Hormonal and environmental regulation of epithelial calcium channel in gill of rainbow trout (Oncorhynchus mykiss)

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Shahsavarani, Arash, and Steve F. Perry. Hormonal and environmental regulation of epithelial calcium channel in gill of rainbow trout (Oncorhynchus mykiss). Am J Physiol Regul Integr Comp Physiol 291: R1490–R1498, 2006. First published June 8, 2006; doi:10.1152/ajpregu.00026.2006.—We indirectly tested the idea that the epithelial Ca^{2+} channel (ECaC) of the trout gill is regulated in an appropriate manner to adjust rates of Ca^{2+} uptake. This was accomplished by assessing the levels of gill ECaC mRNA and protein in fish exposed to treatments known to increase or decrease Ca^{2+} uptake capacity. Exposure of trout to soft water ([Ca^{2+}] = 20–30 nmol/l) for 5 days (a treatment known to increase Ca^{2+} uptake capacity) caused a significant increase in ECaC mRNA levels and an increase in ECaC protein expression. The inducement of hypercalcemia by infusing fish with CaCl_2 (a treatment known to reduce Ca^{2+} uptake) was associated with a significant decrease in ECaC mRNA levels, yet protein levels were unaltered. ECaC mRNA and protein expression were increased in fish treated with the hypercalcemic hormone cortisol. Finally, exposure of trout to 48 h of hypercapnia (~7.5 mmHg, a treatment known to increase Ca^{2+} uptake capacity) elicited an ~100-fold increase in the levels of ECaC mRNA and a significant increase in protein expression. Immunocytochemical analysis of the gills from hypercapnic fish suggested a marked increase in the apical expression of ECaC on pavement cells and a subpopulation of mitochondria-rich cells. The results of this study provide evidence that Ca^{2+} uptake entry through ECaC were indeed an important regulatory step in overall transepithelial Ca^{2+} flux, one would expect predictable changes in the levels of ECaC mRNA and protein in response to physiological cues known to modify branchial Ca^{2+} uptake rates. Thus, in the present study, it was predicted that conditions known to increase Ca^{2+} transport capacity, such as low environmental Ca^{2+} levels (32), cortisol treatment (6), or hypercapnia (21) would be associated with increased ECaC expression. Hypercalcemia, a condition known to reduce branchial Ca^{2+} uptake (19, 30), on the other hand, would be expected to reduce ECaC expression. ECaC mRNA expression was monitored using real-time PCR, whereas protein levels were assessed using Western blot analysis and immunocytochemistry with a homologous polyclonal antibody.

MATERIALS AND METHODS

Animal care. Rainbow trout (O. mykiss) of both sexes were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). The fish were held at the University of Ottawa in large fiberglass tanks supplied with flowing, aerated, and dechlorinated city water, maintained at 13°C on a 12:12-h light-dark photoperiod, and fed daily with a commercial trout diet. All procedures involving animals were carried out according to institutional guidelines, which are in accordance with those of the Canadian Council on Animal Care. The protocols used in this study were approved by the University of

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Ottawa Animal Care Committee in accordance with guidelines provided by the Canadian Council on Animal Care.

Effect of environmental Ca\(^{2+}\). Three aquaria (30 cm high \times 30 cm wide \times 61 cm long, 54-liter volume) were used with water temperature maintained at 13°C by using a recirculating water bath and a stainless-steel cooling coil. All sides of the aquaria were blackened to minimize visual stress. Three environmental Ca\(^{2+}\) levels were established (low = 20–30 nmol/l, normal = 200–300 nmol/l, high = 2–2.5 mmol/l). Ca\(^{2+}\) concentrations were adjusted using calcium nitrate as needed. Six fish [mean mass for all 18 fish: 97.4 ± 10.9 (SE) g] were placed into each environment for a period of 5 days. Water chemistry was monitored on a daily basis to ensure that Ca\(^{2+}\) levels were maintained within the target range. After the 5-day exposure, the animals were euthanized by a sharp blow to the head. The gill basket was quickly removed and gill filaments were isolated. Samples were immediately placed into liquid N\(_2\) and stored at −86°C until use. Tissue samples were also preserved for microscopy using standard procedures (see below). Water [Ca\(^{2+}\)] was determined using flame emission spectrophotometry (model Spectra AA 250 Plus; Varian).

Effect of Ca\(^{2+}\) infusion. Benzocaine (ethyl-p-amino benzole; 2.4 \times 10\(^{-4}\) mol/l) was used to anesthetize each fish (weighing between 180 and 260 g). The fish were then placed on a surgical table, where the gills were irrigated continuously with anesthetic solution. The dorsal aorta was cannulated (37) using a polyethylene cannula (PE-50; Clay-Adams). For recovery, fish were placed into individual opaque acrylic boxes provided with continuous flow of aerated fresh water (13°C) for 24 h before the commencement of the experiments.

After the recovery period, each fish was infused via the dorsal aorta cannula for a period of 24 h with a syringe pump (model 355; Sage Instruments). Fish were infused with either saline (140 mmol/l NaCl, pH 7.8) or Ca\(^{2+}\)-enriched saline (0.01 mol/l CaCl\(_2\)) at a rate of 1.0 ml/h for 24 h. At the conclusion of the experimental period, blood samples were collected for plasma Ca\(^{2+}\) measurements. Blood samples were immediately centrifuged (12,000 g for 1 min), and the plasma was collected and stored at −20°C until analysis. Plasma [Ca\(^{2+}\)] was determined using flame emission spectrophotometry (model Spectra AA 250 Plus; Varian). Each animal was euthanized by a sharp blow to the head, and tissue samples were collected as previously described.

Effect of increased plasma cortisol levels. Fish (60–207 g) were anesthetized and implanted with 5 ml/kg of either cocoa butter (sham) or cocoa butter containing cortisol (22 mg of hydrocortisone 21-hemisuccinate per ml of cocoa butter). The fish were returned to holding tanks, where they were kept for 5 days without disturbance. After the experimental period, fish were quickly collected, euthanized, and sampled as previously described. Blood samples were also collected for cortisol measurements. These samples were centrifuged (12,000 g for 1 min), and the plasma was collected and stored at −80°C until analysis with a commercial RIA kit (ICN Pharmaceuticals).

Effect of 48-h hypercapnia. Fish (205–327 g) were placed into individual opaque acrylic boxes with continuous flow of aerated fresh water (13°C) for 24 h before the start of the experiment. After this acclimation period, one group of fish was exposed to flowing water containing 1% CO\(_2\) in air (hypercapnic water PCO\(_2\) = 7.5 mmHg), while the other group was exposed to normally aerated water for 48 h. The desired level of hypercapnia was achieved by gassing a water equilibration column with appropriate mixtures of CO\(_2\) in air (Camerongas mixer). The PCO\(_2\) of the water exiting the column was continuously measured using a PCO\(_2\) electrode (Camerong Instruments) connected to a blood gas meter (Camerong Instruments). The PCO\(_2\) electrode was calibrated using solutions of water (13°C) equilibrated with mixtures of 0.5 or 1.0% CO\(_2\), achieved using the Camerongas mixer. The final PCO\(_2\) of the water exiting the column was controlled by adjusting the flows of gas and water and the percentage of CO\(_2\) gassing the column. At the conclusion of the exposure period, fish were euthanized and samples were collected as previously described.

Real-time PCR analysis. Tissue samples were removed under liquid N\(_2\) using a mortar and pestle. Tissue total RNA was extracted from 30 mg of tissue using Invitrogen TRIzol reagent. All procedures were followed as per the manufacturer’s instructions with the following modifications. No more than 30 mg of powdered tissue was used per 1 ml of TRIzol, and after the resuspension of the total RNA in 100 μl of nuclease-free water, the RNA was reextracted using 1 ml of TRIzol by repeating the entire procedure. The RNA was then resuspended in 30 μl of nuclease-free water.

Reverse transcription was performed using the Stratascript reverse transcriptase kit (Stratagene). Complementary DNA was synthesized as per the kit manufacturer’s instructions with the following changes. Final reaction volume was adjusted to 12.5 μl, and 0.5 μg of total RNA was used with 0.15 μg of random hexamer primers. Real-time PCR was performed using a MX 4000 Multiplex quantitative PCR system (Stratagene) with Brilliant SYBR Green QPCR master mix (Stratagene) as per the manufacturer’s instructions with the following modifications. The total reaction volume was reduced to 25 μl; 0.5 μl of cDNA template was used, and primer concentrations were 0.150 nmol/l for each primer. All primers (see below) were designed and optimized for the following PCR reaction conditions: 15 min at 95°C, 45 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. At the end of each run, a dissociation curve was established to determine the purity of the amplicons in each reaction. Those samples exhibiting more than one dissociation peak (indicative of multiple products) were eliminated. Control samples (diluted RNA samples) were examined at random to test for the presence of genomic DNA contamination. Primers for real-time PCR were as follows: ECaC-QPCR1 forward, 5′-GGACCCCTTCATGTCACTTTAAT-3′; ECaC-QPCR2 reverse, 5′-ACAGCCATGACAACTTCC-3′; β-actin forward, 5′-CACACAGATGTGGATCGACAA-3′; β-actin reverse, 5′-GGTGCCACAGCTGCTAAGGT-3′.

Tissue preservation and immunocytochemistry. Gill filaments were quickly removed from freshly dissected gill arches collected after an experiment. The filaments were then placed in ice-cold 4% paraformaldehyde (pH 7.4) and kept at 4°C overnight. The filaments were then transferred to phosphate-buffered saline (PBS) containing 15% sucrose for 2 h at 4°C and, finally, transferred to PBS containing 30% sucrose for at least 2 h before sectioning. Tissue samples were embedded in Shandon Cryomatrix embedding medium (Fisher), and thin sections (10 μm) were prepared using a Leica CM 1850 cryostat at −18°C. Sections were placed on SuperFrost\(^++\) (Fisher Scientific) microscope slides, air-dried for 10 min, and stored at −20°C until use.

Sections were incubated in situ (3 × 5 min) with a blocking buffer containing 2% normal goat serum, 0.1 ml/l PBS, 0.9% Triton-X, 1% gelatin, and 2% BSA. They were then incubated for 2 h at room temperature, in a humidified chamber, with one of two primary antibodies diluted in the blocking buffer: α5, a mouse monoclonal antibody against the α1-subunit of chicken Na\(^{+}\)/K\(^{−}\)-ATPase (1:100; University of Iowa Hybridoma Bank) or trout ECaC (1:200). For ECaC, custom polyclonal antibodies were raised in rabbit (Abgent, San Diego, CA) against an 18-amino acid region (SQFRFRLQN-RKGWKEMLD) of rainbow trout ECaC protein. This region corresponded to amino acids 18 through 36 (see Ref. 35). For negative controls, sections were incubated with blocking buffer lacking primary antibodies, with preimmune serum (ECaC), or with antibodies preabsorbed with excess peptide antigen (ECaC).

The α5 antibody has been used in numerous previous studies to localize Na\(^{+}\)/K\(^{−}\)-ATPase in fish tissues (e.g., Ref. 43). The slides were then washed (3 × 5 min) in 0.1 ml/l PBS. For double-immunofluorescence staining, the trout anti-rabbit ECaC was detected with a 1:400 dilution of Alexa 488-coupled goat anti-rabbit IgG (Fisher), and α5 was detected with a 1:400 dilution of Alexa 546-coupled goat anti-mouse IgG (Fisher). Slides were incubated in a humid chamber for 1 h at room temperature. The slides were then washed (3 × 5 min) in 0.1 ml/l PBS and
mounted with a mounting medium (Vector Laboratories) containing 4',6'-diamidino-2-phenylindole (DAPI) to stain nuclei.

Specimens were observed and photographed using a Zeiss Axiophot light microscope and a Hamamatsu C5985 chilled charge-coupled device camera. Images were captured using the Metamorph v4.01 imaging system.

**Western blot analysis.** Proteins were prepared from frozen tissues by homogenization on ice in 1 ml of extraction buffer containing 50 mM Tris·HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS, and protease inhibitor cocktail (Roche). The samples were incubated on ice for 10 min and briefly sonicated to break up any DNA that might have been extracted. The samples were centrifuged at 14,000 g for 10 min at 4°C, and the supernatants were stored at −80°C before use. Protein concentrations were determined using a microbichinonic acid protein assay (Pierce) with BSA as standard. Samples (50 μg of protein) were size-fractionated by reducing SDS-PAGE using 7% separating and 5% stacking polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). After transfer, each membrane was blocked for 1 h in PBST (1× PBS, 0.1% Tween 20)-5% milk and probed with a dilution of 1:3,000 rabbit anti-trout ECaC overnight at 4°C. The membranes were then probed for 1 h at room temperature with 1:4,000 goat anti-rabbit antibody (Pierce). After each exposure to antibody, the membranes were washed 3 × 5 min in PBST. The specific bands were detected using enhanced chemiluminescence (ECL; SuperSignal West Pico chemiluminescent substrate; Pierce), and blots were exposed to Kodak X-Omat, blue XB-1 film (Fisher). The protein size marker used was obtained from Fermentas Life Sciences. To demonstrate specificity of the trout ECaC antibody, we combined primary antiserum with excess (20 μg) of the peptide against which the antibody was raised. Additional negative controls included incubating blots with blocking buffer lacking antibodies or with preimmune serum.

To assess for equal loading, we stripped blots with Re-Blot Plus mild stripping solution (Chemicon). The blot was incubated in 1× stripping solution for 20 min at room temperature and then rinsed for 10 min in PBST. After rinsing, the blot was then blocked twice in 5% PBST-milk for 10 min each. The blot was then probed with an anti-β-tubulin antibody (1:1,000; Sigma-Aldrich Canada) for 1 h at 37°C. The blot was then incubated in anti-mouse Ig, horseradish peroxidase (1:5,000) for 1 h at room temperature. After additional washings, the proteins were visualized using ECL as described above.

The density of the antigenic bands was determined by scanning the films and then analyzing the digital images using commercial software (Quantity One v4.1.1). The results are presented as the ratio of ECaC to tubulin band density.

**Statistical analysis.** Statistical analysis was performed using SigmaStat (version 2.03; SPSS, Chicago, IL). One-way analysis of variance was used to determine the effect of environmental Ca\(^{2+}\) and cortisol implant on ECaC expression. In all other experiments, the Student’s t-test was used; significance was set at P < 0.05.

**RESULTS**

**Localization of ECaC in the gill.** Figure 1 depicts the typical pattern of ECaC expression in the trout gill epithelium that was obtained throughout the course of this study. ECaC was localized to the apical membranes of lamellar PVCs, as well as to cells expressing Na\(^+/K^+\)-ATPase (presumed to be MRCs). Interestingly, not all of the cells expressing Na\(^+/K^+\)-ATPase coexpressed ECaC (Fig. 1A). Preabsorption of the primary antibody with excess peptide antigen eliminated nearly all fluorescence signal (Fig. 1B). On a Western blot (Fig. 1C), the ECaC antibody recognized a single immunoreactive band at 90 kDa; the band was not observed after preabsorption with peptide antigen.

**Effect of environmental Ca\(^{2+}\).** Real-time PCR analysis of rainbow trout gill tissue from fish exposed to low (20–40 nmol/l), normal (200–300 nmol/l), or high (2–2.5 mmol/l) environmental Ca\(^{2+}\) levels revealed a significant 10-fold in-
crease in ECaC mRNA expression in fish exposed to low Ca\(^{2+}\) compared with the control fish (Fig. 2A). ECaC protein levels also were significantly increased in the fish exposed to low levels of Ca\(^{2+}\) but unaltered in the animals kept in high-Ca\(^{2+}\) water (Fig. 2A). Immunocytochemical analysis of gill sections indicated increased amounts of ECaC protein at the tips of the lamellae with an apparent increase in Na\(^{+}/K^{+}\)-ATPase-rich cell population at the base of the lamellae (Fig. 2B). Although not quantified, there also appeared to be a reduction in the intensity and extent of ECaC fluorescence and in the numbers of Na\(^{+}/K^{+}\)-ATPase-enriched cells in the fish kept in high-Ca\(^{2+}\) water (Fig. 2D).

**Effect of intravascular Ca\(^{2+}\) infusion.** Fish infused with CaCl\(_2\) for 24 h exhibited a 2.5-fold increase in plasma Ca\(^{2+}\) levels (from 3.31 ± 0.24 to 8.11 ± 0.74 mmol/l). Gene expression analysis revealed a significant decrease in ECaC...
mRNA expression after 24 h of infusion, yet protein levels were unchanged (Fig. 3).

**Effect of cortisol treatment.** Fish treated with cortisol implants exhibited an ~50-fold increase in plasma cortisol levels compared with the control (untreated) fish (116.2 ± 32.2 vs. 2.3 ± 0.9 ng/ml). The sham-treated fish (cocoa butter only) also demonstrated high plasma cortisol levels (94.4 ± 19.0 ng/ml), and thus the data from these fish were not included in the analysis. The fish given cortisol implants displayed significant threefold increases in relative ECaC mRNA expression and protein levels in relation to the control fish (Fig. 4A). Qualitatively, the gills of cortisol-treated fish possessed greater numbers of Na⁺/K⁺-ATPase-rich cells, especially on lamellae; the majority of these cells appeared to express apical ECaC (Fig. 4B).

**Effect of hypercapnia.** Exposure of fish to hypercapnia (~7.5 mmHg) for a period of 48 h significantly increased ECaC mRNA expression ~100-fold (Fig. 5A), whereas ECaC protein levels were increased only 2.1-fold. Immunocytochemical analysis of gill sections revealed a striking increase in the intensity of apical ECaC expression after hypercapnia on both Na⁺/K⁺-enriched MRCs and lamellar PVCs (Fig. 6, B and C).

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**Fig. 3.** Effects of 24 h of intra-arterial infusion of CaCl₂ on ECaC mRNA and protein expression in rainbow trout gills. Results for mRNA (n = 4 for each treatment) are presented relative to β-actin expression within the same sample. Densitometry results for protein (n = 4 for each treatment) were normalized to the reference protein, β-tubulin. All data are expressed as means ± SE. **P < 0.05, significant difference from the control (infused with 140 mmol/l NaCl) group (Student’s t-test). NS, not significant.**

**Fig. 4.** Effect of increased plasma cortisol concentrations on ECaC mRNA and protein expression or distribution of ECaC and Na⁺/K⁺-ATPase immunoreactivity in rainbow trout gills. Animals were implanted for 5 days with cocoa butter containing cortisol and compared with fish containing no implant. A: results for mRNA (n = 7 for each treatment) are presented relative to β-actin expression within the same sample. Densitometry results for protein (n = 7 for each treatment) were normalized to the reference protein, β-tubulin. All data are expressed as means ± SE. **P < 0.05, significant difference from the control group (Student’s t-test). B: colocalization of ECaC, Na⁺/K⁺-ATPase, and nuclei in fish with no cortisol implant. C: colocalization of ECaC, Na⁺/K⁺-ATPase, and nuclei in fish after 5 days of treatment with cortisol implant. The cell nuclei appear as blue, ECaC appears as green, and Na⁺/K⁺-ATPase appears as red. Yellow represents colocalization of ECaC and Na⁺/K⁺-ATPase. All photos were taken with the same exposure times and adjusted identically for contrast and brightness.
DISCUSSION

The goal of this study was to provide indirect evidence that ECaC is regulated in an appropriate manner to adjust rates of Ca\(^{2+}\) uptake. This was accomplished by assessing the levels of gill ECaC mRNA with real-time PCR and ECaC protein with Western blots and immunocytochemistry in fish exposed to treatments known to increase or decrease Ca\(^{2+}\)-transporting capacity.

The results of real-time PCR analyses clearly demonstrated that the levels of ECaC mRNA varied in direct relation to the Ca\(^{2+}\)-transporting capacity of the gill, increasing in fish exposed to low environmental Ca\(^{2+}\) level, elevated plasma cortisol concentration, or hypercapnia and decreasing in fish experiencing experimentally induced hypercalcemia. Except for the fish experiencing hypercalcemia, there was a matching change in ECaC protein levels associated with the mRNA changes. The changes in protein levels were not always of the same magnitude as the mRNA changes. The results of the immunocytochemistry, although not quantitative, suggest that the altered levels of ECaC detected in this study reflected modifications of ECaC protein within both chloride cells and PVCs.

Low environmental Ca\(^{2+}\) levels. After exposure of trout to low environmental Ca\(^{2+}\) concentrations for 5 days, ECaC gene expression increased ~10-fold, whereas protein levels were increased 2.5-fold. Adult zebrafish, exposed to similar experimental conditions, exhibited a similar response in ECaC gene expression (Shahsavarani A and Perry SF, unpublished data). In addition, Pan et al. (25) recently demonstrated that exposure of zebrafish embryos to water containing low levels of Ca\(^{2+}\) (0.02 mmol/l) caused an increase in ECaC mRNA expression in gills and skin. Thus ECaC clearly is being affected (directly or indirectly) by environmental Ca\(^{2+}\) levels, and this suggests that the increased capacity of the trout gill (32) or zebrafish embryo (25) to absorb Ca\(^{2+}\) after exposure to soft water may indeed reflect increased numbers of apical membrane Ca\(^{2+}\) channels.

The results of previous studies (23, 24) provided evidence that gill MRCs are involved in Ca\(^{2+}\) uptake. On the basis of these and other indirect or correlative studies (18, 26, 28, 32), a model was constructed (27) in which the MRC was implicated as the principal (potentially exclusive) cell type responsible for Ca\(^{2+}\) uptake. In the present study, however, immunocytochemical analysis of gill cross sections in fish exposed...
to soft water failed to demonstrate strong colocalization of Na\(^+\)/K\(^+\)-ATPase (representative of PVCs) and ECaC-positive cells (Fig. 2). Under control conditions, ECaC appeared to be widely distributed to both PVCs and a subset of MRCs. A similar conclusion was reached by Shahsavarani et al. (35) upon examination of both gill sections and cultured cells. During exposure to soft water, there appeared to be a shift in the cell types expressing ECaC. Specifically, ECaC appeared to be highly expressed in a subpopulation of enlarged cells located at the tips of lamellae (Fig. 2B). This regional increase in ECaC expression in fish exposed to a low Ca\(^{2+}\) environment suggests the presence of a previously unidentified cell type that may be playing a significant role in Ca\(^{2+}\) uptake.

Exposure of trout to the varied levels of environmental Ca\(^{2+}\) used in the present study could potentially lead to transient or longer-term alterations in plasma Ca\(^{2+}\) levels. Therefore, the changes in ECaC mRNA expression observed in this study could reflect sensing of ambient and/or internal Ca\(^{2+}\). The presence of a Ca\(^{2+}\)-sensing receptor (CaR) has been demonstrated in the trout gill (34), and thus it is conceivable that CaR is involved in the sensing of low ambient Ca\(^{2+}\) levels and the initiation of events leading to increased transcription of ECaC. Furthermore, it is well established that increased levels of internal Ca\(^{2+}\) can be sensed and can lead to downstream effects to reduce branchial Ca\(^{2+}\) uptake (19). Clearly, further work is required to elucidate the relative importance of external versus internal changes in Ca\(^{2+}\) in promoting transcriptional changes to ECaC expression.

Various studies have clearly demonstrated an increase in MRC surface area (through cellular enlargement, as well as cellular proliferation) with exposure to soft water (14, 32). Similar results were noted in the present study (compare Fig. 2, B and C). The more important observation, however, was that the MRCs in the soft water fish did not display any obvious increase in ECaC expression.

Although the levels of ECaC mRNA and protein were increased in soft water fish, there was no reduction in ECaC mRNA or protein associated with exposing fish to Ca\(^{2+}\)-enriched water. It has been established previously (32) that maximal Ca\(^{2+}\) transport capacity (J\(_{\text{max}}\)) is reduced in trout exposed to high ambient Ca\(^{2+}\). Thus the absence of any regulation of ECaC mRNA (and presumably protein) in these fish suggests that the reduced Ca\(^{2+}\) uptake reflects nontranscriptional control of ECaC or modulation of another component of the overall transepithelial Ca\(^{2+}\) absorption process [basolateral plasma membrane Ca\(^{2+}\) ATPase (PMCA)] or Na\(^+/\)Ca\(^{2+}\) exchange.

**Hypercapnia.** Infusing fish with Ca\(^{2+}\)-enriched saline for 24 h resulted in a marked elevation of plasma Ca\(^{2+}\) levels and a concomitant reduction in ECaC mRNA levels. Because ambient Ca\(^{2+}\) concentration was unchanged, the results provide strong evidence for an internal Ca\(^{2+}\)-sensing mechanism linked to transcriptional control of ECaC. This mechanism may be similar to the one proposed to initiate the release of the hypocalcemic hormone stanniocalcin (STC), in which the CaR is thought to be involved (34). The release of STC from the corporules of Stannius during acute hypercalcemia is a critical mechanism leading to rapid reductions in the rate of branchial Ca\(^{2+}\) uptake (5, 19, 30, 42) thought to involve modification of Ca\(^{2+}\) conductance through existing Ca\(^{2+}\) channels. The results of the present study demonstrate that chronic hypercalcemia may lower Ca\(^{2+}\) uptake by an additional mechanism, a reduction in the number of ECaCs. However, because the reduced levels of ECaC mRNA were not accompanied by a reduction in ECaC protein levels, it is possible that a longer period of hypercalcemia is required for transcriptional changes to significantly impact protein levels.

**Elevated plasma cortisol levels.** Plasma cortisol levels have been shown to increase in fish exposed to soft water (6, 29, 32, 36). Because of the hypercalcemic action of cortisol (6, 32), this response presumably helps to maintain Ca\(^{2+}\) homeostasis in soft water environments. The hypercalcemic actions of cortisol have been attributed to increased branchial Ca\(^{2+}\) uptake associated with MRC proliferation (20) and increased activity of the basolateral PMCA (6). In this study, we have presented evidence for an additional mechanism underlying the hypercalcemic effects of cortisol that involves a transcriptional increase in ECaC. The increased levels of ECaC appeared to be a direct result of MRC proliferation. Thus, although the apparent expression of ECaC per MRC did not change, the number of MRCs expressing ECaC was increased by cortisol treatment. It is possible, therefore, that the increased rates of Ca\(^{2+}\) uptake observed (6) after cortisol treatment in vivo reflect the combined effects of increased PMCA activity and a greater number of apical membrane Ca\(^{2+}\) channels.

**Hypercapnia.** Exposure of trout or bullhead (Ictalurus nebulosus) to hypercapnia has been shown to cause a remodeling of the gill epithelium, whereby the surface area of MRCs exposed to the water is markedly reduced due to their apparent covering by neighboring PVCs (10–13). Assuming that the MRCs are the predominant sites of Ca\(^{2+}\) uptake, one would expect hypercapnia exposure to cause a reduction in the rate of Ca\(^{2+}\) uptake, because MRC apical surface area is being reduced. Indeed, this prediction was tested by MacKenzie and Perry (21), who exposed rainbow trout to hypercapnia while monitoring branchial and renal Ca\(^{2+}\) fluxes. Despite a 68% reduction in the surface area of exposed MRCs, the hypercapnic trout in that study (21) actually exhibited a significant increase in the rate of Ca\(^{2+}\) uptake due to an increase in J\(_{\text{max}}\). The increase in J\(_{\text{max}}\) for Ca\(^{2+}\) uptake was not associated with any changes PMCA, because the ATP-dependent Ca\(^{2+}\)-transporting capacity of basolateral membrane vesicles was unaffected by hypercapnia. Instead, it was suggested that the increased rates of Ca\(^{2+}\) uptake in hypercapnic fish might reflect modification of apical membrane Ca\(^{2+}\) channels. The results of the present study provide compelling evidence that the mechanism underlying the increased rates of Ca\(^{2+}\) uptake observed by MacKenzie and Perry (21) in hypercapnic trout is transcriptional upregulation of branchial ECaC. It is possible that the number of Ca\(^{2+}\) channels on MRC apical membranes is being increased to enhance the transporting capacities of cells still exposed to the water. Alternatively, the numbers of channels may be increasing on PVCs to compensate for the loss of exposed MRC surface area. Given the widespread cellular localization of ECaC (e.g., Fig. 1) and the likelihood that both MRCs and PVCs are involved in Ca\(^{2+}\) uptake (35), it is probable that ECaC expression is being increased in both cell types during hypercapnia. Although it is not possible to distinguish between exposed and covered MRCs from light micrographs, the results of the immunocytochemistry suggest that ECaC expression is increasing in a subset of both MRCs and PVCs.
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Perspectives

The results of this study provide indirect evidence that the passage of Ca\(^{2+}\) through the gill ECaC is a regulated step controlling the overall flux of Ca\(^{2+}\) across the gill of rainbow trout and, presumably, other teleost species. Thus the Ca\(^{2+}\)-transport capacity of the gill can be altered, based on need, by the transcriptional adjustment of ECaC protein levels. The results also suggest that ECaC is expressed in a variety of gill epithelial cells (see also Ref. 35) and not restricted to MRCs as previous models had suggested. This observation is in apparent conflict with the widely held view that the MRC is the exclusive site of branchial Ca\(^{2+}\) uptake in fish. However, because transeellular Ca\(^{2+}\) uptake is assured by the combined actions of Ca\(^{2+}\)-entry across the apical membrane and its exit across basolateral membranes, it is possible that only the MRCs possess ample machinery for the exit step. Clearly, there is a need to reevaluate the roles of the PVCs and MRCs in Ca\(^{2+}\) uptake.

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REFERENCES


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