach and gills express transcript variants, as suggested by the slight difference in Western blot band size with antibody C2.

Before this study, there was some pharmacological evidence for a proton pump of the P-ATPase family in fish gills. For example, Lin and Randall (31) were able to inhibit more than 50% of in vivo net-acid excretion from rainbow trout gills (*Oncorhynchus mykiss*) with vanadate, an inhibitor of P-ATPases. They later showed that 60% of the ATPase activity in gill homogenates from the same species was also inhibited by vanadate. Because azide, ouabain, and Ca²⁺-chelators were present to inhibit mitochondrial H⁺-ATPases, Na α , and Ca²⁺-ATPases, they concluded that the activity was due to a proton pump of the P-ATPase family (30). Using a similar technique, Kormanik et al. (24) concluded that an elasmobranch (little skate, *Raja erinacea*) also had a proton pump of the P-ATPase family in its gills. However, after the first pharmacological studies on rainbow trout, V H⁺-ATPase was identified in the gills of several fish species (29, 35, 46, 47) and no other studies were conducted to identify the potential P-ATPase proton pumps in gills. Therefore, our study provides the first direct evidence for expression of an H⁺-K⁺-ATPase in any fish gill, which may be the P-ATPase that was detected pharmacologically by other studies.

Expression during hypercapnia and salinity acclimation. In previously published experiments, we demonstrated that net-acid excretion rates from seawater stingrays increase to their highest value (5-fold control excretion rates) during the first 4 h of hypercapnia and that blood pH decreases to its lowest value after 2 h of hypercapnia (2). Net acid excretion rates then return to control levels by 8 h and blood pH recovers to near control levels by 8 h. Therefore, the lack of an increase in expression of HK α 1 message while acid transport and blood acidosis is greatest, suggests that increased expression of mRNA for this putative transporter does not have a large quantitative role in compensation for respiratory acidosis. However, our negative results do not exclude the possibility of acute regulation of preexisting HK α 1 protein, as is known to occur in gastric parietal cells (38).

Alternatively, the greater expression of HK α 1 message in freshwater-acclimated stingray gills relative to seawater stingray gills suggests that increased expression of this putative transporter is important for acclimation to a hypotonic environment. The likely function of this increased HK α 1 expression in the gills is active K⁺ absorption from environmental water. Few studies have investigated systemic K⁺ balance in fishes, but it is clear that, in the absence of feeding, freshwater fish require an active mechanism of K⁺ absorption to balance diffusive and renal losses (8, 16, 17). For example, seawater stingray plasma has a [K⁺] of ~7.0 mmol/l, which is slightly lower than ambient seawater (~10 mmol/l) (36). Although freshwater stingray plasma has a slightly lower [K⁺] (~5.2 mmol/l) it is regulated much higher than ambient freshwater (<0.1 mmol/l) (36). The concentration difference between fish plasma and freshwater is far from balanced by a slightly negative inside electrical potential and is even farther from balance between intracellular fluid and freshwater (17). There-

fore, the increase in HK α 1 expression during freshwater acclimation may be a mechanism of increasing active K⁺ absorption.

In summary, we have sequenced the first putative H⁺-K⁺-ATPase from an elasmobranch. This is the first full-length H⁺-K⁺-ATPase transcript in any fish and dates the origin of H⁺-K⁺-ATPases to at least before the division of bony and cartilaginous fishes. The stingray H⁺-K⁺-ATPase shares the most homology with HK α 1 and, therefore, is probably an orthologue of the SCH-28080-sensitive H⁺-K⁺-ATPase found in mammalian parietal cells and renal intercalated cells. We showed that this mRNA transcript is expressed in the stomach and gills of the Atlantic stingray and demonstrated that an HK α 1-like protein is present not only in gastric glands, but also in branchial NaK α -rich cells. We also demonstrated that mRNA expression for this putative HK α 1 increased during acclimation to freshwater, suggesting a role in active K⁺ absorption.

ACKNOWLEDGMENTS

We thank Dr. P. Piermarini, Dr. J. B. Claiborne, J. Catches, C. Fernandez, S. O'Brien, Dr. L. Guillette, and Dr. C. St. Mary for input, assistance, and use of equipment. We especially thank Dr. A. Smolka for donating antibody C2.

GRANTS

This study was funded by National Science Foundation grant IBN-0089943 to D. H. Evans, grants-in-aid of research from Sigma Xi and the Society for Integrative and Comparative Biology to K. P. Choe, The Department of Veteran's Affairs Merit Review Program to C. S. Wingo, and National Institutes of Health Grant DK-49750 to C. S. Wingo.

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