Roles of cytosolic and membrane-bound carbonic anhydrase in renal control of acid-base balance in rainbow trout, *Oncorhynchus mykiss*

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Submitted 15 August 2005; accepted in final form 20 March 2006

Georgalis, T., K. M. Gilmour, J. Yorston, and S. F. Perry. Roles of cytosolic and membrane-bound carbonic anhydrase in renal control of acid-base balance in rainbow trout, *Oncorhynchus mykiss*. Am J Physiol Renal Physiol 291: F407–F421, 2006. First published March 28, 2006; doi:10.1152/ajprenal.00328.2005.—We tested the hypothesis that cytosolic and membrane-associated carbonic anhydrase (CA IV) are involved in renal urinary acidification and bicarbonate reabsorption in rainbow trout. With the use of homologous cloning techniques, a 1,137-bp cDNA was assembled that included an open reading frame encoding for a deduced protein of 297 amino acids. Phylogenetic analysis revealed that this protein was likely a CA IV isoform. With the use of this sequence and a previously described trout cytosolic isoform tCAc (13), tools were developed to quantify and localize mRNA and protein for the two CA isoforms. Unlike tCAc, which displayed a broad tissue distribution, trout CA IV mRNA (and to a lesser extent protein) was highly and preferentially expressed in the posterior kidney. The results of in situ hybridization, immuno-cytochemistry, and standard histological procedures demonstrated that CA IV was likely confined to epithelial cells of the proximal tubule. Thus, although net urinary acid excretion may be a relatively minor route of whole body acid excretion, there must be a pronounced increase in renal acid excretion to prevent accumulated HCO₃⁻ being lost via the urine.

With few exceptions (33, 62), studies that have partitioned whole body acid excretion into branchial and renal components have demonstrated that during acid-base disturbances, the urine is only a minor route of net acid efflux (4, 5, 8, 11, 28, 61, 63, 64). Thus adjustments of plasma HCO₃⁻ levels, and hence pH regulation, are largely dictated by variations in acid output at the gill (3). However, as pointed out previously (39, 56, 63), persistent changes in plasma HCO₃⁻ levels would not be possible without parallel adjustments of renal acid secretion. In particular, the capacity to retain high levels of HCO₃⁻ in the plasma during metabolic compensation of respiratory acidosis could not be achieved without a marked increase in renal acid secretion to facilitate reabsorption of additional filtered HCO₃⁻. Thus, although net urinary acid excretion may be a relatively minor route of whole body acid excretion, there must be a pronounced increase in renal acid secretion to prevent accumulated HCO₃⁻ being lost via the urine.

In mammals, ~80–90% of renal HCO₃⁻ reabsorption occurs within the proximal tubules. According to current models (42, 48), filtered HCO₃⁻ combines with H⁺, derived from an apical membrane sodium proton exchanger isoform 3 (NHE3) (65), to form CO₂, a reaction that is catalyzed by membrane-bound carbonic anhydrase isoform IV (CA IV) (35, 46). The newly formed CO₂ enters the proximal tubule cell where it is hydrated to H⁺ and HCO₃⁻ in the presence of CA isoform II (CA II) (59). Finally, the HCO₃⁻ is moved across the basolateral membrane by a Na⁺-HCO₃⁻ cotransporter (NBC1) (43, 44). Thus, in mammals, HCO₃⁻ reabsorption is reliant on both cytosolic and membrane-bound CA isoforms (45). Although a similar model has been proposed for freshwater fishes [see Fig. 7 in Perry et al. (40)], there is but scant supporting empirical evidence. Indeed, we are aware of only a single study that has experimentally implicated CA in renal HCO₃⁻ reabsorption (34). To our knowledge, no studies have yet attempted to assess the relative involvement of cytosolic and membrane-bound CAs in internal acidosis, branchial net acid excretion is increased (e.g., Ref. 28), whereas during alkalosis, net acid efflux is reduced (e.g., Ref. 4). Such changes in acid excretion promote appropriate adjustments of plasma HCO₃⁻ levels to ultimately regulate blood pH. The branchial mechanisms underlying the changes in net acid excretion during acid-base disturbances are not fully understood and are likely to differ among fish species. However, it is generally accepted that in freshwater fish, net acid movement across the gill is controlled by the relative rates of Cl⁻ and Na⁺ uptake that are linked to HCO₃⁻ and H⁺ effluxes, respectively (7, 32, 37).

IN WATER-BREATHING fish, acid-base regulation is achieved principally by metabolic compensation whereby plasma HCO₃⁻ levels are adjusted at more-or-less constant values of arterial PCO₂ PaCO₂ (6, 25, 26). This is in marked contrast to air-breathers that, in addition to metabolic compensation, also exploit respiratory adjustments to rapidly alter blood PCO₂ levels and hence blood pH. In fish, metabolic compensation reflects an intricate relationship between ionic and acid-base regulation owing to the presence of specific ion transport proteins at the gill and kidney that link ion fluxes to the transepithelial movements of acidic and basic equivalents (7, 37, 40). It is well established that the gill is an important site of metabolic compensation owing to its capacity to dynamically regulate net acid excretion (16, 24). During periods of
renal HCO₃⁻ reabsorption in fish. Thus the goal of the present study was to test the hypothesis that both cytosolic and membrane-bound CA (CA IV) are involved in urinary acidification and HCO₃⁻ reabsorption in rainbow trout (*Oncorhynchus mykiss*). This was accomplished by cloning and molecular characterization of a putative trout renal CA IV and by comparing the effects of its selective inhibition in vivo using a membrane-impermeant CA inhibitor vs. the effects of total CA inhibition.

**Materials and Methods**

**Experimental animals.** Adult rainbow trout (*Oncorhynchus mykiss; ~250 g; n = 80) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish were maintained on a 12:12-h light-dark photoperiod in large fiberglass aquariums supplied with flowing, aerated, and dechlorinatedated City of Ottawa tapwater at 13°C. They were fed daily with a commercial trout diet. Animals were allowed to acclimate to the holding conditions for at least 2 wk before any experiments were performed. All procedures involving animals were in accordance with guidelines from the Canadian Council on Animal Care and were approved by the University of Ottawa Animal Care Committee.

**Molecular cloning of CA IV.** Fish were killed by a blow to the head and kidney tissue was harvested and immediately frozen in liquid nitrogen before storage at ~80°C. Total RNA was extracted from kidney using TRIzol (Invitrogen) according to the instructions of the manufacturer. RNA concentrations were verified using spectrophotometry (Eppendorf BioPhotometer; VWR International). cDNA was synthesized from 5 μg of total RNA using Stratascript reverse transcriptase (Stratagene) and random hexamer primers. Initially, a 290-bp cDNA fragment was amplified by PCR using degenerate primers, forward 5'-CARWSICNATHAAYAHTG-3' and reverse 5'-RTTIACDATRTGIARYTCCAT-3', designed from regions of vertebrate CA IV’s exhibiting a high degree of amino acid conservation. PCR was performed using 1 μl of cDNA template in 25-μl reaction mixtures containing 2.5 mM MgCl₂, 200 μM each of dNTPs, 200 nM of each primer, and 1 U of Taq polymerase (Life Technologies) in PCR buffer supplied with the enzyme. The template for the reactions was 1 μl of kidney cDNA. PCR conditions consisted of an initial denaturation at 94°C for 30 s followed by 40 cycles of: 94°C for 30 s; annealing temperature for 60 s: 72°C for 90 s, and ending with a final extension at 10 min at 72°C. The PCR product was gel-purified and cloned into pCR-II TOPO vector (TOPOII TA cloning kit, Invitrogen) and sequenced. A search of GenBank protein databases using BLASTX revealed that the cloned 290-bp cDNA exhibited highest amino acid identity with known CA IV sequences. Based on this sequence, primers were designed to be used for 3’ and 5’ Rapid Amplification of cDNA Ends (RACE). For 3’ RACE, total kidney RNA was isolated using TRIzol reagent (Invitrogen) and reverse-transcribed to cDNA using a 3’ RACE adapter primer (GIBCO) and Superscript II reverse transcriptase (GIBCO). Seminested PCR was performed on the cDNA using abridged universal amplification primer (Invitrogen) for the second round. PCR products were cloned into pCR2.1 vectors using TOPO TA cloning kits (Invitrogen). All RACE product sequences were confirmed by overlap with the initial 290-bp cDNA. After repeated bidirectional sequencing of both RACE products, a consensus sequence was created by multiple sequence alignment (DNAMAN; Lynnon Biosoft). Owing to possible sequence ambiguities, a small portion of the 3’-end was recloned and its sequence confirmed using primers 5’-CTGTAAGTTTGGACAGGGTTC-3’ (forward) and 5’-AGTAAGAGTGGCGAAAGTGC-3’ (reverse).

**Phylogenetic analysis.** The rainbow trout CA IV amino acid sequence was aligned with GenBank sequences of CA IV from selected vertebrates using the default setting in CLUSTAL W version 1.8 (51). The accession numbers for sequences used in the phylogenetic analysis are presented in Fig. 2. Maximum likelihood phylogenetic analysis was performed using PUZZLE version 4.0.2 (49). The following program settings were used: quartet puzzling tree search, compute exact quartet likelihood, 1,000 puzzling steps, use of the human CA VII sequence as the out-group, branch lengths are not clocklike, JTT model of substitution, amino acid frequencies were estimated from the data and the model of rate heterogeneity was I + 8 gamma rates. The selection of the human CA VII sequence as the out-group was based on a previous publication (13).

**RNA analyses and Northern blots.** Total RNA was isolated using TRIzol Reagent (Invitrogen). Poly A+ RNA was then made from total RNA using a PolyAtract mRNA Isolation System III kit (Promega). Poly A+ RNA samples (1 μg) were incubated in loading buffer at 65°C and electrophoresed through 1.5% (wt/vol) agarose gel in MOPS buffer containing 0.65 mol/l formaldehyde. RNA was transferred to GeneScreen+ membranes (New England Nuclear Life Sciences) by capillary action for 24 h. Ambion millennium RNA markers were used to estimate transcript size. Membranes were prehybridized at 65°C for 4 h in a buffer containing 6× SSC (0.9 mol/l NaCl, 0.09 mol/l sodium citrate, pH 7.0), 5× Denhardt’s (1× Denhardt’s is 0.1% Ficoll 400,000, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 μg/ml single-strand herring sperm DNA, 1% SDS, and 10% dextran sulfate (Amersham Pharmacia Biotech). The probe was prepared by PCR amplification of the 290-bp insert followed by incorporation of 32P-labeled dCTP (using DNA polymerase fragment I; GIBCO), purification (SigmaSpin Post Reaction Purification Column; Sigma), and thermal denaturation. The probe (specific activity 10⁸ cpm/μg DNA) was then added to the hybridization buffer and the hybridization proceeded for 20 h at 65°C in the same solution. After hybridization, the membrane was washed several times at 65°C with 0.1× SSC, 0.1% SDS, and exposed to Bio Max Film plus intensifying screen (Kodak) at ~80°C for up to 2 days. To confirm equal loading between samples, the membrane was reprobed with a homologous β-actin probe (514-bp PCR product) under the same conditions but with an exposure time of few hours (data not shown).

**Real-time PCR.** Tissues were homogenized to powder under liquid N₂ using a mortar and pestle. Total RNA was extracted from 30-mg aliquots of powdered tissue samples using an Absolutely RNA RT-PCR Miniprep Kit (Stratagene). To remove any remaining genomic DNA, the RNA was treated on-column using RNase-free DNase (5 μl; Invitrogen) for 15 min at 37°C. The RNA was eluted in 70 μl of nuclease-free H₂O, and its quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf Biophotometer). cDNA was synthesized from 2 μg of RNA using random hexamer primers and Stratascript reverse transcriptase (Stratagene).

The relative RNA levels of trout CA IV (tCA IV) and trout cytosolic CA (tCcAC 13) were assessed by real-time PCR on samples of cDNA using a Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) and a Stratagene MX-4000 multiplex quantitative PCR system. ROX (Stratagene) was used as reference dye. The PCR conditions (final reaction volume = 25 μl) were as follows: cDNA template = 0.5 μl; forward and reverse primers = 300 nmol/l; 2× Master Mix = 12 μl; ROX = 1:30,000 final dilution. The annealing and extension temperatures over 40 cycles were 58°C (30 s) and 72°C.
were then quickly chilled on ice and centrifuged for 1 min at 7,500 g, respectively. The following primer pairs were designed using Primer3 software: β-actin forward 5'-CCAACAGGATCTGATCAGCA-3', β-actin reverse 5'-GGTGGGAGAGCCATTGAAGTG TA-3', tCA IV forward 5'-ATACGATCTGCACTGATAAATGCT-3', tCA IV reverse 5'-CGGAAATGGTCTGATCATATTCTG-3', tCac forward 5'-CAGTCTCCATT GACATCGTA-3' and tCac reverse 5'-CGTTGTGCTG GG TGTAGGT-3'.

The specificity of the primers was verified by the cloning (TOPO TA cloning kit; Invitrogen) and sequencing of amplified products. To ensure that SYBR green was not being incorporated into primer dimers or nonspecific amplicons during the real-time PCR runs, the PCR products were analyzed in initial experiments by gel electrophoresis. Single bands of the expected size were obtained in all cases. The construction of SYBR green dissociation curves after completion of 40 PCR cycles revealed the presence of single amplicons for each primer pair. To ensure that residual genomic DNA was not being amplified, control experiments were performed in which reverse transcriptase was omitted during cDNA synthesis. Relative expression of mRNA levels was determined (using actin as an endogenous standard) by using the delta-delta Ct method (41). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

In situ hybridization. Antisense riboprobes for tCA IV and tCac were generated from kidney cDNA using primers designed to yield PCR products of ~600 bp. For CA IV, primers were forward 5'-CCATCGTTGGAAGATCTCAAGG-3' and reverse 5'-ACAGCGGAGTTGGTGAAGAG-3'. For tCac, primers were forward 5'-ATGCTCTGATCAGGGATAC-3' and reverse 5'-CACCTCTCCAGCA-3'. PCR products were cloned into pCR 2.0 vector using TOPO TA cloning kit (Invitrogen). Plasmids were sequenced to confirm product and their orientation. Two micrograms of plasmid DNA were digested using XhoI (Invitrogen) under conditions recommended by the manufacturer. Products were phenol/chloroform purified and resuspended in 10 μl of diethylpyrocarbonate-treated H2O. Digest concentrations were verified using an Eppendorf BioPhotometer spectrophotometer. The probe labeling assay was carried out using SP6 RNA polymerase (New England Biolabs) along with DIG RNA labeling mix (Roche) as described by Roche.

Small pieces (~200 mg) of posterior kidney (final 1.5 cm of trunk kidney) tissue were fixed in 4% paraformaldehyde (pH 7.4) overnight at 4°C. Tissues were then rinsed several times in PBS followed by immersion in 15% sucrose for 2 h and 30% sucrose, where they were left at 4°C until sectioning. Cryoprotected tissue pieces were frozen in immersion in 15% sucrose for 2 h and 30% sucrose, where they were left at 4°C until sectioning. Cryoprotected tissue pieces were frozen in immersion in 15% sucrose for 2 h and 30% sucrose, where they were left at 4°C until sectioning. Tissue was then homogenized by passing through a needle and syringe a number of times. Samples were stored on ice for 15 min and centrifuged at 10,000 rpm for 10 min at 4°C; the supernatant containing soluble proteins was frozen and stored at −80°C until subsequent use.

Protein concentration was determined using a BioRad protein assay kit (Bio-Rad Laboratories) using BSA as a standard. Proteins (120 μg per lane) were separated by SDS-PAGE on 10.5% tris-tricine polyacrylamide gels and then transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories) using a wet transfer unit. The blots were blocked in 5% PBST-milk for 1 h at room temperature. Following blocking, the first blot was then probed with an anti-tCA IV antibody (1:1,000) for 1 h at 37°C. To demonstrate specificity of the tCA IV antibody, a second blot was incubated simultaneously with the tCA IV antibody in the presence of an excess (20 μg) of the peptide against which the antibody was raised.

Tissue distribution and antibody specificity. Proteins from tissues were extracted using RIPA buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) with protease inhibitors [complete Mini protease inhibitor cocktail tablets (Roche) and 2 μg/ml pepstatin A (Sigma)]. The tissues were then first incubated with 5× more unlabeled probe in hybridization buffer for 3 h at 58°C. This solution was then removed from sections and replaced with hybridization buffer containing 5× unlabeled probe and ~900 pg of probe and incubated overnight at 58°C.

Protein analyses and Western blots. Custom rabbit polyclonal antibodies (Abgent, San Diego, CA) raised against trout CA IV and tCac (13) were generated using synthetic peptide antigens conjugated to keyhole limpet protein. For CA IV, the synthetic peptide TRRTPDLRITPPFTTGGY corresponded to amino acids 57–74 of the rainbow trout protein sequence (GenBank accession AAR99330). For tCac, the synthetic peptide WNTKYPSFGDAASKSDGLA corresponded to amino acids 122–141 of the rainbow trout protein sequence (GenBank accession AAR99329). Both antisera were purified by protein G affinity chromatography; the CA IV antiserum was purified further by peptide affinity purification (Abgent).

Dilution of plasmid DNA.
Effects of hypercapnia on protein expression. Proteins were prepared from frozen tissues (0.5 g/ml homogenization buffer) by homogenization on ice in 10 ml Tris-SO4 buffer (25 mmol Tris-SO4, 0.9% NaCl, pH 7.4) containing protease inhibitors (complete Mini protease inhibitor cocktail tablets, Roche) and 2 μg/ml pepstatin A (Sigma). Samples were stored on ice for 15 min and centrifuged at 10,000 rpm for 10 min at 4°C; the supernatant containing soluble proteins was frozen and stored at −80°C until subsequent analysis.

Protein concentrations were determined using a microcinchonic acid protein assay (Pierce) using BSA as standard. Samples (100 μg protein) were size fractionated by reducing SDS-PAGE using 10–14% separating and 4% stacking polyacrylamide gels. Fractionated proteins were transferred to nitrocellulose membranes (BioRad, Hercules, CA) by using a transblot electrophoretic transfer cell (Bio-Rad) according to instructions of the manufacturer. After transfer, each membrane was blocked for 1 h in Tris-buffered Tween 20 (TBS-T)-5% milk and probed with a dilution of 1:100 rabbit anti-trout CA IV or CAC for 1.5 h at 37°C. The membranes were then probed for an additional 1 h at room temperature with 1:2,000 goat anti-rabbit antibody (Pierce). After each exposure to antibody, the membranes were washed 3 × 5 min in TBST. The antigenic bands were visualized by enhanced chemiluminescence (ECL; Pierce; SuperSignal West Pico Chemiluminescent Substrate) using a digital gel documentation system (Bio-Rad ChemDoc). The digital images were processed using commercial software (Quantity One v4.1.1). The protein size marker used was obtained from Fermentas Life Sciences.

Immunocytochemistry. Tissue sections were prepared as described for in situ hybridization. A hydrophobic barrier was created around each section with a PAP pen (Electron Microscopy Sciences, Fort Washington, PA). Sections were incubated in situ (3 × 5 min) with a blocking buffer containing 2% normal goat serum, 0.1 mol/l PB, 0.9% Triton X-100, 1% gelatin, and 2% BSA. They were then incubated for 2 h at room temperature, in a humidified chamber, with one of four primary antibodies diluted in the blocking buffer: α5, a mouse monoclonal antibody against the α5 subunit of chicken 20 (TBS-T)-5% milk and probed with a dilution of 1:100 rabbit anti-trout CA IV or CAC for 1.5 h at 37°C. The membranes were then probed for an additional 1 h at room temperature with 1:2,000 goat anti-rabbit antibody (Pierce). After each exposure to antibody, the membranes were washed 3 × 5 min in TBST.

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Figs. 1A and 1B: deduced amino acid sequence of Tr (Onchorhynchus mykiss) CA IV (CA IV) showing the position (circled) of residues known to be conserved in the active site of other vertebrate CAs; presumptive zinc binding histidines are denoted with *.

Exposure of fish to hypercapnia. Fish were placed into opaque acrylic boxes supplied with flowing and aerated water. They were allowed to recover for 24 h and were then exposed to external hypercapnia [target final water PCO2 (PwCO2) was ~6.0 mmHg] for 24 h. To reach a final PwCO2 of ~6.0 mmHg, a water equilibrium column was gassed with mixtures of CO2 and air (Sierra CI100L Smart-trak mass flow controllers; SRB Controls). PwCO2 was monitored using a CO2 electrode that was linked to a CO2 meter (Cameron Instruments). Deviations from the target PwCO2 were corrected by adjusting the gas and/or the water flows through the equilibration column.

For all experiments except those involving determination of mRNA using real-time PCR, fish were euthanized by a blow to the head and tissues were sampled and processed after 24 h of exposure of fish to hypercapnia (n = 6) or normocapnia (controls; n = 6). To assess the temporal changes in CA mRNA levels using real-time PCR, tissues were collected at 1, 2, 3, 6, 12, and 24 h (n = 6 at each time point) of exposure to hypercapnia, or at 3 h (representing control points for 1–3 h hypercapnic fish), 6, 12, and 24 h (n = 6 at each time point) of exposure to normocapnia.

Effects of CA inhibition on urinary acidification and HCO3 reabsorption. Fish were anesthetized by immersing them in an oxygenated solution of benzocaine (ethyl-p-aminobenzoate; 0.1 g/l) and then placed on a surgical table that allowed continuous irrigation of the gills with the same anesthetic solution. All trout were fitted with dorsal aortic cannulas (Clay-Adams PE50 polyethylene tubing) according to the basic method of Soivio et al. (47). Additionally, the intestine was ligated and an external urinary catheter was attached (10). After revival, fish were placed into opaque acrylic boxes supplied with flowing and aerated dechlorinated tapwater, where they were allowed to recover for 24 h. Cannulas were flushed daily with heparinized [100 IU/ml ammonium heparin (Sigma)] Cortland saline (60).

Fig. 1A: deduced amino acid sequence of rainbow trout (Onchorhynchus mykiss) CA IV (CA IV) showing the position (circled) of residues known to be conserved in the active site of other vertebrate CAs; presumptive zinc binding histidines are denoted with *.

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\begin{align*}
\text{Trout:} & \quad YSN\text{NRVSVGDIHHEHEHEHVNYLPITTPDFPWV} \\
\text{Zebrafish:} & \quad YSN\text{HTHTVCHYEHEHEHVNYLPITTPDFPWV} \\
\text{Pufferfish:} & \quad YSN\text{HHSVCHYEHEHEHVNYLPITTPDFPWV} \\
\text{Dogfish:} & \quad YSN\text{HHSCHYEHEHEHVNYLPITTPDFPWV} \\
\text{Xenopus:} & \quad YSN\text{HHSCHYEHEHEHVNYLPITTPDFPWV} \\
\text{Chicken:} & \quad YSN\text{HHTTVCHYEHEHEHVNYLPITTPDFPWV} \\
\text{Rat:} & \quad YSN\text{NEEYHVEHEHEHVNYLPITTPDFPWV} \\
\text{Rabbit:} & \quad YSN\text{NEEYHVEHEHEHVNYLPITTPDFPWV} \\
\text{Human:} & \quad YSN\text{NEEYHVEHEHEHVNYLPITTPDFPWV}
\end{align*}
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Experimental protocol. After the 24-h postsurgery recovery period, urine was collected continuously during a 27-h period of experimentation. This was accomplished by allowing the urinary catheters to drain, by gravity, into vials located outside the holding boxes 5 cm below water level. To check for leaks, catheters were raised 5 cm above water level; any fall of the urine level in the catheter indicated a leak and the results from these fish were not used. Fish were exposed to either hypercapnia ($P_WCO_2 = 6.0 \text{ mmHg}$) or normocapnia (air-saturated water; $P_WCO_2 = 0 \text{ mmHg}$) for 21 h. Urine was collected for two consecutive 3-h periods (21–24 and 24–27 h) representing a control period and a period immediately following an arterial injection of a permeant (acetazolamide; 30 mg/kg) or membrane-impermeant (F3500; 50 mg/kg) CA inhibitor. Unlike acetazolamide, which inhibits both cytosolic and membrane-associated (extracellular) forms of CA, F3500, a polymerized form of aminobenzolamide, is restricted to the extracellular environment. F3500 is filtered by the kidney, however, and thus was able to selectively inhibit luminal CA IV activity (9, 31). To ensure that the dead space volume within the urinary catheter was cleared, any urine collected over the first 30 min after drug injection was discarded.

Urine flow rates were determined gravimetrically. Urine pH, total CO$_2$, and titratable acidity were determined immediately on collection; remaining urine was frozen for later analysis of Na$^+$, K$^+$, Ca$^{2+}$, Cl$^-$, and ammonia. Blood samples (0.6 ml) were withdrawn from the dorsal aortic cannula at 21 and 27 h. After determination of hematocrit, the blood was centrifuged and plasma was removed for immediate determination of pH and total CO$_2$. The remaining red blood cells were resuspended in Cortland saline and reinjected into the fish.

**Blood and urine analysis.** Following withdrawal, blood samples were centrifuged (~10,000 g for 1 min) to obtain plasma. Plasma total CO$_2$ concentration was determined in duplicate on 50-$\mu$l samples using a Capnicon total CO$_2$ analyzer (CC501; Cameron Instruments), while pH was assessed using a pH electrode and calomel reference (Cameron Instruments-E301 glass pH electrode) housed in a temperature-controlled (13°C) low-volume pH chamber (Cameron Instruments) and connected to a PHM 72 acid-base analyzer (Radiometer Copenhagen). PCO$_2$ and [HCO$_3^-$] were calculated by rearranging the Henderson-Hasselbalch equation using appropriate values of $\alpha$CO$_2$ and $pK_a$ for rainbow trout plasma (2).
Total ammonia concentration was determined using a micromodification of the salicylate-hypochlorite technique of Verdouw et al. (55). K\+/H1\(^+\), Ca\(^{2+}/H1\(^+\), and Na\(^+/H1\(^+\) levels were determined using atomic absorption spectroscopy (Varian) and Cl\(^-/H1\(^-\) levels (66) were measured colorimetrically using mercuric thiocyanate and ferric nitrate and quantified by spectrophotometry (Spectramax; 340BC). Urine pH and total CO\(_2\) concentration were determined as described for plasma above, and urine [HCO\(_3^-\)] was calculated using constants derived for freshwater.

Net renal acid efflux was calculated according to Wood and Caldwell (62) by summing ammonia efflux and titratable acidity (TA – HCO\(_3^-\)) efflux. Urinary [TA – HCO\(_3^-\)] was measured by adding a known volume of 0.02 mol/I HCl to 200 \(\mu\)l of urine to lower the pH below 5.0; the sample was then aerated for 20 min to remove CO\(_2\). While aeration was continued, NaOH (0.02 mol/I) was added gradually using a precision microburet (Gilmore) to restore urine to the pH of blood representative of the particular sampling period. The difference between the quantities of acid and base added to the urine yields the titratable component of net renal acid efflux and, when added to ammonia efflux, gives the total amount of acid excreted.

Statistical analyses. Data are reported as means ± SE. The effect of exposure to hypercapnia on gill tCAc mRNA expression determined by real-time PCR was analyzed using one-sample Student’s \(t\)-tests. Two-way repeated-measures ANOVA with sampling period (pre- and postinjection of acetazolamide or F3500) and treatment group (control or hypercapnia-exposed) as factors were used to analyze the effects of acetazolamide or F3500 injection (independently) on blood acid-base variables and net acid-base fluxes. Differences in band intensities on Western blots were assessed using unpaired Student’s \(t\)-tests.

RESULTS

Molecular cloning of trout CA IV. With the use of homology cloning techniques, a 1,137-bp cDNA was assembled that included an open reading frame encoding for a deduced protein of 297 amino acids (Fig. 1A; GenBank accession AAR99330). The trout protein most closely resembled CA IV, sharing 38% identity and 55% similarity to human CA IV (GenBank accession AAA35630). Moreover, like other CA IVs, the trout sequence contains an NH\(_2\)-terminal signal peptide (AA As 1–17) with the mature peptide beginning at position 18 (Fig. 1A) as well as two predicted disulfide bridges between cysteines 21–28 and 32–45 (Fig. 1A). The predicted active site of the trout sequence closely resembled the active site of vertebrae CA IVs (Fig. 1B). Finally, phylogenetic analysis revealed that the trout gene grouped closely with vertebrae CA IVs and that it was clearly isolated from other membrane-bound isoforms, CA IX, XII, and XIV (Fig. 2). Notably, the trout gene did not group with previously cloned fish cytosolic CAs. Thus we have tentatively identified the trout gene as CA IV (tCA IV).

Tissue distribution of tCA IV. The distribution of CA IV was examined using Northern blots, real-time PCR, and Western blots of saline-perfused (to eliminate blood contamination) tissues. Analysis of Northern blots revealed a single band with a transcript size of approximately 2.1 kb that was clearly detectable in kidney but not in
blood (Fig. 3A). The absence of any detectable tCA IV RNA in blood indicated that the source of the kidney CA IV was not contaminating red blood cells. The results of real-time PCR confirmed high levels of tCA IV mRNA in kidney (mostly posterior kidney; Fig. 3B) but also revealed lower levels of expression in the brain and spleen. The results of Western blot analysis revealed that the molecular mass of the tCA IV protein was tissue dependent. In brain and heart, the molecular mass was estimated to be 43 kDa, whereas in the kidney, the mass was estimated to be 45 kDa (Fig. 3C). In each case, the molecular mass of tCA IV was higher than the predicted mass of 34 kDa for the translated cDNA. Although several bands were observed in the Western blots, the specificity of the tCA IV antibody was demonstrated by elimination of a single immunoreactive band following preabsorption of antibody with excess blocking peptide (Fig. 3E). The expression profile of tCA IV protein differed somewhat from the mRNA results with highest levels apparent in brain followed by heart and kidney; tCA IV protein was not detectable in gill, liver, or muscle (Fig. 3C). However, when adjusted for differences in protein loading (using tubulin as a reference protein), the kidney clearly demonstrated the highest level of tCA IV (ratio of tCA IV to tubulin = 6.6) followed by heart (ratio = 2.2) and brain (ratio = 0.9).

Localization of tCAc and tCA IV in trout kidney. tCAc and tCA IV mRNA and protein expression patterns in the kidney were assessed using in situ hybridization and immunocytochemistry (Figs. 4 and 5). Positive hybridization signals for tCAc mRNA were detected in selected renal tubules (Fig. 4, A and B). Minimal or no staining (and hence hybridization) occurred in the absence of probe (Fig. 4C) or when tissues were pretreated with excess unlabeled probe (Fig. 4D). tCAc protein appeared to be localized to the apical regions of renal tubules (arrows) and was colocalized (yellow/orange) with Na\(^+\)-K\(^+\)-ATPase in the basolateral regions. Specific fluorescence was prevented by (G and H) omission of primary antibodies. Scale bars = 25 μm.
tubule cells and also appeared to be colocalized with \( \text{Na}^+\text{-K}^+\text{-ATPase} \) in the basolateral regions (Fig. 4, E and F). Immuno-fluorescence for tCAc and \( \text{Na}^+\text{-K}^+\text{-ATPase} \) was prevented by omission of primary antibodies (Fig. 4, G and H). tCA IV mRNA also was visualized by in situ hybridization in a subset of renal tubules (Fig. 5, A and B) and as with tCAc, staining was virtually eliminated in the absence of probe (Fig. 5C) or when tissues were pretreated with excess unlabeled probe (Fig. 5D). tCA IV protein was expressed in the vicinity of the apical membrane in cells coexpressing basolateral membrane \( \text{Na}^+\text{-K}^+\text{-ATPase} \) (Fig. 5, E-G). Depending on the tubule being examined, there was a variable degree of colocalization of tCA IV and \( \text{Na}^+\text{-K}^+\text{-ATPase} \) on the basolateral membrane. In some instances, \( \text{Na}^+\text{-K}^+\text{-ATPase} \)-positive cells did not express tCA IV (Fig. 5E). tCA IV immunofluorescence was eliminated by preincubating the antibody with excess immunizing peptide (Fig. 5H) or by omission of primary antibodies (Fig. 5I).

By posttreatting slides previously viewed for immunocytochemistry with PAS, it was possible to identify the tubule types expressing tCAc and tCA IV (Fig. 6). tCAc protein was present in both proximal and distal tubules, although there appeared to be a greater percentage of distal tubules expressing tCAc than proximal tubules (Fig. 6, A and B). In contrast, tCA IV appeared to be exclusively localized to proximal tubule cells (Fig. 6, C and D).

Expression of renal tCAc and tCA IV during hypercapnia. Exposure to hypercapnia resulted in a transient relative increase (≈5-fold) in renal tCAc mRNA that was statistically significant after 3 h (Fig. 7A). Renal tCA IV mRNA was markedly elevated (≈20-fold) but only after 24 h of continuous hypercapnia.
Analysis of Western blots (Fig. 7, B–D) demonstrated that tCAc protein was increased by 2.2 times (Student’s t-test, $P = 0.012$) after 24 h of exposure to hypercapnia; tCA IV protein levels were unaltered (data not shown).

Effects of CA inhibition on blood gases and renal acidification. Fish exposed to hypercapnia for 24 h exhibited total pH compensation owing to large increases in plasma $\text{HCO}_3^-$ levels (Fig. 8). In one series of experiments, acetazolamide was used to inhibit total (intracellular and extracellular) CA activities, whereas in the second, F3500 was used to selectively inhibit extracellular CA activity. The urine of some fish ($n = 12$) used in the present study was assessed for CA inhibitory activity before and following F3500 treatment. These experiments demonstrated that 50 μl of urine after intravascular F3500 injection were able to reduce the activity of a standard amount of bovine CA by 95% (from $29,779 \pm 2,636$ to $1,524 \pm 226 \mu \text{mol CO}_2/\text{ml} \cdot \text{min}^{-1}$, $P < 0.01$, paired Student’s t-test). Thus, clearly F3500 is being filtered by the kidney of rainbow trout while retaining its inhibitory properties.

Acetazolamide, whether administered during normocapnia or hypercapnia, caused a rapid increase in $\text{Paco}_2$ and a reduction of arterial blood pH ($\text{pHa}$; Fig. 8, A and B). Despite the respiratory acidosis, plasma $\text{HCO}_3^-$ levels were unaffected by acetazolamide (Fig. 9C). Analysis of the data using a pH-$\text{HCO}_3^-$ diagram revealed that acetazolamide caused a mixed respiratory/metabolic acidosis (data not shown). The base deficits induced by acetazolamide were calculated to be 3.0 and 2.2 mmol/l during normocapnia and hypercapnia, respectively. Unlike acetazolamide, F3500 was without effect on blood acid-base status during either normocapnia or hypercapnia (Fig. 8).

Urinary net acid efflux was significantly reduced by acetazolamide to a similar extent during normocapnia or hypercapnia (Fig. 9) owing to changes in TA-$\text{HCO}_3^-$ flux; ammonia fluxes were unaffected. Urine pH, $\text{HCO}_3^-$, and $\text{Na}^+$ levels were markedly increased by acetazolamide (Fig. 10), although for $\text{HCO}_3^-$ and $\text{Na}^+$, the changes were statistically different only during hypercapnia. Urine flow rate was increased by acetazolamide but only during normocapnia; urine levels of $\text{K}^+$, $\text{Cl}^-$, and $\text{Ca}^{2+}$ were unaffected by acetazolamide treatment or hypercapnic exposure (Table 1).

Treatment of fish with F3500 reduced net renal acid efflux owing to a reduction of TA-$\text{HCO}_3^-$ efflux (Fig. 9). Because the statistical analysis (2-way ANOVA) did not reveal any significant interactions between factors, it was not possible to statistically distinguish differences in net acid excretion between normocapnia and hypercapnia. Thus the apparently greater effect of F3500 on net acid flux during hypercapnic conditions could not be verified statistically. Urine pH and $\text{HCO}_3^-$ levels were significantly increased by F3500 (Fig. 10). The levels of $\text{Na}^+$ in the urine were increased by F3500, but only during hypercapnia (Fig. 10). Urine flow rate and other measured urinary ions were unaffected by F3500 (Table 1).

DISCUSSION

Previous studies have demonstrated that the kidney of rainbow trout and presumably other freshwater fishes plays an essential role in systemic pH regulation during periods of acidosis owing to its ability to increase tubular $\text{HCO}_3^-$ reabsorption (39, 56, 63). In mammals, such increases in tubular $\text{HCO}_3^-$ reabsorption are reliant on the stimulation of tubular
HCO₃⁻ associated (CA IV) isoforms of CA (45, 50). In contrast, only membrane-tetrapod CA II isoform, phylogenetic analysis clearly demonstrated that the trout genes for cytosolic CAs originated before a gene duplication event that gave rise to CAs I, II, and III in tetrapods (13). Interestingly, there are several entries in GenBank for CA II, and to lesser extent CA I, derived from teleost species. Indeed, identical sequences corresponding to tCAc and tCAb have been entered in GenBank as CA I (accession BAD36835) and CA II (accession BAD36836), respectively (Tohse H, Murayama E, Ohira T, Takagi Y, and Nagasawa H, direct submissions to GenBank). We believe that it is probably inappropriate to apply the tetrapod nomenclature to the cytosolic CAs of teleost fish (53).

In this study, we cloned an additional CA isoform from rainbow trout kidney that most closely resembles a mammalian membrane-bound isoform, CA IV. Although no functional characterizations were performed, the predicted presence of a signal peptide leader sequence and two disulfide bridges in addition to its close grouping with other vertebrate CA IVs (17, 35, 58) provide strong evidence that the trout gene is indeed a CA IV homolog. The presence of disulfide bridges in mammalian CA IV is thought to confer resistance to the detergent sodium dodecyl sulphate (SDS) (35). It is noteworthy that the two trout cytosolic isoforms of CA lack disulfide bridges and consequently exhibit sensitivity to SDS treatment (13). Another feature which distinguishes CA IV from other membrane-associated CA isoforms (i.e., CA IX, XII, and XIV) is the presence of a glycosylphosphatidylinositol (GPI) anchor site near the COOH terminus that serves to tether the enzyme to the apical plasma membrane (45). A probable anchor site in the trout sequence is a serine residue at position 277 just before a hydrophobic domain (AAs 282–292) (12).

Using the recently acquired tCA IV sequence (this study) and the previously published sequence of tCAc (13), various tools were developed to examine the expression of these genes in trout kidney and their responses to respiratory acidosis.

Localization of tCAc and tCA IV in trout kidney. Although homologous polyclonal antibodies were developed for both tCAc and tCA IV, we opted to use a commercial antibody raised against human CA II to assess the levels of tCAc protein. Although both antibodies yielded bands at 29 kDa (the expected size of tCAc) on Western blots, the amount of background staining (especially for immunocytochemistry) was significantly less when the human antibody was used. This same human antibody was used successfully in a previous study to localize cytosolic CA in the inner ear of salmon (52).

tCAc and tCA IV were both expressed in the posterior kidney. Interestingly, for tCA IV, slightly different results were obtained when evaluating mRNA vs. protein levels. When mRNA by real-time PCR was assessed, the posterior kidney was the predominant site of tCA IV expression with much lower levels being expressed in brain and spleen. Although Western blots confirmed high levels of tCA IV in kidney (when normalized to tubulin levels), they also revealed high levels in heart and brain. Although we are currently unable to explain the discrepant results, the important finding for the purposes of this study was that tCA IV is abundant in the kidney and thus potentially able to participate in renal HCO₃⁻ reabsorption. On the basis of its nucleotide sequence, the predicted molecular mass of tCA IV is 34 kDa. However, as in the rabbit (45) the actual molecular masses of tCA IV exceed the predicted sizes ranging from 43 to 45 kDa with the kidney expressing the largest protein.
The larger than predicted masses of tCA IV likely reflect post-translational addition of glycosyl and sialic acid residues (45).

While this is the first study to directly demonstrate the presence of CA IV in fish, several previous studies have provided indirect evidence for the presence of CA IV-like activity in a variety of tissues including the gills of dogfish (Squalus acanthias) (22) and two Antarctic species (Chaenocellulus aceratus and Notothenia coriiceps) (54), the heart of lamprey (Petromyzon marinus) (15), the intestine of eel (Anguilla anguilla) (29), and the kidney of the marine winter flounder (Pleuronectes americanus) (36). Additionally, we recently cloned and sequenced CA IV from the gill of dogfish (Perry SF et al., GenBank accession number DQ092628) where it is believed to play an important role in CO2 excretion (21).

Current models for CO2 excretion in trout and other teleosts contend that CO2 excretion depends exclusively on cytosolic CA (38). Thus the lack of detectable CA IV in the trout gill (this study) is consistent with the results of previous indirect studies that also were unable to provide evidence for membrane-associated extracellular CA in teleosts (23, 27; reviewed in Refs. 19, 20).

The results of in situ hybridization, immunocytochemistry, and conventional histology demonstrated that tCAc and tCA IV were localized to a subset of renal tubules. tCA IV appeared to be localized to the apical membrane/brush border of proximal tubules and in some instances was colocalized with Na⁺-K⁺-ATPase on the basolateral membrane. tCAc appeared to be associated with the apical and
basolateral regions of proximal and distal tubules. Although it has been shown that the proximal tubule of trout, like that of mammals, is composed of discrete sections (e.g., P1 and P2 in fish) (1), we were unable to reliably distinguish P1 from P2 segments based on standard morphological criteria (thickness of the brush border and position of nuclei). In mammals, CA IV is expressed on the apical and basolateral membranes of S2 and S1 proximal tubules (36, 46). The presence of tCA IV within the brush border of proximal tubules is consistent with the role of this segment of the nephron in reabsorbing the majority of HCO$_3$ from the renal filtrate. The results of selective inhibition of extracellular CA activity suggest that the apical membrane CA IV is externally oriented and thus able to catalyze reactions within the lumen of the proximal tubule.

Cytosolic and membrane-associated CAs are involved in renal HCO$_3$ reabsorption. Having established that the trout kidney contains both cytosolic (tCAc) and membrane-associated (tCA IV) forms of CA, we designed experiments to assess their relative involvement in renal HCO$_3$ reabsorption under normocapnic and hypercapnic conditions. In these experiments, we compared the renal and blood acid-base responses of trout to a permeant CA inhibitor, acetazolamide (30), and a nonpermeant inhibitor, F3500 (9). F3500 is a polymer of aminobenzolamide that is small enough to be filtered by the kidney yet too large to enter cells (9). Previous research on mammals (31) and fish (21) showed that it does not inhibit red cell CA activity. Thus, in this study, we assumed that acetazolamide inhibited total (cytosolic and extracellular) CA activity, whereas F3500 selectively inhibited the extracellular luminal CA IV. Indeed, in contrast to acetazolamide treatment, the lack of any respiratory acidosis associated with F3500 injection provided further evidence that F3500 was not inhibiting red blood cell CA activity.

The results of the present study provide strong data to support the view (40) that both cytosolic and membrane-associated CAs are involved in renal acid secretion and HCO$_3$
Fig. 10. Effects of total CA inhibition using acetazolamide (A–C) or specific inhibition of extracellular CA using F3500 (D–E) administered to rainbow trout (O. mykiss) under control conditions or during hypercapnia on urine pH (A and B), urine HCO₃⁻ levels (C and D), and urine sodium levels (E and F). Filled bars represent values for a 3-h period before administration of CA inhibitors; open bars represent values for a 3-h period beginning immediately after administration of inhibitors. All values are represented as means ± SE; n = 7–8 for acetazolamide-treated trout and n = 5–7 for F3500-treated trout. Data were analyzed statistically using a 2-way repeated-measures ANOVA with sample time (pre- or post-inhibitor injection) and treatment (control or hypercapnia) as factors. Groups that do not share a letter were significantly different from one another; where the interaction term of the 2 factors was not significant, preinjection vs. postinjection comparisons (indicated by horizontal lines) or control vs. hypercapnia (indicated by letters placed between the bars) comparisons only were carried out as appropriate.

P values for sample time, treatment, and the interaction of these 2 factors, respectively, were A: <0.001, 0.006, 0.887; B: <0.001, 0.001, 0.001; C: <0.001, 0.065, <0.001; D: 0.009, 0.066, 0.340; E: 0.007, 0.007, 0.140; and F: <0.001, 0.325, 0.005.

Table 1. Interactive effects of exposure to hypercapnia and CA inhibition on UFR and urine concentrations of K⁺, Ca²⁺, and Cl⁻ in rainbow trout (Oncorhynchus mykiss)

<table>
<thead>
<tr>
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<th>Acetazolamide</th>
<th>Hypercapnia</th>
<th>F3500</th>
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<tr>
<td></td>
<td>Preinjection</td>
<td>Postinjection</td>
<td>Preinjection</td>
</tr>
<tr>
<td>UFR</td>
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<td>7.76±1.00*</td>
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<tr>
<td>[Ca²⁺]</td>
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<td>1.66±0.12</td>
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<tr>
<td>[Cl⁻]</td>
<td>10.06±3.32</td>
<td>8.35±3.28</td>
<td>5.79±1.61</td>
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Values are means ± SE; n values are indicated in parentheses. Statistical differences from the preinjection values are indicated with *. 1Data were analyzed individually for acetazolamide-treated and F3500-treated fish using a 2-way repeated-measures ANOVA with sampling time (pre- or postinjection) and treatment (control or hypercapnia) as factors. Acetazolamide was used to inhibit total carbonic anhydrase (CA) activity, whereas F3500 was used to selectively inhibit apical membrane-associated CA. UFR, urine flow rate (ml·kg⁻¹·h⁻¹).
The CO₂ thus formed diffuses into the tubule cells where CO₂ by cytosolic CA. During hypercapnia, acid-base regulation is tubule, a process that is dependent on the catalyzed hydration of the kidney must concomitantly adjust net acid secretion and balances, fish possess an additional site of metabolic compensation of acid-base disturbances. Thus CA isoforms in both gill and kidney play important roles in acid-base regulation in fishes. The gills of fish and kidneys of tetrapods are considered to be functionally equivalent with respect to their roles in acid-base balance, electrolyte regulation, and nitrogenous waste excretion. However, an important difference between the two sites of CA-assisted acid secretion is that there does not appear to be the equivalent of a luminal membrane CA isofrom in the fish gill.

ACKNOWLEDGMENTS

We are grateful to B. McNeill and M. Bayaa for invaluable technical support. We thank Dr. E. Swenson for helpful advice on the choice of CA inhibitors to use.

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