Cortisol alters carbonic anhydrase-mediated renal sulfate secretion

Ryan M. Pelis, James E. Goldmeyer, Joseph Crivello, and J. Larry Renfro. Cortisol alters carbonic anhydrase-mediated renal sulfate secretion. Am J Physiol Regul Integr Comp Physiol 285: R1430–R1438, 2003. First published August 7, 2003; 10.1152/ajpregu.00331.2003.—Active transepithelial sulfate secretion rate by winter flounder renal proximal tubule epithelium in primary culture (fPTC) is dependent on intracellular carbonic anhydrase (CA) and enhanced by cortisol. To further evaluate this relationship, a partial cDNA clone (327 bp) of carbonic anhydrase II (CAII) with high sequence similarity to CAII from numerous species including fish, chicken, and human was obtained from fPTCs. The majority of CA activity and CAII protein was present in the cytosol of fPTCs; however, significant amounts of both (in addition to SDS-resistant CA activity, i.e., CAIV-like isoform) were present in concentrated plasma membranes. CAII from concentrated membranes migrated differently than purified CAII on nondenaturing PAGE gels, suggesting that CAII associates with another membrane component. Treatment of fPTCs with the cell-soluble CA inhibitor methazolamide (100 μM) caused a 58% reduction in active transepithelial SO4\(^2^-\) secretion. fPTCs that were previously cultured under high-cortisol concentrations, when subjected to 5 days of low physiological levels of cortisol, had decreased CA activity (28%), CAII protein abundance (65%), and net active SO4\(^2^-\) secretion (28%), with no effect on epithelial differentiation. Methazolamide and low-cortisol treatment in combination inhibited net active SO4\(^2^-\) secretion 56%, which was not different than the effect of methazolamide treatment alone. These data indicate that cortisol directly increases renal CA activity, CAII protein abundance, and CA-dependent SO4\(^2^-\) secretion in the marine teleost renal proximal tubule.

Renal proximal tubule CA has also been linked to transepithelial SO4\(^2^-\) reabsorption in chickens (12), and while not directly linked to SO4\(^2^-\) transport, CA activity in the mammalian renal proximal tubule facilitates HCO3\(^-\) reabsorption and H\(^+\) secretion thereby promoting urinary acidification (42). More than 95% of renal CA activity in mammals is attributed to cytosolic CA, with the remaining ~5% membrane bound (32, 59). Four CA isoforms (CAII, CAIV, CAXII, and CAXIV) have been identified in the mammalian renal proximal tubule. CAII is cytosolic (4, 42), whereas CAIV (8), CAXII (33), and CAXIV (24) are localized to the basolateral membrane (CAXII) or both basolateral and apical membranes (CAIV and CAXIV). Whereas CAII has been demonstrated in chicken proximal tubule (12), its presence in marine flounder proximal tubule is yet to be determined.

Several studies suggest that CA expression may be regulated; for example, the avian CAII gene contains a vitamin D3 response element in the promoter regions (34). Metabolic acidosis in rabbits increases CAII and CAIV activity (6, 7, 58) and mRNA expression (43, 53, 58) in the kidney, changes that function to enhance acid excretion. Similarly, renal CAII mRNA levels increase following acidosis in a cyprinid teleost (Tribolodon hakonensis) (22). Glucocorticoid levels increase during metabolic acidosis (31, 57), which may implicate cortisol in the control of renal CA expression during acidosis. Sexual maturation induces an increase in CA activity in red blood cells and kidneys of rats (6), and dexamethasone (a cortisol agonist) treatment (in vivo) of rats accelerates the maturation-induced increase in CA activity in the brain (13). CA activity in red blood cells is increased by in vitro and in vivo cortisol treatment (39).

SO4\(^2^-\) secretion, reabsorption, and urinary acidification are all affected, directly or indirectly, by glucocorticoids.
ticoïdes (3, 12, 36), respectively. Teleosts that tolerate varying salinities obviously must regulate renal excretion. Bern (2) implicated cortisol in the adaptation of teleosts to seawater; indeed, it was termed the “seawater-adapting” hormone. Renfro (35) showed that acclimation of seawater winter flounder to 10% seawater (SO4²⁻ free) reduced SO4²⁻ clearance ratios from 12 to less than 1 and concurrently decreased renal epithelial cell apical SO4²⁻/HCO₃⁻ exchange. Clearance ratios greater than one and enhanced apical SO4²⁻/HCO₃⁻ exchange were restored by daily injections of dexamethasone (60 µg/100 g body wt) for 5 days. Glucocorticoids also increase SO4²⁻ excretion in birds and mammals through effectively decreasing apical Na-dependent SO4²⁻ reabsorption (12, 40). The observations that renal tubular SO4²⁻ transport is cortisol dependent, metabolic acidosis stimulates renal CA expression, and CA activity is required for full proximal tubular SO4²⁻ secretion prompted the present investigation. The results suggest that cortisol stimulates renal proximal tubule CA activity, CAII protein abundance, and CA-dependent SO4²⁻ secretion.

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Animals. Winter flounder, Pleuronectes americanus, were obtained by otter trawl in Frenchman’s Bay, Maine, or in Long Island Sound, Connecticut. Animals (250–400 g) were held in flowing seawater (17–19°C) or in Living Stream Units (Toledo) filled with artificial seawater (Utikem) at 12°C. Animal use followed the newest guiding principles for research (1).

Solutions and chemicals. Modified medium 199 with Earle’s salts (M199, Sigma Chemical, St. Louis, MO) was supplemented with (in mM) 30.0 NaCl, 4.2 NaHCO₃, 1.0 l-glutamine, 25.0 HEPES, 14.75 NaOH (pH 7.5, 347 mosmol/kgH₂O), and 20 mg/l tetracycline. Modified M199 was supplemented with 10 µg/ml insulin, 5 µg/ml hydrocortisone, and 10% flounder serum to form the final plating medium. Ca- and Mg-free solutions (CMF) used for removing extrarenal tissues contained the following (in mM): 150.0 NaCl, 4.0 KCl, 0.5 NaH₂PO₄, 4.2 NaHCO₃, 25.0 HEPES, 5.5 glucose, 0.3 ethylenediaminetetraacetic acid, 14.75 NaOH (pH 7.5), and 20 mg/l tetracycline. Flounder saline (FS) contained (in mM) 150.0 NaCl, 4.0 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 0.4 NaH₂PO₄, 4.2 NaHCO₃, 25.0 HEPES, 5.5 glucose, 1.0 l-glutamine, and 14.75 NaOH (pH 7.5).

Preparation and primary culture of flounder renal proximal tubule epithelium. The method shown here was first described by Dickman and Renfro (11) and then modified by Gupta and Renfro (19). Briefly, kidneys were perfused with modified M199, removed, and tubules were teased apart. Hematopoietic and lymphoid tissues were removed by brief incubation of tubules in CMF containing 0.2% trypsin at 22°C. Epithelial cells were released from tubule fragments by 3 days of trypsinsization (5°C) in M199 supplemented with 0.06% trypsin. Dissociated cells were washed, suspended in culture medium, and plated on native rat-tail collagen. Collagen gels were released after 4 days, and after 12 days, the epithelial monolayers had contracted from the initial 55-mm diameter to 17 mm and had assumed fully differentiated transepithelial transport capabilities.

Cortisol levels in plasma and culture medium were measured by radioimmunoassay (Diagnostic Products). Plasma cortisol levels from winter flounder sampled in October and February were highly variable (8.4 × 10⁻⁹ – 3.5 × 10⁻⁷ M, n = 15). Primary cultures of flounder renal proximal tubule epithelium (fPTCs) were supplemented with cortisol (7.3 × 10⁻⁶ M) and removal of added cortisol from culture medium 5 days before experimental analysis resulted in levels that were at the lower end of the physiological range (6.3 × 10⁻⁹ M, n = 7).

Preparation of total mRNA from fPTCs. A single fPTC containing ~10⁷ cells was homogenized to a fine powder under liquid nitrogen using mortar and pestle. Total RNA was isolated with QiaGen RNeasy mini kit. Purified RNA was stored in 200 µl of RNase-free water. Total RNA was quantified with Ribogreen (Molecular Probes) and by comparison to RNA standard curve. The final concentration of RNA was adjusted to 100 ng/µl.

Isolation of mRNA from 1.0 mg of total RNA obtained was carried out as described by the QiaGen Oligotex mRNA kit. mRNA was eluted into 40 µl of elution buffer yielding a final mRNA concentration of 1 ng/µl.

RT-PCR method for detecting CAII in fPTCs. The isolated mRNA was used for RT-PCR as described in the Qiagen one-step-RT-PCR kit. Briefly, reverse transcription of mRNA and the subsequent PCR of CAII sequences were carried out in a single step with degenerate primers provided by Dr. Steven Gehnrich, Salisbury State, Maryland. The forward and reverse primer sequences were 5’CATTTCCATYTKCACTGG and 5’GNGGGNGTNAGNNGANC respectively. The primers (2 µM) and 2 ng of template mRNA were used in the RT-PCR reaction. The reverse transcription reaction was carried out at 50°C for 30 min followed by a 15-min incubation at 95°C to denature the reverse transcriptase activity. The PCR reaction was run for 37 cycles with a denaturing temperature of 94°C, an annealing temperature of 55°C, and an elongating temperature of 72°C. PCR products were separated on a 2% agarose gel and stained with Gel-Star (Fisher). A single band was predominant at 329 bp.

Cloning and sequencing of RT-PCR product from fPTC total mRNA. Cloning of PCR products was carried out according to the protocol of Hamilton et al. (20) with a few minor modifications. The RT-PCR product was cleaned with Qiaquick spin tubes and ligated to SNX linker fragments, after digestion with Mung bean exonuclease to create blunt ends and dephosphorylation with calf intestinal phosphatase. Ligation to the SNX linker occurred in the presence of XMN 1. NHE 1 was used to cut the SNX linker for insertion into p-Bluescript. The 329-bp product was chosen for sequencing. Positive colonies from white/blue X-gal/IPTG-based selection on ampicillin-positive plates were selected at random and sequenced by the Yale University HHMI/KECK Biotechnology Resource Laboratory, New Haven, CT. Blast searches were used to compare the cloned CAII DNA fragment to published DNA and peptide sequences. The partial P. americanus CAII cDNA was deposited in GenBank with accession no. AY321311.

Measurement of CA biochemical activity. Culture medium from individual fPTCs was aspirated and fPTCs were rinsed in 1 ml of 150 mM NaCl. Individual fPTCs were transferred to 200 ml of ice-cold 150 mM NaCl and rinsed for 30 min. This step was repeated once. Epithelial cells were scraped from the collagen substrate using a glass microscope slide, aspirated, and placed in a microcentrifuge tube. Cells were pelleted by centrifugation (11,000 g, 90 s) and resuspended in 100 µl of H₂O. The method for determining CA biochemical activity is a colorimetric assay that monitors change in pH (5). The assay was conducted at 0.5°C. The sample mixture was prepared by adding 10 µl of cell suspension to 185 µl H₂O containing 5 µl of 1-octanol. CAIV is a membrane-bound
CA, which unlike other CA isozymes is resistant to SDS (0.2–10%; 7). SDS (1%) and 15 µl 1-octanol (to prevent foaming) were included in the sample mixture when examining CAIV activity only. CO₂ was bubbled into the sample mixture and 200 µl of buffer/indicator mix (5.0 mM Tris·HCl, 20 mM imidazole, and 0.4 mM para-nitrophenol) were added to the sample mixture. A yellow-to-clear color change indicated the reaction endpoint, and a stopwatch was used to monitor the time for this to occur. The uncatalyzed reaction was monitored in the absence of sample. Samples were run with and without 100 µM methazolamide (CA inhibitor) to determine total CA activity in the sample. Protein concentration among samples was determined by a protein assay (Bio-Rad). Total CA activity is expressed in enzyme units per milligram of protein, and 1 enzyme unit (EU) equals one-half the time for the uncatalyzed reaction endpoint.

Isolation of fPTC cell lysates, plasma membranes, and cytosolic fractions for determination of CA activity and CAII protein expression. Culture medium from individual fPTCs was aspirated, and fPTCs were rinsed in 1 ml of 150 mM NaCl followed by two additional washes (30 min each) in 200 ml of 150 mM NaCl. Cells were removed from the collagen substratum by scraping with a glass microscope slide and placed in 50 ml of ice-cold 150 mM NaCl, pelleted at 5,000 x g for 10 min, lysed in 300 µl of water, and a sample (cell lysate) was saved. The cell lysate was centrifuged at 30,000 x g for 30 min at 4°C. The supernatant (cytosolic fraction) was removed and the pellet (membrane fraction) was resuspended in 100 µl of water. CA activities of cell lysate, plasma membranes, and cytosolic fraction were assayed. The cell lysate, plasma membranes, and cytosolic fraction were also diluted in Kaman buffer for SDS-PAGE.

Preparation of plasma membranes from fPTCs. fPTCs were rinsed in FS and the epithelium was scraped from the collagen substratum with a glass microscope slide. Cells were suspended and pelleted in FS by centrifugation at 5,000 x g for 10 min at 4°C. Cells were lysed in a 1,000-fold dilution of lysis buffer (5 mM NaH₂PO₄, pH 7.0) by syringing with a 23-gauge needle. Lysed cells were further diluted 1:40 before centrifugation at 49,000 x g for 45 min at 4°C. Pelleted membranes were diluted 1:1,000 with lysis buffer, syringed, and further diluted 1:40. Cell membranes were centrifuged at 49,000 x g and resuspended in H₂O (±1% SDS) for measurement of CA biochemical activity, Kaman buffer (2.3% SDS, 5% -mercaptoethanol, 10% glycerol, 0.5% saturated bromophenol blue, 62.5 mM trizma base, pH 6.8) for SDS-PAGE, or solubulized with 0.5% -mercaptoethanol containing protease inhibitors (in µM) 100 4-(2-thiophenyl)benzenesulfonyl fluoride HCl, 0.08 aprotinin, 5.0 benzamidine, 1.5 E-64, 2.0 leupeptin, and 1.0 pepstatin A.

Preparation of rat red blood cell membranes. Packed red cells (500 µl) were diluted in 5 ml of ice-cold lysis buffer (5 mM sodium phosphate, pH 8.0) containing the aforementioned protease inhibitors and centrifuged at 5,000 x g for 5 min. This step was repeated five times. Membranes were incubated in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3 with HCl) for 30 min on ice and solubulized by incubation in lysis buffer containing 0.5% Triton X-100. Solubulized membranes were centrifuged at 11,000 x g for 90 s, and the supernatant was boiled for 5 min. SDS-PAGE was conducted using 4–12% polyacrylamide gels. For nondenaturing PAGE, plasma membranes (fPTCs and rat red cell membranes) were solubulized in 0.5% Triton X-100 were placed in Kaman buffer without SDS or -mercaptoethanol. Samples for nondenaturing PAGE were not boiled and were run on 5% polyacrylamide gels. After SDS-PAGE and nondenaturing PAGE, proteins were transferred to polyvinylidine fluoride membranes (Milipore, Bedford, MA) that were then incubated in blocking buffer (PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween 20), 0.01% antifoam A, 0.02% NaN₃, and 10% nonfat dry milk) overnight at 4°C followed by 1-h incubation at room temperature with primary antibodies diluted 1:500 in blocking buffer. Primary antibodies included sheep anti-human CAII IgG (Accurate, Westbury, NY) and a monoclonal directed against human actin (Sigma). Membranes were washed three times with PBS (10 min each) and once in phosphate-free buffer (150 mM NaCl, 10 mM Tris-base, 40 mM Tris·HCl, pH 7.5). Membranes were incubated for 1 h at room temperature with secondary antibodies diluted 1:1,000 in phosphate-free buffer containing 10% nonfat dry milk. Secondary antibodies included alkaline phosphatase-conjugated donkey anti-sheep IgG (Sigma) and alkaline phosphatase-conjugated goat anti-mouse IgG (StressGen, Victoria BC, Canada). Membranes were washed four times in phosphate-free buffer and signals were detected by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) according to the manufacturer’s protocol. CAII protein expression among treatment groups was determined using densitometric analysis (National Institutes of Health image 1.60) and actin expression was used to control for differences in protein loading among samples.

Ussing chamber studies and determination of transepithelial SO₄²⁻ fluxes. fPTCs were mounted in Ussing chambers with an aperture size of 0.332 cm². Fluid volume was 1.2 ml/hemichamber, and temperature was maintained at a constant 20°C with water circulated on the outside surface of the chambers by a Lauda RM6 Electronic water bath. Fluid inside the chambers was constantly and vigorously stirred with small magnetic stir bars turned by external stir-plates. Chambers were insulated with humidified 99% O₂-1% CO₂. Transepithelial potential difference (TPD) was determined with Ag/AgCl electrodes connected to the luminal and peritubular compartments with 3 M KCl-2% agar bridges. Electrical properties were determined with a pair of computer-controlled, high-impedence automatic dual-voltage clamps (DVC 1,000; World Precision Instruments, Sarasota, FL). Electrode asymmetry was corrected at the beginning and end of each experiment and fluid resistance was compensated. Short-circuiting electrodes were connected to the luminal and peritubular solutions with 3 M KCl-2% agar bridges. Transepithelial resistance (TER) was determined from the change in TPD produced by a brief 10-µA pulse controlled by the voltage clamps.

Tissues were continuously short-circuited during flux determinations. Unidirectional tracer fluxes were initiated by the addition of 1.0–2.0 µCi³⁵S to the appropriate hemichamber. Duplicate 50-µl samples were taken from the unlabeled side at 30-min intervals over a period of 1.5 h and replaced with equal volumes of unlabeled solution. The specific activity of the labeled solution was determined at the beginning and end of each experiment. Net flux was calculated as the difference between unidirectional secretory and reabsorptive fluxes. Tissues used in a given experiment were prepared from the same culture batch. Proximal tubule-like function
and integrity were assessed by measurement of TPD, TER, and phlorizin-sensitive glucose current (I_{glu}).

**Statistics.** Experimental results are expressed as means ± SE, except flux data, which are presented as means ± SD. For examining differences in CA biochemical activity and CAII protein abundance, paired comparisons of sample means were done using Student’s t-test. One-way ANOVA was used to test the effects of methazolamide, low cortisol, and methazolamide and low cortisol in combination. Tukey’s HSD test was used for pairwise comparison of individual treatments. All statistical analyses were done using STATISTICA (StatSoft, Tulsa, OK) and deemed significant if P < 0.05.

**RESULTS**

Reverse transcription of fPTC mRNA with CAII-specific primers produced a single PCR product of 329 bp (Fig. 1A). The 329-bp fragment was cloned (Fig. 1B) and exhibited high sequence (nucleotide) homology to CAII from the European flounder (*Platichthys flesus*, 93%), Atlantic salmon (*Salmo salar*, 72%), big-scaled redfin (*T. hakonensis*, 71.1%), longnose gar (*Lepisosteus osseus*, 68.1%), chicken (*Gallus gallus*, 64.2%), and human (64%). The cloned fragment encodes 109 amino acids (aa), which represents 42% of the peptide (260 aa).

When probed with an anti-human CAII antibody, both rat red blood cells and winter flounder red blood cells exhibited intense immunoreactive signals corresponding to 29 kDa on immunoblots (Fig. 2A). Although less intensely stained than in red blood cells, CAII was present in fPTC cell lysates and in the cytosolic fraction. CAII appeared to be almost entirely absent from the plasma membrane (Fig. 2A). CA enzymatic activity measurements showed that ~85% of total CA activity (fPTC lysate, 2.638 EU) was in the cytosolic fraction (2.24 EU). When plasma membranes were concentrated, an intense signal corresponding to CAII was present (Fig. 2B). Compared with the cell lysate, enzymatic activity in the concentrated plasma membranes was enriched 1.65-fold (4.86 vs. 8.05 EU/mg protein). Of the CA biochemical activity in the concentrated plasma membranes, 56% was inhibited by 1% SDS (data not shown). Under nondenaturing conditions (nondenaturing PAGE), both fPTC plasma membranes and rat red blood cell membranes were immunoreactive for CAII (Fig. 2C). Unlike SDS-PAGE, CAII from rat red blood cells and fPTC plasma membranes migrated differently (the 5% gel alone does not allow molecular weight determination) than purified CAII, which migrates as three charge isomers on nondenaturing PAGE gels (Sigma Technical Bulletin No. MKR-137).

Analysis of immunoblots indicated that CAII protein abundance in fPTC cell lysates was 65% lower in fPTCs exposed to low cortisol for 5 days (Fig. 3). In addition, exposure to low cortisol caused a 28% reduction in CA enzymatic activity in fPTC cell lysates (Fig. 3).

Control fPTCs actively secreted SO$_4^{2-}$ at a net rate of 114.4 ± 5.81 nmol·cm$^{-2}$·h$^{-1}$ (Fig. 4). Treatment of fPTCs with the cell-soluble CA inhibitor methazolamide significantly reduced net active SO$_4^{2-}$ secretion to 47.9 ± 3.44 nmol·cm$^{-2}$·h$^{-1}$. The 58% inhibition in net active SO$_4^{2-}$ secretion following methazolamide treatment was due to a decrease in the unidirectional secretory flux (118.4 ± 6.43 to 52.6 ± 3.47 nmol·cm$^{-2}$·h$^{-1}$). Exposure to low cortisol reduced the unidirectional secretory flux to 92.9 ± 9.07 nmol·cm$^{-2}$·h$^{-1}$, which inhibited net active SO$_4^{2-}$ secretion 28%. Low cortisol and methazolamide treatment in combination reduced net active SO$_4^{2-}$ secretion to 43.8 ± 5.06 nmol·cm$^{-2}$·h$^{-1}$. The 61% inhibition in net active SO$_4^{2-}$ secretion following exposure to low cortisol and methazolamide together was not significantly different from methazolamide treatment alone. The unidirectional reabsorptive flux was not altered by methazolamide treatment, low cortisol, or methazolamide treatment and low cortisol in combination. None of the

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**Fig. 1.** A: RT-PCR for flounder renal carbonic anhydrase II (CAII). mRNA isolated from flounder renal proximal tubule epithelium in primary cultures (fPTCs) was reverse transcribed and amplified using degenerate primers to CAII. The RT-PCR product was run on a 2% agarose gel and stained with Gel-Star. A single product of 329 bp was obtained (CAII). No product was obtained when cDNA template was omitted (control). Molecular weight markers (MW) are labeled in bp. B: partial cDNA clone of winter flounder CAII and its deduced amino acid sequence. Amino acids are identified by standard 1-letter code. Amino acid numbers begin at 92 with glutamine and end with proline at position 200.
treatments had an effect on TER, TPD, or $I_{pu}$ (Table 1). In the case of low-cortisol treatment, this is especially noteworthy because this steroid is a known differentiating factor (15), and after 12 days in low cortisol considerable dedifferentiation occurs in fPTCs (19).

**DISCUSSION**

Previous work (36) demonstrated CA enzymatic activity in fPTCs, and unlike other studies that had used whole kidney tissue to establish whether marine teleost renal tubule contained CA activity (16, 17, 30, 51, 52), the finding was not confounded by the presence of hematopoietic tissue. Furthermore, it was shown that the rate of renal tubular $SO_4^{2-}/H_2O_2$ secretion is dependent on intracellular CA activity. With the use of the same culture system, the present study revealed 1) that proximal tubular CA activity includes at least CAII and, probably, CAIV, 2) that CA activity and CAII protein are both cytosolic and membrane associated and increased by cortisol, and 3) that increased CAII protein and CA activity in this tissue are correlated with an increase in a component of $SO_4^{2-}$ transport that is sensitive to cortisol.

**Fig. 2.** A: immunoblot showing CAII protein expression from rat (rRBC) and flounder (fRBC) red cell lysates and the lysate, plasma membrane (mem.), and cytosolic fractions from fPTCs (6 fPTCs). The cell lysate, plasma membrane, and cytosolic fractions were prepared from the same fPTCs. All tissues were run on 4–12% SDS-PAGE gels. A band corresponding to 29 kDa (CAII) was present in all tissues examined. Differences in protein loading are reflected in the staining intensity of actin (45 kDa). B: immunoblot showing purified bovine CAII and CAII from concentrated fPTC plasma membranes. The plasma membranes were concentrated by using more fPTCs (10 fPTCs) and by suspending the membranes in a smaller volume of Kaman buffer. Tissues were run on 4–12% SDS-PAGE gels. C: immunoblot showing CAII protein expression from purified bovine CAII, rat red blood cell plasma membranes, and fPTC plasma membranes. Purified CAII, rat red blood cell plasma membranes, and fPTC plasma membranes were solubilized with 0.5% Triton X-100 before run out on 5% nondenaturing polyacrylamide gels.

In the present study, RT-PCR and degenerate primers to CAII were used to clone a 329-bp fragment from fPTCs that exhibited high sequence (nucleotide) similarity to CAII from numerous species including human Fig. 3. Differences in CAII protein expression and CA biochemical activity in fPTCs either exposed to normal levels (control) or low physiological levels of hydrocortisone for 5 days (low cortisol). Values are means ± SE of $n = 3$ (CA activity) and $n = 4$ (CAII protein expression) fPTC cell lysate preparations. CAII protein levels are expressed as the ratio of CAII/actin. Whereas this ratio could be affected by changes in actin levels, no functional dedifferentiation of the epithelium was detected in this time frame (see Table 1). The 5-day period with low cortisol resulted in very faint CAII staining, and more tissue (total protein) was loaded to compensate resulting in a greater actin signal in the low-cortisol group. Cell lysates were run on 4–12% SDS-PAGE gels. CA biochemical activity is expressed as enzyme units (EU)/mg protein.*Significantly different from control ($P < 0.05$, paired t-test).}

Fig. 4. Effects of methazolamide (Mthz; 100 $\mu$M) supplementing with low physiological levels of hydrocortisone for 5 days (low cortisol), and methazolamide and low hydrocortisone in combination (Mthz + low cortisol) on the unidirectional secretory [peritubular-to-lumen ($P$-$L$)], unidirectional reabsorptive [lumen-to-peritubular ($L$-$P$)], and net $SO_4^{2-}$ fluxes across fPTCs. Control and methazolamide-treated fPTCs were supplemented with normal levels of culture medium hydrocortisone throughout. Fluxes shown were obtained at steady state following tracer addition ($t = 1.5$ h) and are means ± SE (vertical line) of 10 preparations. Different letters indicate significant differences among treatment groups ($P < 0.05$, Tukey's HSD).
Table 1. Effects of various treatments on transepithelial electrical properties generated by fPTCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>TER, Ω × cm²</th>
<th>TPD, mV</th>
<th>Iphlor, µA/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>39.4 ± 4.31</td>
<td>-0.2 ± 0.26</td>
<td>2.7 ± 0.33</td>
</tr>
<tr>
<td>Mthz</td>
<td>10</td>
<td>30.3 ± 3.19</td>
<td>-0.2 ± 0.05</td>
<td>2.2 ± 0.36</td>
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<tr>
<td>Low cortisol</td>
<td>10</td>
<td>44.2 ± 10.3</td>
<td>-0.3 ± 0.08</td>
<td>2.5 ± 0.39</td>
</tr>
<tr>
<td>Mthz + low cortisol</td>
<td>10</td>
<td>50.7 ± 5.48</td>
<td>-0.3 ± 0.05</td>
<td>1.9 ± 0.26</td>
</tr>
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Values are means ± SE. Treatments included methazolamide (Mthz; 100 µM), exposure to low cortisol for 5 days, and Mthz and low-cortisol treatments in combination. Control and Mthz-treated flounder proximal tubule in primary cultures (fPTCs) were supplemented with normal levels of culture medium hydrocortisone throughout. Values were obtained at 1.5 h in Ussing chambers. TER, transepithelial resistance; TPD, transepithelial electrical potential difference (lumen negative); Iphlor, phlorizin-sensitive glucose current.

(64%). This unequivocally demonstrates that CAII is specifically expressed in proximal tubule epithelium of winter flounder. Several key amino acid residues that are essential for the catalytic activity of CAII were present in the cloned fragment (Fig. 1B). H94, H96, and H119 reside in the active site pocket and are essential for binding Zn²⁺, which when complexed with OH⁻ (Zn-OH) forms the nucleophile required for enzymatic catalysis (25). Q92, E117, and T199 are considered second-shell residues that accept hydrogen bonds from H94, H119, and the Zn-OH, respectively, thereby functioning to fine-tune protein-zinc affinity (9, 26).

Measurements of CA activity in fPTCs indicated that 85% of enzymatic activity was in the cytosol with the remaining 15% membrane associated. More than 95% of CA activity in the rat and human kidney is contained within the cytosol (32, 59). In contrast, only 86% of CA activity in the dog kidney is attributable to cytosolic CA (41). In the mammalian kidney, CAII is cytosolic, whereas CAIV, CAXII, and CAXIV are tethered to the plasma membrane (42). When immunoblots were probed with a polyclonal antibody against human CAII, a single band of 29 kDa was apparent from winter flounder red blood cells and fPTC cell lysates and was identical in size to CAII from rat red blood cells. When cytosolic and membrane fractions were isolated, there was strong immunoreactivity for CAII in the cytosolic fraction, whereas the membrane fraction displayed weak immunoreactivity. Taken together, these data indicate that the total amounts of CA activity and CAII protein expression in renal proximal tubule epithelial cells of marine teleosts are highest in the cytosol.

When plasma membranes from fPTCs were concentrated, CA activity was enriched 1.65-fold. The enrichment of CA activity in plasma membrane preparations from dog renal tissue has been previously demonstrated (41). CA activity in fPTC plasma membranes (8.05 EU/mg protein) was similar to the enzymatic activity found in apical (12.2 EU/mg protein) and basolateral (13.0 EU/mg protein) membranes of rat renal tubular cells (59). Approximately 50% of the CA activity in the fPTC plasma membranes was inhibited by 1% SDS. CAIV is an extracellular glycosylphosphatidylinositol-anchored CA that contains two disulfide bridges allowing it to maintain its structure and catalytic activity in the presence of 0.2–10% SDS (7). The presence of SDS-resistant enzymatic activity in fPTC plasma membranes may suggest that ~50% of CA activity is due to a CAIV-like isoform. Membrane-associated CA (including CAIV) is essential for HCO₃⁻ reabsorption in the mammalian renal proximal tubule (28, 54). At this time, a function for CAIV in the marine teleost renal tubule is uncertain because CA inhibition (in vivo) has no effect on HCO₃⁻ excretion or urinary pH (23) and an extracellular CA inhibitor, F3500, has no effect on net SO₄²⁻ secretion in fPTCs (36). CAII protein was shown to be present in concentrated fPTC plasma membranes and migrated identically to purified bovine CAII (29 kDa) on SDS-PAGE. Although CAII appears to be present at the plasma membrane, it remains to be determined if CAIV, CAXII, and/or CAXIV are present in renal proximal tubule epithelial cells of marine teleosts.

The question that remains, however, is why CAII occurs at the plasma membrane when it is supposedly a soluble isoform? One possible explanation for membrane-associated CAII is that, due to nonspecific binding, soluble CAII had contaminated the plasma membranes during their preparation. Wistrand and Kinne (59), however, showed that when exogenous CAII was added to renal homogenates, the additional enzyme was readily washed out, suggesting that CA activity in the plasma membranes was not due to contamination with soluble CAII. When rat red blood cell membranes and fPTC plasma membranes were run on nondenaturing PAGE gels, CAII migrated differently than purified CAII, raising the possibility that CAII is physically associated with an integral membrane protein. AE1 (band 3) has been shown to physically associate with CAII in the mammalian red blood cell (56) and is likely the reason why CAII from rat red blood cell membranes migrated differently than purified CAII on nondenaturing PAGE gels (see Fig. 2C). Both the Na⁺:HCO₃⁻ transporter (NBC1; 18) and the Na⁺/H⁺ exchanger (NHE1; 27) have also now been shown to reversibly associate with CAII.

The marine teleost renal proximal tubule is characterized by a pronounced SO₄²⁻ secretory process that is partially inhibited (>50%) by concentrations of methazolamide and ethoxzolamide (10–100 µM) that inhibit 100% of CA enzymatic activity (36). Similarly, in the present study, methazolamide treatment (100 µM) of fPTCs resulted in a 58% reduction in SO₄²⁻ secretion. Presumably, CA inhibition reduces the dehydroxylation rate of HCO₃⁻ (HCO₃⁻ → CO₂ + OH⁻) thereby reducing the driving force for apical SO₄²⁻/HCO₃⁻ and(or) basolateral SO₄²⁻/OH⁻ exchange. An explanation for only partial inhibition of renal tubular SO₄²⁻ secretion may include the fact that the slower, uncatalyzed reaction can contribute and, although luminal HCO₃⁻ serves as the most efficient substrate, luminal Cl⁻ can also serve as a counteranion for the apical SO₄²⁻/HCO₃⁻ (Cl⁻) exchanger (38). In the latter event, Na⁺–K⁺-ATPase and Na⁺/H⁺ exchanger activity are a likely source of...
the OH\(^-\) gradient necessary for basolateral \(\text{SO}_4^{2-}/\text{OH}^-\) exchange and the movement of \(\text{SO}_4^{2-}\) into the cell across the basolateral membrane (36).

As noted above, cortisol has been shown to stimulate CA activity in the mammalian brain (13) and red blood cell (39). Reducing cortisol to low physiological levels reduced CA activity 28% and CAII protein abundance 65% in fPTC cell lysates. The difference in cortisol effect on CA activity and CAII protein levels (see Fig. 3) is likely explained by the fact that enzymatic activity is not a linear function of CAII abundance and total CA activity is due to a CAII-like isoform and possibly other CA isoforms as well. There may also be a nonlinear relationship between cytosolic CA abundance and membrane-associated CA. The latter may have a higher molar activity. The DADD peptide sequence in AE1, for example, strongly activates human CAII (44). Thus a reduction in soluble CAII protein may have less effect on total CA activity than a reduction in membrane-associated CAII. Concurrent with the reductions in CA activity and CAII protein abundance was a 28% inhibition of this enzyme and the physiological effect. He points out that, based on CA biochemical assays, inhibitor concentrations sufficient to produce fractional enzyme inhibition of 0.992 have no apparent physiological effect on several standard functional parameters of kidney, eye, pancreas, and stomach. However, at inhibitor concentrations sufficient to cause fractional inhibition of 0.9976, renal \(\text{HCO}_3^-\) excretion increases and intraocular pressure decreases. That is, there appears to be a 500-fold excess of the enzyme in vivo because less than 1% of the enzyme is enough to maintain normal function in the majority of tissues. If CA is in such excess, why should a 30–50% change in total CA activity cause physiologically important changes in \(\text{HCO}_3^-\) and \(\text{SO}_4^{2-}\) transport, as seems to occur at the tubule level, for example, during chronic metabolic acidosis in rabbit kidney (7, 53) and following exposure of renal proximal tubule epithelium to low cortisol (present study)?

In mammalian red blood cells, the NH\(_2\) terminus of CAII contains five histidine residues that form an electrostatic association with three aspartate residues (LDADD motif) in the intracellular COOH terminus of the Cl\(^-\)/\(\text{HCO}_3^-\) exchanger AE1 forming a transport “metabolon” (55, 56). The function of a transport metabolon is likely one of proximity, where the production of substrate occurs near the substrate’s transport site, or reciprocally, there is more rapid removal of substrate from the transport site, thereby preventing competitive substrate buildup in an unstirred layer. This may be particularly important for exchangers where there is little asymmetry in the intra- and extracellular binding sites. Furthermore, although the mechanism is not known, CAII enzymatic activity is enhanced by synthesized peptides mimicking the COOH terminus of AE1 (44). Formation of such metabolons allows maximal transport activity and may provide a regulatory site (18, 47). In addition to AE1, Sterling et al. (47) showed that many other \(\text{HCO}_3^-\) transporters, including those found in the kidney, contain potential CAII-binding sites at their COOH termini. In the present study, CAII from fPTC plasma membranes migrated differently than purified bovine CAII on nondenaturing PAGE gels, which suggests that CAII associates with an integral membrane protein in renal proximal tubule cells. Although we did not clone the NH\(_2\) terminus of winter flounder CAII, CAII from other fish species contains histidine residues that match the five histidine residue binding sites for \(\text{HCO}_3^-\) transporters.

Because the most likely model of \(\text{SO}_4^{2-}\) secretion in the marine teleost involves two anion exchangers in series (brush-border membrane, \(\text{SO}_4^{2-}/\text{HCO}_3^-\); basolateral membrane, \(\text{SO}_4^{2-}/\text{OH}^-\)), one could reasonably posit roles for a CAII metabolon at both poles of the cells. It is unlikely that CAII at the apical membrane would enhance exchange through dissipation of \(\text{HCO}_3^-\)

Perspectives

In his classical review of the physiology of CA, Maren (29) explores the relationship between fractional inhibition of this enzyme and the physiological effect. He points out that, based on CA biochemical assays, inhibitor concentrations sufficient to produce fractional enzyme inhibition of 0.992 have no apparent physiological effect on several standard functional parameters of kidney, eye, pancreas, and stomach. However, at inhibitor concentrations sufficient to cause fractional inhibition of 0.9976, renal \(\text{HCO}_3^-\) excretion increases and intraocular pressure decreases. That is, there appears to be a 500-fold excess of the enzyme in vivo because less than 1% of the enzyme is enough to maintain normal function in the majority of tissues. If CA is in such excess, why should a 30–50% change in total CA activity cause physiologically important changes in \(\text{HCO}_3^-\) and \(\text{SO}_4^{2-}\) transport, as seems to occur at the tubule level, for example, during chronic metabolic acidosis in rabbit kidney (7, 53) and following exposure of renal proximal tubule epithelium to low cortisol (present study)?

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following exchange for intracellular SO\textsubscript{4}\textsuperscript{2-} because at physiological intracellular pH, the ratio of HCO\textsubscript{3} to CO\textsubscript{2} is \textasciitilde 20:1 (14). However, it is entirely possible that CO\textsubscript{3}\textsuperscript{2-}, rather than HCO\textsubscript{3}, is being transported on the apical SO\textsubscript{4}\textsuperscript{2-} /anion exchanger (SO\textsubscript{4}\textsuperscript{2-} /CO\textsubscript{3}\textsuperscript{2-}). The mammalian Na\textsuperscript{+}/HCO\textsubscript{3} cotransporters can be electrogenic including a stoichiometry of 1 Na\textsuperscript{+}:1 CO\textsubscript{3}\textsuperscript{2-}:1 HCO\textsubscript{3} (46). In fPTCs, a CAII-SO\textsubscript{4}\textsuperscript{2-} /CO\textsubscript{3}\textsuperscript{2-} association would be stimulatory because CAII could rapidly supply the H\textsuperscript{+} necessary for the conversion of CO\textsubscript{3}\textsuperscript{2-} to HCO\textsubscript{3}, thereby preventing an increase in local [CO\textsubscript{3}\textsuperscript{2-}] near the transporter. An association of CAII with the basolateral SO\textsubscript{4}\textsuperscript{2-} /OH\textsuperscript{-} exchanger could also potentially stimulate transport by localized dehydroxylation of HCO\textsubscript{3} (HCO\textsubscript{3} \rightarrow CO\textsubscript{2} + OH\textsuperscript{-}) near the OH\textsuperscript{-} transport site. The majority of CA activity (85%) and CAII protein (see Fig. 2) were found in the cytosol. CAII has been shown to accelerate the rate of CO\textsubscript{2} diffusion (14, 48), and therefore, cytosolic CAII is likely a “facilitation factor” in substrate diffusion. Future work will be directed at identifying the renal proximal tubule SO\textsubscript{4}\textsuperscript{2-} transporters at the molecular level (SLC26 gene family members, e.g., Sat-1, DTDST, DRA, etc.), determining if CAII associates with these SO\textsubscript{4}\textsuperscript{2-} transporter(s), and determining whether this association stimulates SO\textsubscript{4}\textsuperscript{2-} transport and/or CAII activity.

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DISCLOSURES

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