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The Roles of Cytosolic and Membrane-Bound Carbonic Anhydrase in Acid-Base Regulation in Rainbow Trout, *Oncorhynchus mykiss*

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THE ROLES OF CYTOSOLIC AND MEMBRANE-BOUND CARBONIC ANHYDRASE IN ACID-BASE REGULATION IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

By

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THE ROLES OF CYTOSOLIC AND MEMBRANE-BOUND CARBONIC AHNYDRASE IN ACID-BASE REGULATION IN RAINBOW TROUT, ONCORHYNCHUS MYKISS

Abstract

Carbonic anhydrase (CA) is an important enzyme that catalyzes the reversible hydration/dehydration reactions of CO_2. In mammals, there are at least 15 isoforms of CA with diverse tissue distributions, widely varying kinetic properties and numerous physiological functions. The research reported in this thesis focused on the molecular characterization and physiological roles of two rainbow trout (*Oncorhynchus mykiss*) CA isoforms, the membrane-associated CAIV and a cytosolic form (tCAc). More specifically, I evaluated the roles of CAIV and tCAc in renal acidification and bicarbonate reabsorption at rest and during respiratory acidosis. In addition, the localization of these proteins within the kidney and gill was investigated, and the role of tCAc in branchial acid-base regulation was assessed.

Using an array of techniques that included Northern analysis, Western analysis, real time RT-PCR, immunohistochemistry and *in situ* hybridization, I demonstrated that trout CAIV is specifically localized to renal tubule cells, whereas tCAc is widely distributed across different tissues such as gill, kidney and brain. Within the gills, *in situ* hybridization using a riboprobe specific to tCAc and immunoreactivity using a polyclonal antibody raised against tCAc revealed tCAc mRNA and protein expression in both pavement cells in the interlamellar region, and along the lamellae. The levels of mRNA and protein for both CA genes (in gill, and kidney for tCAc and CAIV, respectively) increased during respiratory acidosis induced by exposure to hypercapnia (final nominal water CO_2 tension of 7.5 Torr), suggesting that both play a role in regulating systemic acid-base disturbances.

By using a membrane-impermeant inhibitor of CA, F3500, in concert with blood and urine analyses, I demonstrated that CAIV plays an important role in renal bicarbonate reabsorption, and thereby contributing to whole body acid-base regulation. This role was enhanced during hypercapnic acidosis, a reflection of HCO_3^- accumulation for acid-base compensation and hence a greater requirement for HCO_3^- reabsorption from the filtrate. A comparison of the effects on renal acidification of the rapidly permeating CA inhibitor, acetazolamide, with those of F3500 so as to partition the contributions of tCAc and CAIV suggested that tCAc plays the quantitatively larger role in renal acidification. The results of this analysis support a model for HCO_3^- reabsorption in the renal tubule in which the dehydration of filtrate HCO_3^- by H^+ added to the nephron lumen is catalyzed by CAIV. Cytoplasmic CA (tCAc) catalyses the hydration of CO_2 to provide the H^+ that are added to the nephron lumen.

Cytoplasmic CA (tCAc) also plays a role in branchial net acid excretion, as inhibition of branchial tCAc using acetazolamide resulted in a significant reduction in branchial net acid excretion. This role was enhanced during hypercapnic acidosis, a reflection of increased branchial net acid excretion for the purposes of acid-base regulation.

Résumé

Les anhydrases carboniques (CA) sont d'importantes enzymes qui catalysent les réactions réversibles d'hydrations/déshydrations du CO_2. Chez les mammifères, on retrouve au moins 15 isoformes de CA connues distribuées dans divers organes, variant en propriétés cinétiques et possédant différentes fonctions physiologiques. La recherche retrouvée dans cette thèse se concentre sur la caractérisation moléculaire et les rôles physiologiques de deux isoformes de CA chez la truite arc-en-ciel (*Oncorhynchus mykiss*), la CAIV qui s'associe à la membrane et la forme cytoplasique (tCAc). Plus spécifiquement, j'ai évalué les rôles de la CAIV et de la tCAc dans l'acidification rénale et la réabsorption de bicarbonates au repos et lors d'une acidose respiratoire. De plus, j'ai localisé ces protéines dans le rein et les branchies, et j'ai identifié le rôle de la tCAc dans l'ajustement branchial acido-basique. En utilisant divers techniques tels que le Northern blot, le Western blot, le QPCR, l'immunohistochimie et l'hybridation *in situ,* j'ai démontré que chez la truite, la CAIV est spécifiquement localisée au niveau des cellules des tubules rénales, tandis que la tCAc est largement distribué sur différents tissus tels que les branchies, le rein et le cerveau. Au niveau des branchies, l'hybridation *in situ* utilisant une sonde ribozomale spécifique à la tCAc et l'immunochimie utilisant un anticorps polyclonal agissant contre la tCAc, ont indiqué une expression d'ARNm et de proteine de la tCAc sur divers cellules pavementeuses dans la region interlamellaire et tout le long des lamelles. Les niveaux d'ARNm et de potéines pour les deux différents gènes de CA (dans les branchies et le rein pour la tCAc et la CAIV, respectivement) ont augmenté lors d'une acidose respiratoire induite par exposition à l'hypercapnie (tension nominale finale de CO_2 de l'eau de 7.5 torr), suggérant que les deux jouent un rôle dans

l'ajustement systémique des perturbations acido-basiques. En utilisant un inhibiteur de CA qui est imperméable à la membrane, tel que le F3500, et à partir d'analyses de sang et d'urine, j'ai pu démontrer que la CAIV joue un rôle important dans la réabsorption rénale de bicarbonate et de ce fait, contribue aux ajustements acido-basiques de tout le corps. Ce rôle a été plus important lors d'une acidose hypercapnique, qui reflète une accumulation de HCO_3^- pour la compensation acido-basique, et requiére par conséquent une plus grande exigence de la réabsorption de HCO_3^- à partir du filtrat. Une comparaison des effets sur l'acidification rénale en utilisant un inhibiteur de CA qui s'infiltre rapidement, l'acétazolamide, avec les effets obtenus en utilisant le F3500, pour identifier les contributions de la tCAc et de la CAIV, a suggéré que la tCAc joue un rôle quantitatif plus important dans l'acidification rénale. Les résultats de cette analyse supportent le modèle sur le réabsorption de HCO_3^- dans les tubules rénaux où la déshydratation des HCO_3^- du filtrat par les H^+ qui sont ajoutés au lumen du néphron, est catalysée par la CAIV. La CA cytoplasmique (tCAc) catalyse l'hydratation du CO_2 pour fournir les H^+ qui sont ajoutés au lumen de néphron.

La CA cytoplasmique (tCAc) joue également un rôle dans l'excrétion nette d'acides branchiaux. Ceci a été démontré par une réduction significative d'excrétion nette d'acides branchiaux après inhibition de la tCAc branchiale en utilisant de l'acétazolamide. Ce rôle a été plus important lors d'une acidose hypercapnique, qui reflète une augmentation d'excrétion nette d'acides branchiaux pour les ajustements acido-basiques.

Table of Contents

Esbaugh A.J., Perry S.F., Bayaa M., Georgalis T., Nickerson J., Tufts B.L., Gilmour K.M. 2005. Identification and characterization of a novel cytoplasmic carbonic anhydrase isozyme from rainbow trout, *Oncorhynchus mykiss,* and comparison to the erythrocyte specific isozyme. Journ. Exp. Biology. 208: 1951:61.

List of Figures

List of Tables

Abbreviations

CA, carbonic anhydrase
BSA, bovine serum albumin
PCO_2, partial pressure of carbon dioxide
$PaCO_2$, arterial partial pressure of carbon dioxide
pHa, arterial pH
Rbc, red blood cell
SEM, standard error of the mean

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Carbonic anhydrase (CA) is a ubiquitous zinc metalloenzyme that catalyzes the reversible hydration/dehydration of CO_2 and water:

$$CO_2 + H_2O \underset{}{\overset{CA}{\rightleftharpoons}} H^+ + HCO_3^-$$

This enzyme has been found in a variety of tissues, cell types, and subcellular organelles, from unicellular cyanobacteria through to mammals (reviewed by Henry, 1996; Swenson 2000).

The active site of CA contains a Zn atom coordinated by three histidine residues that hydrolyses water to H^+ and a highly reactive Zn-OH. The highly reactive Zn-OH makes CA one of the fastest enzymes known. The rapid catalysis of CO_2 to HCO_3^- enhances the CO_2 carrying of blood and ensures complete elimination of CO_2 by diffusion. The catalytic hydration of CO_2 occurs as fast as the substrate can diffuse into the active site, therefore it is thought that a proton transfer between CA and buffer occurs (Schwartz 2002). This would lead to two forms of the active site; a high pH form that is active in the hydration of CO_2 and a low pH form that is active in the dehydration of the bicarbonate (reviewed by Henry and Swenson 2000; Schwartz 2002).

Because the reactants and products include both gaseous and ionic chemical species, CA could potentially be important for any physiological or biochemical process in which these species are used (reviewed by Henry 1996). CA's are often distributed in a heterogeneous way within both tissue and cell types (i.e. cytoplasm, organelles, and the extracellular surface of membranes), and these different subcellular CA pools are known to be involved in different physiological and biochemical processes (reviewed by Henry and Swenson 2000). CA was first discovered in vertebrate red blood cells (rbc; Brinkman

at al. 1932), where it plays a role in a variety of processes ranging from respiration to intermediary metabolism (reviewed by Henry et al. 1996).

There are three distinct CA gene families (α, β and γ). All CA's characterized to date in the animal kingdom belong to a single gene family referred to as the α-carbonic anhydrase (Hewett-Emmett and Tashian 1996). Fifteen different α-CAs isoforms have been discovered in mammals (Peterson et al. 1997; Schwartz 2002). The α-CA's include CA's that are glycosylphosphatidylinositol (GPI)-anchored to the plasma membrane (CA IV), as well as integral membrane proteins (CA IX, CA XII, CA XIV), mitochondrial enzymes (CA V), cytoplasmic enzymes (CA I, CA II, CA III, CA VIII and CA XIII), and enzymes in secretory granules (CA VI) (Peterson et al. 1997). In comparison to the situation in mammals, much less is known about the CA isoforms and their evolution in non-mammalian vertebrates. The evolution of vertebrates was probably associated with an increase in the catalytic efficiency of the rbc CA (Tufts et al. 2003, Esbaugh et al. 2005). Henry et al. (1993) suggested that here could be an important link between the evolution of rbc CA activity and the appearance of the anion exchanger such as the Cl^- /HCO_3^- exchanger, in the vertebrate rbc membrane. Elasmobranches may represent an intermediate step in the evolution of these two proteins because their rbc's posses the anion exchange protein, but also contain a CA isoform with low catalytic capacity (Maren et al. 1980; Tufts et al. 2003).

Ancestral cytoplasmic CA's appear to have given rise to mammalian CA's through duplication events over the course of 600 million year (Peterson et al. 1997). CA VII is thought to closely resemble the ancestral enzymes based on a low rate of evolution,

active site conversion, and phylogeny. CA I, II and III have arisen owing to recent gene duplications occurring as early as the reptilian era (Sanyal et al. 1984).

Distribution and function of branchial CA

Carbonic anhydrase activity was reported to be present in the gills of fish 60 years ago (Sobotka and Kann 1941). Because the gill functions both as the organ of respiratory gas exchange and extracellular fluid ion regulation, the physiological role of CA has been examined in relation to both of those processes. The majority of studies on CA function involve two approaches: 1) localization of the enzyme to specific tissues; and 2) physiological measurements after treatment with one or more CA inhibitors. High levels of CA activity have been found in all the gills of all species that have been examined (Henry et al. 1997; reviewed by Henry and Swenson 2000), but very little information on the physiological role of branchial CA can be gained from activity measurements alone. Total branchial CA activity has been reported to be altered by certain environmental factors (reviewed by Henry and Swenson 2000). Immunocytochemisty has been used to localize CA to specific cell types within the gill and even to distinct regions within the cells. Those data showed that among teleosts, all cell types appear to stain at least weakly for CA, with the heaviest concentration of reaction product being found in the chloride cells and the pavement cells of the respiratory epithelium, and in the mucous cells of the filament epithelium (Rahim et al. 1988; Perry and Laurent 1990; Henry et al. 2000).

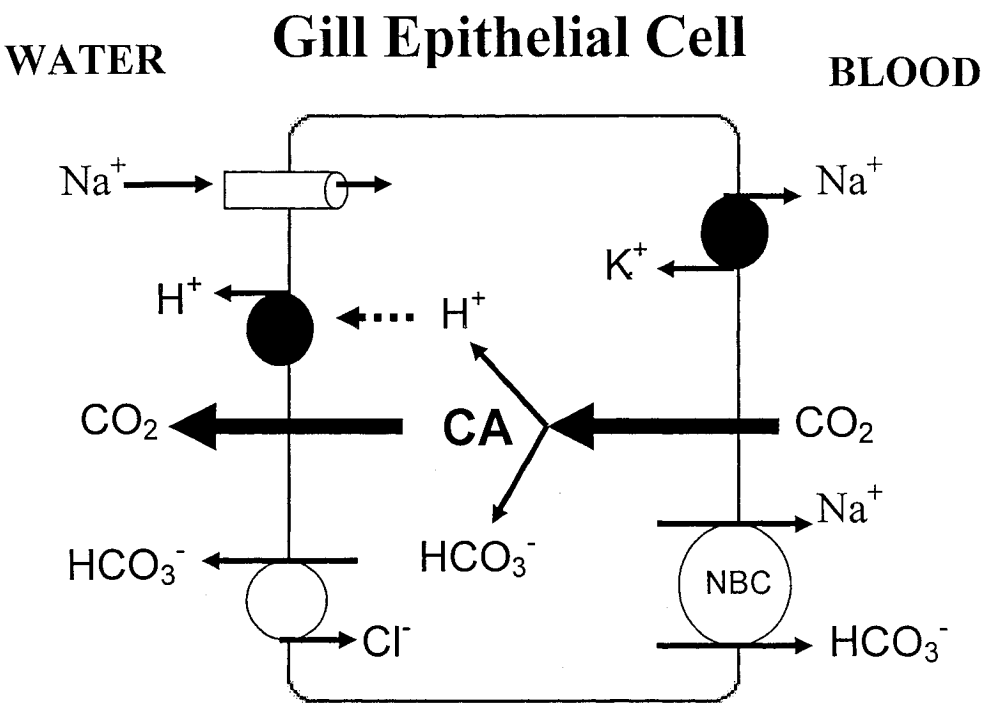
The subcellular distribution of branchial CA has also been studied biochemically. CA activity in homogenates of gill tissue, subjected to differential centrifugation in order to isolate various subcellular fractions, has been shown to be heterogeneously distributed.

While every fraction has some detectable CA activity, the highest activities are found consistently within the cytoplasmic pool (Henry et al. 1997; reviewed by Henry and Swenson 2000). The cytoplasmic CA plays a major role in regulating pH within the cell. Meanwhile, Gilmour and co-workers (Gilmour and Perry 1994; 1996; Gilmour et al. 2002), by demonstrating a post-branchial pH disequilibrium in trout blood, confirmed the absence of branchial membrane-associated CA in the gill of teleosts. In elasmobranches and channichthyidaes there is a CA IV-like activity present in the gill which is oriented towards the plasma, and functions in CO_2 excretion (Perry et al. 1999; Gilmour et al. 2001; 2002; Maffia et al. 2001; Tufts et al. 2002). The differences between elasmobranchs and teleosts suggest a divergence between those two types of fish with respect to the distribution of branchial and plasma CA activities.

Blood acid-base regulation in fish is linked to branchial ion uptake (Cameron 1976). It is believed that in response to an acid-base disturbance branchial Na^+/H^+ and/ or Cl^-/HCO_3^- exchange rates are modulated in order to compensate for acid or base loads and restore resting blood pH values. Thus, there is a clear relationship between the branchial cytoplasmic CA activity and the ion transport: the counter ions (H^+ and HCO_3^-) are formed via the CA catalysed hydration of intracellular CO_2.

An overview of the role of cytoplasmic CA in the gill is shown in Figure 1-1.

Figure 1-1: A model showing the proposed role of cytoplasmic carbonic anhydrase (CA) in the mitochondria rich (MR) cells of rainbow trout gill. According to this scheme, CA catalyses the hydration of intracellular CO_2 leading to the formation of HCO_3^- for Cl^- /HCO_3^- exchange and H^+ pumping across the apical membrane. NBC refers to sodium-bicarbonate co-transporter; modified from Perry (2003).


WATER
Gill Epithelial Cell
BLOOD
Na+
Na+
K+
H+
H+
CO2
CA
CO2
Na+
HCO3-
HCO3-
NBC
Cl-
HCO3-

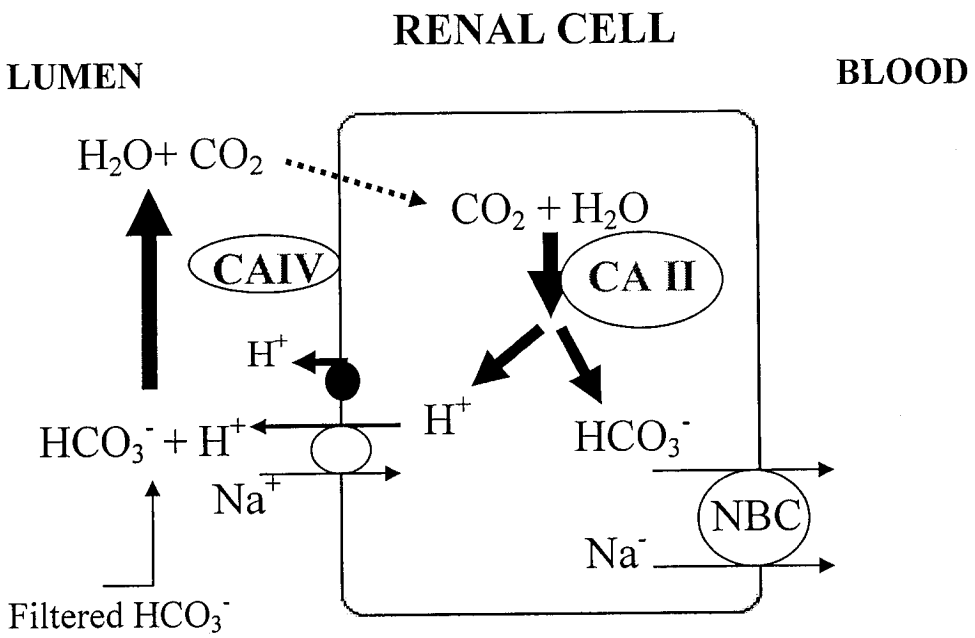
Plasma CO_2 entering in the gill epithelium cell is hydrated by the cytoplasmic CA to produce H^+ and HCO_3^- which are then excreted by the different pumps and/ or transporters on the apical membrane to regulate the acid-base status of the blood. Branchial CA activity was found to be increased by environmental hypercapnia, consistent with its role in acid-base regulation (Dimberg and Hoglund 1987).

Distribution and function of renal CA

CA involvement in renal function is well documented in mammals where three kinetically distinct forms of the enzyme are known to occur (Dodgson 1991). In addition to the cytoplasmic isoform CAII, which is mostly involved in H^+ secretion and intracellular pH maintenance (Maren et al. 1980), a membrane-bound isoform, CAIV, first found in the human kidney, is thought to function in bicarbonate reabsorption (Wistrand and Kinne 1977). This type of CA facilitates the dehydration of carbonic acid that is generated from the combination of secreted protons and filtered HCO_3^- (reviewed by Swenson 2000; Schwartz 2002; Perry et al. 2003). This reaction keeps free H^+ at low levels.

An overview of what happens in a kidney cell is shown in Figure 1-2.

Figure 1-2. A model of a mammalian proximal tubular cell showing acid-base reactions occurring in the lumen and in the cytoplasm. Notice the two types of CA found in kidney (CA IV associated with the apical membrane and CA II within the cytosol). Modified from Schwartz (2002).


RENAL CELL
LUMEN
BLOOD
$H_2O + CO_2$
$CO_2 + H_2O$
CAIV
CA II
H^+
H^+
$HCO_3^- + H^+$
HCO_3^-
Na^+
NBC
Na^-
Filtered HCO_3^-

In mammals, CA II, IV, V, XII and CA XIV have been identified at the kidney, however CA II represents >95% of the activity (Kyllonen et al., 2003). Proximal tubules of a few mammalian species have CA II and CA IV while only some species have CA in the straight portions of the proximal tubule. The CA IV in the apical brush border and basolateral membrane comprises only 3-5% of total renal CA activity (reviewed by Swenson 2000; Schwartz, 2002).

Goals and hypothesis

The objective of this project is to increase our understanding of the distribution and function of CA in fish by using molecular and physiological tools. For that, the use of the rainbow trout was appropriate for this study.

In Chapter 2, I test the hypothesis that cytoplasmic CA (tCAc) and membrane-associated CA IV are present in kidney where they play complimentary roles in renal acidification and systemic acid-base balance. The basic experimental protocol will be to localize the CA IV and tCAc mRNA and proteins within the kidney and to compare their expression in control versus hypercapnic conditions. In addition, Chapter 2 will examine the role of the CA IV and the tCAc in renal bicarbonate reabsorption, using CA inhibitors (acetazolamide for total inhibition of the CA activity and F3500 to specifically inhibit extracellular CA), and comparing their effects under normocapnic and hypercapnic conditions.

In Chapter 3, I will examine the hypothesis that cytosolic CA (tCAc) is present in trout gill where it plays a role in branchial net acid excretion. Moreover, utilizing the same techniques as in Chapter 2, I will localize the branchial tCAc mRNA and proteins

within the gill epithelium, and will compare their expression under control and hypercapnic conditions. To determine the role of the branchial tCAc in acid-base regulation, I will inhibit its action with acetazolamide and analyze the impact on blood acid-base status and branchial net acid excretion.

CHAPTER 2

THE ROLES OF CYTOSOLIC AND MEMBRANE BOUND CARBONIC ANHYDRASE IN THE RENAL CONTROL OF ACID-BASE BALANCE IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

Chapter 3 has been submitted to *Journal of Experimental Biology*, authors Georgalis, Gilmour, Yorston and Perry.

Abstract

Experiments were performed to test the hypothesis that cytosolic and membrane-associated carbonic anhydrase (CA IV) are involved in renal urinary acidification and bicarbonate reabsorption in rainbow trout both at rest and during respiratory acidosis. Using homology cloning techniques, an 1137 base pair 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. Using this sequence and a previously described trout cytosolic isoform (tCAc; Esbaugh et al. 2005), tools were developed to quantify and localize mRNA and proteins for the two CA isoforms. Unlike tCAc that displayed a broad tissue distribution, trout CA IV mRNA (and to a lesser extent proteins) was highly and preferentially expressed in the posterior kidney. The results of *in situ* hybridization, immunocytochemistry and standard histological procedures demonstrated that CA IV was likely confined to epithelial cells of the proximal tubule with the protein exhibiting an apical localization. The CA IV containing tubule cells were enriched with basolateral Na^+/K^+-ATPase. Similar results were obtained for tCAc except that it appeared to be present in both proximal and distal tubule cells.

The levels of mRNA and proteins for both CA genes increased significantly during respiratory acidosis (hypercapnia). By using a membrane-impermeant (yet filtered) inhibitor of CA, F3500, in concert with blood and urine analyses, we demonstrated that CA IV plays an important role in urinary acidification and renal bicarbonate reabsorption and thus contributes to whole body acid-base regulation; the

role of CAIV was enhanced during hypercapnic acidosis. A comparison of results obtained using a membrane permeant inhibitor, acetazolamide, revealed that tCAc is likely the key renal isoform contributing to urinary acidification and bicarbonate reabsorption.

Introduction

In water-breathing fish, acid-base regulation is achieved principally by metabolic compensation whereby plasma HCO_3^- levels are adjusted at more-or-less constant values of arterial PCO_2 (Heisler 1984; 1993; Claiborne 1998). This is in marked contrast to air-breathers that in addition to metabolic compensation also exploit respiratory adjustments to rapidly alter blood PCO_2 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 (Claiborne et al. 2002; Perry et al. 2003a; 2003b). It is well established that the gill is an important site of metabolic compensation owing to its capacity to dynamically regulate net acid excretion (Goss et al. 1992; Evans et al. 2005). During periods of internal acidosis, branchial net acid excretion is increased (e.g. Kobayashi and Wood 1980) whereas during alkalosis, net acid efflux is reduced (e.g. Cameron and Kormanik 1982). Such changes in acid excretion promote appropriate adjustments of plasma HCO_3^- 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 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 HCO_3^- and H^+ effluxes, respectively (Claiborne et al. 2002; Marshall 2002; Perry et al. 2003a).

With few exceptions (Wood and Caldwell 1978; McDonald and Wood 1981), 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 (Kobayashi and Wood 1980; Wood and Jackson 1980; McDonald and Wood 1981; Cameron and Kormanik 1982; Smatresk and Cameron 1982; Wheatly et al. 1984; Perry et al. 1987; Hyde and Perry 1987; Wood 1988; Curtis and Wood 1992; Claiborne et al. 1994; Wood et al. 1999; Choe and Evans 2003). Thus, adjustments of plasma HCO_3^- levels, and hence pH regulation, are largely dictated by variations in acid output at the gill (reviewed by Cameron 1978). However, as pointed out previously (Wood and Jackson 1980; Wheatly et al. 1984; Perry et al. 1987), persistent changes in plasma HCO_3^- levels would not be possible without parallel adjustments of renal acid secretion. In particular, the capacity to retain high levels of HCO_3^- 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 HCO_3^-.

In mammals, approximately 80 - 90% of renal HCO_3^- reabsorption occurs within the proximal tubules. According to current models (Romero and Boron 1999; Soleimani 2002), filtered HCO_3^- combines with H^+ (derived from an apical membrane sodium proton exchanger isoform 3 [NHE3 (Wu et al. 1996)] to form CO_2, a reaction that is catalysed by membrane bound carbonic anhydrase isoform IV [CA IV (Okuyama et al. 1992; Schwartz et al. 2000)]. The newly formed CO_2 enters the proximal tubule cell where it is hydrated to H^+ and HCO_3^- in the presence of CA isoform II [CA II (Wistrand and Wahlstrand 1977)]. Finally, the HCO_3^- is moved across the basolateral membrane by

a Na^+-HCO_3^- co-transporter [NBC1 (Romero et al. 1998; Schmitt et al. 1999)]. Thus, in mammals, HCO_3^- reabsorption is reliant on both cytosolic and membrane bound CA isoforms (reviewed by Schwartz 2002). Although a similar model has been proposed for freshwater fishes [see Figure 7 in Perry et al. 2003b], there is but scant supporting empirical evidence. Indeed, we are aware of only a single study that has experimentally implicated CA in renal HCO_3^- reabsorption (Nishimura 1977). To our knowledge, no studies have yet attempted to assess the relative involvement of cytosolic and membrane bound CA's in renal HCO_3^- 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 HCO_3^- reabsorption in rainbow trout (*Oncorhynchus mykiss*). This was accomplished by cloning and molecular characterization of a putative trout renal CA IV and its selective inhibition *in vivo* using a membrane impermeant CA inhibitor.

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 12h: 12h light:dark photoperiod in large fiberglass aquaria supplied with flowing, aerated and dechloraminated City of Ottawa tapwater at 13°C. They were fed daily with a commercial trout diet. Animals were allowed at acclimate to the holding conditions at least 2 weeks before performing any experiments.

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 prior to storage at -80° C. Total RNA was extracted from kidney tissue using Trizol (Invitrogen) according to the instructions of the manufacturer. RNA concentrations were verified using spectrophotometry (Eppendorf BioPhotometer). 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 primer; FptC (5'-CARWSICCNATHAAYATHGT-3') and SFPCAIVR (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 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of each dNTP, 200 nmol l^{-1} of each primer and 1 unit 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 for 10 min at 72° C. The PCR product was gel-purified and cloned into pCRII-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' RACE (Rapid Amplification of cDNA Ends). 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 Supercript II reverse transcriptase (Gibco). Semi-nested PCR was performed on the cDNA using abridged universal amplification primers (Gibco) with 3'RACE primers; first round primer 3F1N (5'-GCCTTACACCATTCACATTGG-3') and second round primer 3F2N (5'-CCATACAAGGCAAGGCAATACAGCTC-3'). For 5′ RACE, kidney cDNA was synthesized using an oligo dT primer using Superscript II reverse trascriptase (Invitrogen), and purified using a PCR purification kit (Sigma). The cDNA was then tailed with dCTP using a terminal transferase TdT (Invitrogen) with final reaction conditions; 10 mmol l^{-1} Tris-HCl (pH 8.4), 25 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 200 umol l^{-1} dCTP, 1 µl cDNA, 1 ul TdT. The tailed cDNA was used for two rounds of nested PCR using the following primers, 5R1 (5'-TTGTATGGCACAGCCACAT-3') and abridged anchor primer (Invitrogen) for the first round, and 5R2 (5'-

AGACTGTGGAAGGCCTCTTG-3') and 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 bi-directional sequencing of both RACE products, a consensus sequence was created by multiple sequence alignment (DNAMAN; Lynnon Biosoft^{R}). Owing to possible sequence ambiguities, a small portion of the 3' end was re-cloned and its sequence confirmed using primers tCA4F2 (5'CTGTAGTTTGGACCGTGTTC-3') and tCAc4R (5'-AGTAAGAGAGTGGCAAAGTGC-3').

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 (Thompson et al. 1994). The accession numbers for sequences used in the phylogenetic analysis are presented in Figure 2. Maximum likelihood phylogenetic analysis was performed using PUZZLE version 4.0.2 (Strimmer and von Haeseler 1996). The following program settings were used: quartet puzzling tree search, compute exact quartet likelihood, 1000 puzzling steps, use 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 set, and the model of rate heterogeneity was 1 invariable + 8 gamma rates. The selection of the human CA VII sequence as the out-group was based on a previous publication (Esbaugh et al. 2005).

RNA analyses

Northern blots

Total RNA was isolated using Trizol Reagent (Invitrogen). Poly A+ RNA was then isolated from total RNA using a PolyATtract 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% (w/v) agarose gel in MOPS buffer containing 0.65 mol l^{-1} formaldehyde. RNA was transferred to GeneScreen^{+} membranes (NEN Life Sciences) by capillary action for 24 h. Ambion millennium RNA markers were used to estimate transcript size. Membranes were pre-hybridised at 65° C for 4 h in a buffer containing 6X SSC (0.9 mol l^{-1} NaCl, 0.09 mol l^{-1} sodium citrate, pH 7.0), 5X Denhardt's (1X Denhardt's is 0.1% Ficoll 400.000, 0.1% polyvniylpyrrolidone, 0.1% bovine serum albumin), 100 μg ml^{-1} single-stranded herring sperm DNA, 1% sodium dodecyl sulfate (SDS) and 10% dextran sulfate (Amersham Pharmacia Biotech). The probe was prepared by PCR amplification of the 290-bp insert followed by incorporation of ^{32}P libeled dCTP (using DNA polymerase fragment I; Gibco), purification (SigmaSpin Post Reaction Purification Column; Sigma) and thermal denaturation. The probe (specific activity 10^{9} cpm μg^{-1} 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 0.1X 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 re-probed with a homologous β-actin probe (514 bp PCR product) under the same conditions but with an exposure time of a few hours (data not shown).

Real time PCR

Tissues were homogenized to powder under liquid N_2 using a mortar and pestle. Total RNA was extracted from 30 mg aliquots of powdered tissue samples using the 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 H_2O 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 (tCAc; Esbaugh et al., 2005) 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^{-1}; 2X Master Mix = 12 µl; ROX = 1:30000 final dilution. The annealing and extension temperatures over 40 cycles were 58° C (30 s) and 72° C (30 s), respectively. The following primer pairs were designed using Primer3 software: β-actin forward (5'-CCAACAGATGTGGATCAGCAA-3'), β-actin reverse (5'-GGTGGCACAGAGCTGAAGTGG TA-3'), tCA IV forward (5'-ATACGAATCCATCATCATAAATGCT-3'), tCA IV reverse (5'-CGGAAGTAGCTGGTCATATTACTCTG-3'), tCAc forward (5'-CAGTCTCCCATT GACATCGTA-3') and tCAc reverse (5'-CGTTGTCGT 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 incorporated into primer dimers or non-specific amplicons during the real time PCR runs, the PCR products were analysed 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 was determined (using actin as an endogenous standard) by a modification of the delta-delta Ct method (Pfaffl 2001). 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 approximately 600 bp. For CA IV, primers were forward 5'-CCATCATTGTGAAGATTCAAGG-3' and reverse 5'-ACAGGGAGGTGTGGTAAGAGAG-3'. For tCAc, primers were forward 5'-ATGTCTCATGCATGGGGATAC-3' and reverse 5'-CAGGTGACACTCTCCAGCA-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. 2 μg of plasmid DNA was digested using Xba I (Invitrogen) under conditions recommended by the manufacturer. Products were phenol/chloroform purified and resuspended in 10 μl of DEPC H_2O. Digest concentrations were verified using an Eppendorf BioPhotometer

spectrophotometer (VWR International). The probe labelling assay was carried out using SP6 RNA polymerase (New England Biolabs) along with DIG RNA labelling mix (Roche) as described by Roche.

Small pieces (~200mg) 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 emersion in 15% sucrose for 2 h and 30% sucrose where they were left at 4° C until sectioning. Cryoprotected tissue pieces were frozen in Shandon Cryomatrix embedding medium (Fisher) and thiner sections (10 μm) were prepared using a Leica CM 1850 cryostat at -15° C. Frozen sections were placed onto electrostatically charged slides (SuperFrost Plus, VWR) and sections were air-dried for 30 min and then stored at -20° C.

Sections on slides were hydrated (2 X 15 min) in 1X PBST (Phosphate buffer saline (PBS) with 0.1% Tween 20). Tissues were de-proteinated using 20 μg ml^{-1} of proteinase K (Gibco BRL, Orand Island, NY) in PBST for 20 min at room temperature and then rinsed (2 X 10 min)in 1X PBST. After digestion, tissues were re-fixed in 4% formaldehyde in PBS for 5 min, rinsed (2 X 10 min) in 1X PBST and air-dried at 58°C for 15 min. Probes (approximately 4 ug) were denatured for 3 min at 94° C in a solution containing 250 μg ml^{-1} of salmon sperm DNA and 250 μg of Poly A, topped to 12.5μl with DEPC H_2O. Probes were then quickly chilled on ice and centrifuged for 1 min at 7,500g. 100 μl of hybridization buffer (50 % deionized formamide, 1X Denhardt's, 0.2% SDS, 5% dextran sulphate, 0.75 mol l^{-1} NaCl, 25 mmol l^{-1} EDTA and 25 mmol l^{-1} PIPES) were added to each probe, vortexed and placed on sections. Sections were hybridized overnight at 58° C in a humid chamber.

Subsequently, sections were thoroughly washed at 58° C in 2X SSC (2 X 15 min) and 0.2X SSC (2 X 15 min), followed by one wash in 0.1X SSC for 10 min at room temperature and twice in 0.1M PBS for 10 min at room temperature. For detection of hybridization, sections were incubated first with 1% goat serum, 2 mg ml^{-1} BSA in 0.1 mol^{-1} PBS with 0.3% Triton-X at room temperature for 1 h, followed by an incubation in anti-digoxigenin conjugated to alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:1000 in above solution overnight at 4°C. After 2 washes in 0.1 mol l^{-1} PB at room temperature for 15 min and a brief rinse in water, the slides were washed twice for 5 min in coloration buffer (100 mmol l^{-1} Tris pH 9.5, 50 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 0.1% Tween-20). Nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolyl phosphate (BCIP) tablets (Sigma) were dissolved in 10 ml of H_20 and layered over the sections. Colour was allowed to develop in a humid chamber for at least 4 h at room temperature in the dark until satisfactory coloration was achieved. The slides were then washed (2 X 15 min) with 0.1 mol l^{-1} PBS. The slides were mounted with 60% glycerol and cover slipped. Sections were viewed using a Zeiss Axiophot light microscope and a Hamamatsu C5985 chilled CCD camera. Images were captured using Metamorph imaging system (Version 4.01).

Two types of negative control experiments were performed to assess the specificity of hybridization. In one case, probe was omitted from the hybridization protocol and in the other sections were pre-treated with excess unlabelled probe. For the latter, sections were first incubated with 5X more unlabeled probe in hybridisation buffer for 3 h at 58° C. Solution was removed from sections and replaced with hybridization

buffer containing 5X unlabeled probe and approximately 4 ug of probe and incubated overnight at 58° C

Protein analyses

Western blots

Custom rabbit polyclonal antibodies (Abgent, San Diego, USA) raised against trout CA IV and tCAc (Esbaugh et al. 2005) were generated using synthetic peptide antigens conjugated to keyhole limpet protein. For CA IV, the synthetic peptide TRRTLPDERLTPFTFTGY 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).

Proteins were prepared from frozen tissues (0.5 g ml^{-1} homogenization buffer) by homogenization on ice in10 ml Tris-SO4 buffer (25 $mmol^{-1}$ Tris-SO4, 0.9% NaCl, pH 7.4) containing the protease inhibitors (complete™ Mini protease inhibitor cocktail tablets, Roche) and 2 μg ml^{-1} pepstatin A (Sigma). Samples were stored on ice for 15 min and centrifuged at 10000 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 micro bicinchoninic acid protein assay (Pierce) using BSA as standard. Samples (100 – 150 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) by using a transblot electrophoretic transfer cell (Bio-Rad, Hercules, CA) 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 1:2000 goat anti-rabbit antibody (Pierce). After each exposure to antibody, the membranes were washed 3 X 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 (BIORAD Chemi Doc). The digital images were processed using commercial software (Quantity One, version 4.1.1). The protein size marker used was obtained from Fermentas Life Sciences. To demonstrate specificity of the tCAIV antibody, sections were incubated with the tTCAIV antibody in the presence of an excess (20 μg) of the peptide against which the antibody was raised. Negative control sections were incubated with blocking buffer lacking antibodies or with pre-immune serum.

Immunocytochemistry

Tissue sections were prepared as described for *in situ* hybridization (see above). A hydrophobic barrier was created around each section with a PAP pen (Electron Microscopy Suppliers, Fort Washington). Sections were incubated in situ (3 X 5 min) with a blocking buffer containing 2% normal goat serum, 0.1mol l^{-1} PB, 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 four primary antibodies, diluted in the blocking buffer: α5, a mouse monoclonal antibody against the α_1 sub-unit of chicken Na^+-K^+-ATPase

(University of Iowa Hybridoma Bank; 1:100), trout CA IV (1:100), trout CAc (1:200) or human CA II (1:200; Rockland). Alternatively, negative control sections were incubated with blocking buffer lacking primary antibodies, pre-immune serum (trout CA IV) or antibodies pre-absorbed with excess peptide antigen (trout CA IV and CAc). The α_5 antibody has been used in numerous previous studies to localize Na^+/K^+-ATPase in fish tissues (e.g.Wilson et al. 2000). The human CA II antibody was used previously to localize cytosolic CA in salmon both by immunocytochemistry and western blot (Tohse et al., 2004). The human CA II antibody was used rather than the homologous tCAc antibody because preliminary experiments demonstrated that it produced less background fluorescence. The slides were then washed (3 X 5 min) in 0.1 mol l^{-1} PB. For double immunofluorescence staining, the trout anti-rabbit CA IV was detected with Alexa 488-coupled goat anti-rabbit IgG (1:400; Fisher) and α_5 was detected with Alexa 546-coupled goat anti-mouse IgG (1:400; Fisher). Slides were incubated in a humid chamber for 1 h at room temperature. The slides were then washed (3 X 5 min) in 0.1 mol l^{-1} PB (phosphate buffer) and mounted with a mounting medium (Vector Laboratories) containing DAPI to stain nuclei. The sections were examined with a conventional epifluorescence microscope (Zeiss Axiophot) and CCD camera. Final images were obtained using Metamorph software (4.01).

Some kidney sections (normocapnic and hypercapnic) on which immunocytochemistry was done, were treated with aqueous periodic acid for 10 min at room temperature, rinsed well in distilled water, and covered with Schiff solution for 4 min. The periodic acid Schiff (PAS)-positive tubules were then compared with the corresponding CA IV or human CAII immunoreactive tubules and photographed. When

treated with periodic acid, glycols are oxidized to aldehydes. After reaction with Schiff's reagent, a parasosaniline adduct is realed that stains tha glycol-containing cellular components.

Exposure of fish to hypercapnia

Fish were placed into acrylic boxes supplied with flowing and aerated water. They were allowed to recover for 24 h and then exposed to external hypercapnia (target final water PCO_2 ($PwCO_2$) was approximately 6.0 mm Hg) for 24 h. To reach a final $PwCO_2$ of ~ 6.0 mm Hg, a water equilibrium column was gassed with mixtures of CO_2 and air (Sierra Instruments, Inc, Smart track, flowmeters). $PwCO_2$ was monitored by using a CO_2 electrode that was linked to a CO_2 meter (Cameron Instruments Inc). Deviations from the target $PwCO_2$ 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 t1, 2, 3, 6, 12 and 24 h (N = 6 at each time point) Control fish (N = 6) were also placed into boxes but were maintained under normocapnic conditions for 24 h. Tissues were removed at 3 h (representing control points for 1- 3 h hypercapnic fish), 6, 12 and 24 h.

The effects of CA inhibition on urinary acidification and HCO_3^- reabsorption

Fish were anaesthetized by immersing them in an oxygenated solution of benzocaine (ethyl-p-aminobenzoate; 0.1g l^{-1}) and then placed on a surgical table that allowed continuous irrigation of the gills with the same anaesthetic solution. All trout were fitted with dorsal aortic cannulae (Clay-Adams PE50 polyethylene tubing) according to the basic method of Soivio et al. (1975). Additionally, the intestine was ligated and an external urinary catheter was attached (Curtis and Wood 1991). After revival, fish were placed into opaque boxes supplied with flowing and aerated tapwater where they were allowed to recover for 24 h. Cannulae were flushed daily with heparinised [100 IU ml^{-1} ammonium heparin (Sigma)] Cortland saline (Wolf 1963)].

Experimental protocol

After the 24 h post surgery 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 approximately 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 either exposed to hypercapnia ($PwCO_2$ = 6.0 mm Hg) or normocapnia 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^{-1}) or membrane impermeant (F3500; 50 mg kg^{-1}) CA inhibitor. Unlike acetazolamide, which inhibits both cytosolic and membrane-associated (extracellular) forms of CA, F3500, a polymerized form of aminobenzolamide, is filtered by the kidney and thus able to selectively inhibit luminal

CA IV activity (Conroy et al. 1996). 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 pH, total CO_2 and titratable acidity were determined immediately upon collection; urine flow rates were determined gravimetrically. 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 haematocrit, the blood was centrifuged and plasma removed for immediate determination of pH and total CO_2. The remaining red blood cells were re-suspended in Cortland saline and re-injected 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 µl samples using a Capnicon total CO_2 analyser (CC501; Cameron Instruments), while pH was assessed using a pH electrode and calomel reference electrode (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 αCO_2 and pK' for rainbow trout plasma (Boutilier et al. 1984).

Total ammonia concentration was determined using a micro-modification of the salicylate-hypochlorite technique of Verdouw et al. (1978). K^+, Ca^+, and Na^+ levels were determined using atomic absorption spectroscopy (Varian) and Cl^- levels were measured

colorimetrically using mercuric thiocyanate and ferric nitrate and quantified by spectrophotometry (Spectramax; 340BC).

Net renal acid efflux was calculated according to Wood and Caldwell (1978) 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 l^{-1} HCl to 200 µl of urine to lower the pH below 5.0; the sample was aerated for 20 min to remove CO_2. While continuing to aerate, NaOH (0.02 mol l^{-1}) was added gradually using a precision microburet (Gilmont) 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 mean values ± 1 standard error of the mean (SEM). 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. The statistical significance of a two-way repeated measures analysis of variance (ANOVA) with sampling period (pre, normocapnia/hypercapnia and post-Az and/or F3500) and treatment group (control or hypercapnia-exposed) as factors was used to analyse the effects of acetazolamide/F3500 injection and treatment group (control versus hypercapnia-exposed) on blood acid-base variables and net acid-base fluxes. Differences in band intensities on western blots were assessed using unpaired Student's *t*-test.

Results

Molecular cloning of trout CA IV

Using homology cloning techniques, an 1137 base pair cDNA was assembled that included an open reading frame encoding for a deduced protein of 297 amino acids (Figure 2-1; 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 IV's, the trout sequence contains an N-terminal signal peptide (AA's 1 - 17) with the mature peptide beginning at position 18 (Figure 2-1) as well as two predicted disulfide bridges between cysteines 21 - 28 and 32 - 45 (Figure 2-1). The predicted active site of the trout sequence closely resembled the active site of vertebrate CA IV's (Figure 2-1). Finally, phylogenic analysis revealed that the trout gene grouped closely with vertebrate CA IV's and that it was clearly isolated from other membrane bound isoforms, CA IX, XII and XIV (Figure 2-2). Notably, the trout gene did not group with previously cloned fish cytosolic CA's. Thus, we have tentatively identified the trout gene as CA IV (tCA IV).

Tissue distribution of tCA IV

The tissue 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 ~2.1 kb that was detectable only in kidney (Figure 2-3A). The results of real time PCR confirmed high levels of tCA IV mRNA in kidney (mostly posterior kidney; Figure 2-3C) but also revealed expression in the brain and spleen. The expression profile of tCAIV protein

differed somewhat from the mRNA results with highest levels apparent in brain followed by heart, kidney and white muscle; tCA IV protein was not detectable in blood, gill or liver (Figure 2-3D). The molecular mass of tCA IV in all tissues containing the protein is 37.5 kDa, slightly higher than the predicted molecular mass of 34 kDa. The specificity of the tCA IV antibody was demonstrated by elimination of the immunoreactive band following preabsorption of antibody with excess blocking peptide (Figure 2-3E).

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. Figures 2-4 and 2-5 depict positive hybridization signals for tCAc and tCA IV mRNA respectively, in selected renal tubules. Minimal or no staining (and hence hybridization) occurred in the absence of probe (Figures 2-4C and 2-5C) or when tissues were pre-treated with excess unlabelled probe (Figures 2-4D and 2-5D).

tCAc protein was predominantly localized to the apical membrane of renal tubule cells co-expressing Na^+/K^+-ATPase on the basolateral membrane (Figure 2-6A, B). Immunofluorescence for both tCAc and Na^+/K^+-ATPase was prevented by omission of primary antibodies (Figure 2-6C, D). tCA IV also was expressed on the apical membrane in cells co-expressing basolateral membrane Na^+/K^+-ATPase (Figure 2-7 A - C). In some instances, Na^+/K^+-ATPase positive cells did not express tCA IV (Figure 2-7A). tCA IV immunofluorescence was eliminated by pre-incubating the antibody with excess immunizing peptide (Figure 2-7D) or by omission of primary antibodies (Figure 2-7E).

By post-treating slides previously viewed for immunocytochemistry with PAS, it was possible to identify the tubule types expressing tCAc and tCA IV (Figure 2-8). 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 (Figure 8A, B). In contrast, tCAIV appeared to be exclusively localized to proximal tubule cells (Figure 2-8C, 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 (Figure 2-9A). Renal tCA IV mRNA was markedly elevated (~20 fold) but only after 24 h of continuous hypercapnia (Figure 2-9B).

Levels of CA proteins were assessed by western blots and immunocytochemistry after 24 h of hypercapnia. Analysis of western blots (Figure 2-10A and B) demonstrated that tCAc protein was increased by 2.2 fold ($P = 0.012$) during hypercapnia. These results were confirmed qualitatively by immunofluorescence (Figure 2-10C and D). tCA IV was increased 2 fold ($P = 0.024$) during hypercapnia (Figure 2-11A and B), a result that was consistent with examination of immunofluorescence micrographs (Figure 2-11 C and D).

Effects of CA inhibition on blood gases and renal acidification

In one series of experiments, acetazolamide was used to inhibit total (intracellular and extracellular) CA activities whereas in another, F3500 was used to selectively inhibit extracellular CA activity. Acetazolamide, whether administered during normocapnia or

hypercapnia, caused a rapid increase in $PaCO_2$ and a reduction of pHa (Figure 2-12A, B). Despite the respiratory acidosis, plasma HCO_3^- levels were unaffected by acetazolamide (Figure 2-12C). The placement of the data on a pH-HCO_3^- diagram is consistent with acetazolamide causing a mixed respiratory/metabolic acidosis (Figure 2-13A). The base deficits induced by acetazolamide were calculated to be 3.0 and 2.2 mmol l^{-1} during normocapnia and hypercapnia, respectively. Unlike acetazolamide, F3500 was without effect on blood acids-base status either during normocapnia or hypercapnia (Figures 2-12 and 2-13). Urinary net acid efflux was significantly reduced by acetazolamide to a similar extent during normocapnia or hypercapnia (Figure 2-14) owing to changes in TA-HCO_3^- flux; ammonia fluxes were unaffected. Urine pH, HCO_3^- and Na^+ levels were markedly increased by acetazolamide (Figure 2-15) although for HCO_3^- and Na^+, the changes were statistically different only during hypercapnia. Urine flow rate was increased by acetazolamide both during normocapnia and hypercapnia; urine levels of K^+, Cl^- and Ca^{2+} were unaffected (Table 2-1).

During hypercapnia, the treatment of fish with F3500 reduced net renal acid efflux to a similar extent as did acetazolamide, an effect solely attributable to reduction of TA-HCO_3^- efflux (Figure 2-14). During normocapnia, F3500 did not affect net renal acid excretion. Levels of HCO_3^- and Na^+ in the urine were increased by F3500 but only during hypercapnia (Figure 2-15). Urine flow rate and other measured urinary ions were unaffected by F3500 (Table 2-1).

Figure 2-1. A) The deduced amino acid sequence of trout CA IV (tCA IV) showing the position (in red font) of residues known to be conserved in the active site of other vertebrate CA's; presumptive zinc binding histidines are denoted with asterisks. A signal peptide typical of CA IV isoforms is shaded [predicted using web-based software (http://www.cbs.dtu.dk/services/SignalP/index.php)]. Disulfide bridges are indicated at cysteines 21 - 28 and 32 - 45 (PredictProtein; (http://www.predictprotein.org/). Potential glycosylation sites are underlined (http://www.cbs.dtu.dk/services/NetNGlyc/). B) A comparison of the amino acid residues comprising the active site of vertebrate CA IV's including rainbow trout (GenBank accession AAR99330), zebrafish (AAH78387), pufferfish (*Tetraodon nigroviridis*,CAF98532), dogfish (*Squalus acanthias*, DQ092628), *Xenopus laevis* (AAH54242), chicken (XP_415893), rat (NP_062047), rabbit (P48283) and human (AAA35630). Highly conserved regions of the CA IV active site are shaded.

A

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MHLFISFFFA IIVKIQGADW CYQSQVTCGG NCTGPDGWAT VAGTCDGRAQ   50
SPINIVTRRT LPDGRLTPFT FTGYQEAFHG FITNNGHTVQ VDLPATAKVQ  100
GGDLAVPYKA VQLHLHWGKD GGPGSEHTID GERYPMELHI VNIKEEYNSL  150
AEALKDTAGV GVLGFFYEES RSSNKKYDAI VNALNTIKHP SSNTTLSEVS  200
LDMLIPSQSN MTSYFRYQGS LTTPPCEEAV VWTVFKNTIP LSRQQLDAFS  250
RLQFADGKPM VGTYRPVQPL NGRPVYRSGS QVILVSTLPL SY
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B

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Trout       YSNNHTVQDIQHHEHEHVLVVGFYLTTPPEAVWVVR
Zebrafish   YSNHHTAHTVQHHEHEHVLVVAYYLTTPSEAVWVFR
Pufferfish  YSNNHSVQDVQHHEHEHVLVVGYYLTTPGTSVWLQR
Dogfish     YSNNHSAQTVQHHEHEHVILVGYYLTTPGFAVWVFR
Xenopus     YSNNHSAQDIQHHEHEHVDLVGYYLTTPPNTVWLFR
Chicken     YSNNHTVKTIEHHEHEHVLIVAYYLTTPDHTVWVFR
Rat         YSNNHSVESIQHHEHEHVVIVAFYLTTPGDTVWVVR
Rabbit      YSNNHSVMSTQHHEHEHVVIVAFYLTTPASTVWVVR
Human       YSNNHSVMLKQHHEHEHVVIVAFYLTTPTDKVWVYR
```

Figure 2-2. Phylogenetic tree of selected vertebrate cytoplasmic and membrane associated CA isoforms using the following GenBank protein accession numbers: human CA IV (AAA35630), rainbow trout CA IV (AAR99330), mouse CA IV (Q64444), rat CA IV (NP_062047), bovine CA IV (NP_776322), rabbit CA IV (P48283), *Xenopus* CA IV (AAH54242), chicken CA IV (XP_415893), pufferfish (*Tetraodon nigroviridis*) CA IV type 1 (CAF98532) and type 2 (CAG08972), human CA XII (AAP35302), mouse CA XII (AAH31385), rabbit CA XII (Q9MZ30), rat CA XII (AAX50191), mouse CA XIV (Q9WVT6), human CA XIV (Q9ULX7), mouse CA IX (AAL14193), human CA IX (CAI13455), gorilla CA I (Q7M316), mouse CA I (AAH11223), human CA I, (AAH27890), sheep CA I (NP_001009771), monkey CA I (P35217), rat CA II (NP_062164), chicken CA II (NP_990648), human CA II (AAH11949), mouse CA II (AAA37357), mouse CA III (NP_031632), human CA III (AAA52293), rat CA III (AAA40846), rainbow trout cytoplasmic CA (tCAc; AAR99329), rainbow trout red blood cell CA (AAP73748), zebrafish cytoplasmic CA (NP_571185), Japanese dace cytoplasmic CA (BAB83090), gar cytoplasmic CA (AAM94169). The phylogenetic tree was constructed using neighbour joining analysis (see Materials and Methods for more details). The tree was ordered using human CA VII (AAH33865) as a monophyletic outgroup. Horizontal branch lengths are scaled to represent the relative number of amino acid substitutions occurring along a branch and support values at the nodes are indicated as a percentage from 1000 puzzling steps.

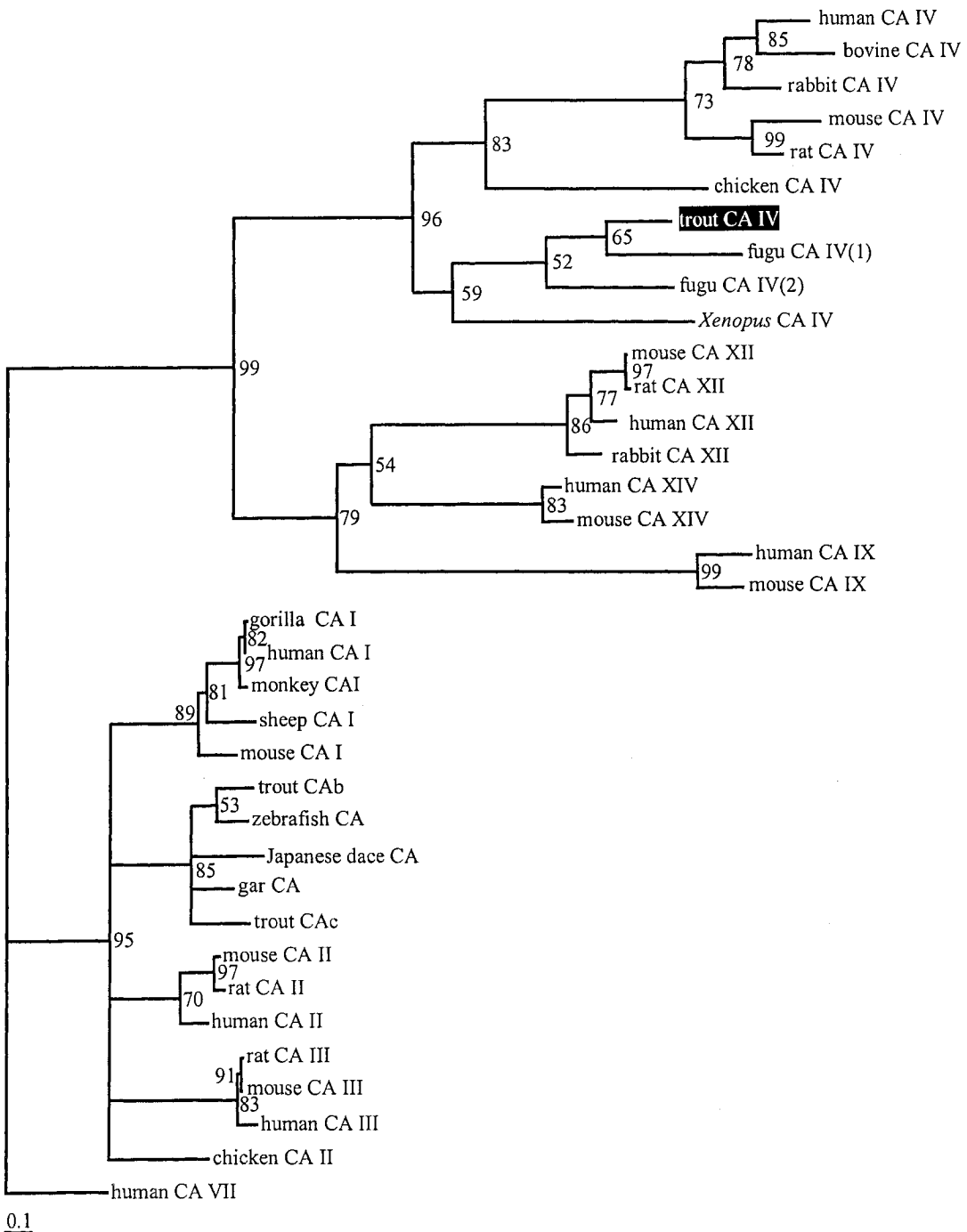

human CA IV
85
bovine CA IV
78
rabbit CA IV
73
mouse CA IV
99
rat CA IV
83
chicken CA IV
96
trout CA IV
65
fugu CA IV(1)
52
fugu CA IV(2)
59
Xenopus CA IV
99
mouse CA XII
97
rat CA XII
77
human CA XII
86
rabbit CA XII
54
human CA XIV
83
mouse CA XIV
79
human CA IX
99
mouse CA IX
gorilla CA I
82
human CA I
97
monkey CAI
81
89
sheep CA I
mouse CA I
trout CAb
53
zebrafish CA
Japanese dace CA
85
gar CA
trout CAc
95
mouse CA II
97
rat CA II
70
human CA II
rat CA III
91
mouse CA III
83
human CA III
chicken CA II
human CA VII
0.1

Figure 2-3. Tissue distribution of rainbow trout CA IV mRNA and proteins using (A, B) northern blots, (C) real time PCR and (D, E) western blots. Northern transfers of poly A+ RNA isolated from selected tissues were hybridized with a homologous trout CA IV cDNA probe (the 290 base pair clone). Gels were loaded with 5 μg per lane of poly A+ RNA and stained with ethidium bromide (B) prior to transfer. Except for heart RNA, samples displayed rougly equivalent levels of residual 28s and 18s RNA. For (C) real time PCR, data were compared to posterior kidney, which was given a relative value of 1. The western blots (D, E) depicted an immunoreactive band at 37.5 kDa in bran, heart, kidney and white muscle (w. muscle). The 37.5 kDa immunoreactive band in brain was eliminated after preabsorbing the primary antibody with excess peptide antigen (panel E).

Figure 3

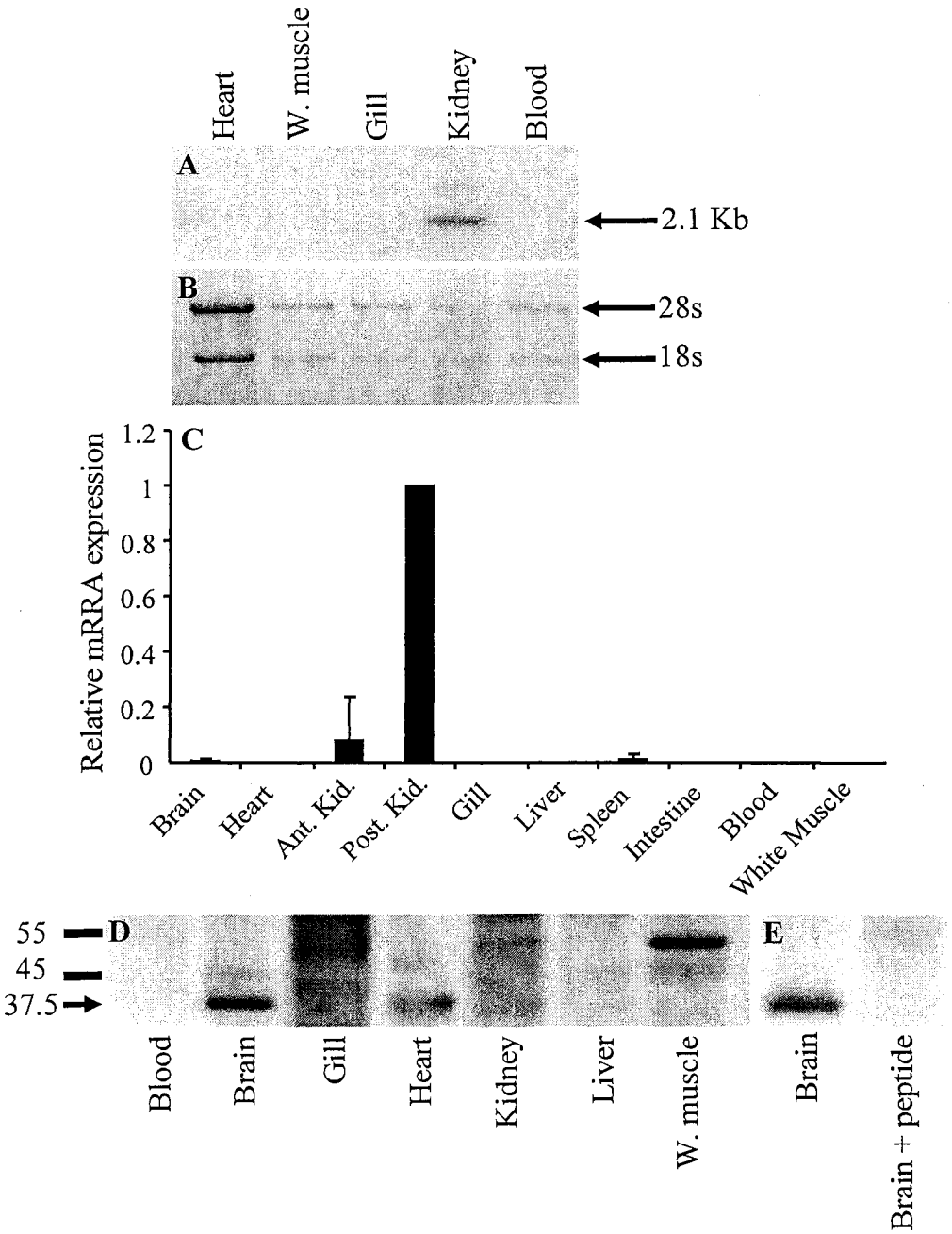

Heart
W. muscle
Gill
Kidney
Blood
A
2.1 Kb
B
28s
18s
C
Relative mRRA expression
1.2
1
0.8
0.6
0.4
0.2
0
Brain
Heart
Ant. Kid.
Post. Kid.
Gill
Liver
Spleen
Intestine
Blood
White Muscle
55
45
37.5
D
E
Blood
Brain
Gill
Heart
Kidney
Liver
W. muscle
Brain
Brain + peptide

Figure 2-4. Representative light micrographs revealing the presence of trout cytoplasmic CA (tCAc) mRNA in renal tubules by *in situ* hybridization. Panels A and B show two different tubules viewed at 40 and 100X magnification, respectively. Colour development was prevented by (C) omission of probe during hybridization and (D) pre-treatment of tissue with excess unlabeled probe. Scale = 25 μm.

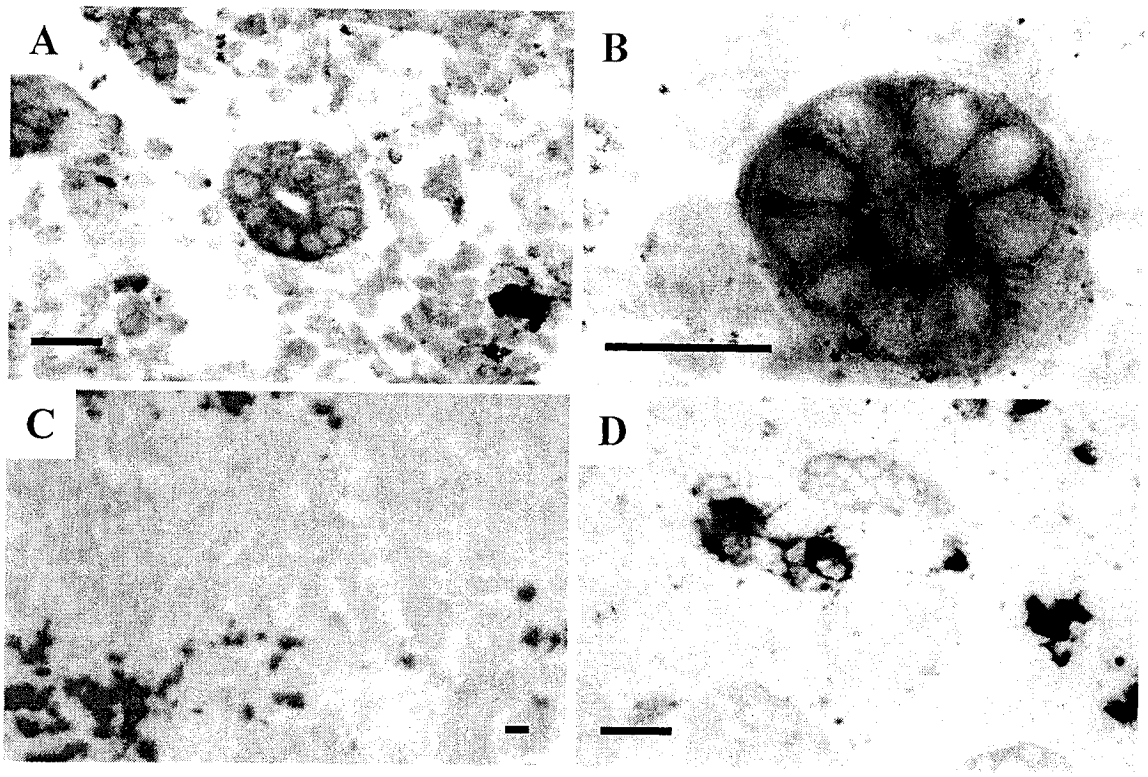

A
B
C
D

Figure 2-5. Representative light micrographs revealing the presence of trout CA IV mRNA in renal tubules by *in situ* hybridization. Panels A and B show two different tubules viewed at 40 and 100X magnification, respectively. Colour development was prevented by (C) omission of probe during hybridization and (D) pre-treatment of tissue with excess unlabeled probe. Scale = 25 μm.

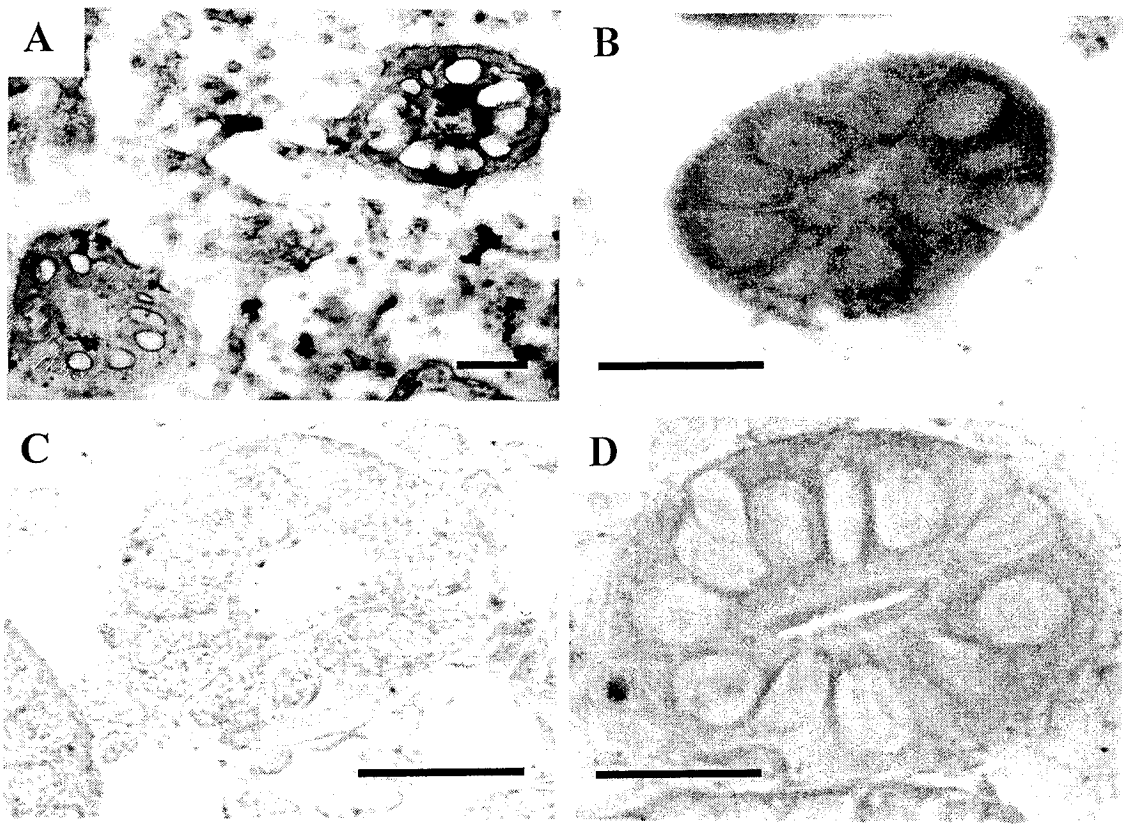

A
B
C
D

Figure 2-6. Representative light micrographs revealing the presence of trout cytoplasmic CA (tCAc) and Na^+/K^+-ATPase proteins in renal tubules by fluorescence immunocytochemistry. Green colour indicates tCAc, red colour indicates Na^+/K^+-ATPase and nuclei are stained blue (DAPI). Panels A and B show different tubules viewed at 40 and 100X magnification, respectively. tCAc was localized to the apical regions of tubules (arrows) whereas Na^+/K^+-ATPase was confined to basolateral regions. Specific fluorescence was prevented by (C, D) omission of primary antibodies. Scale = 25 μm.

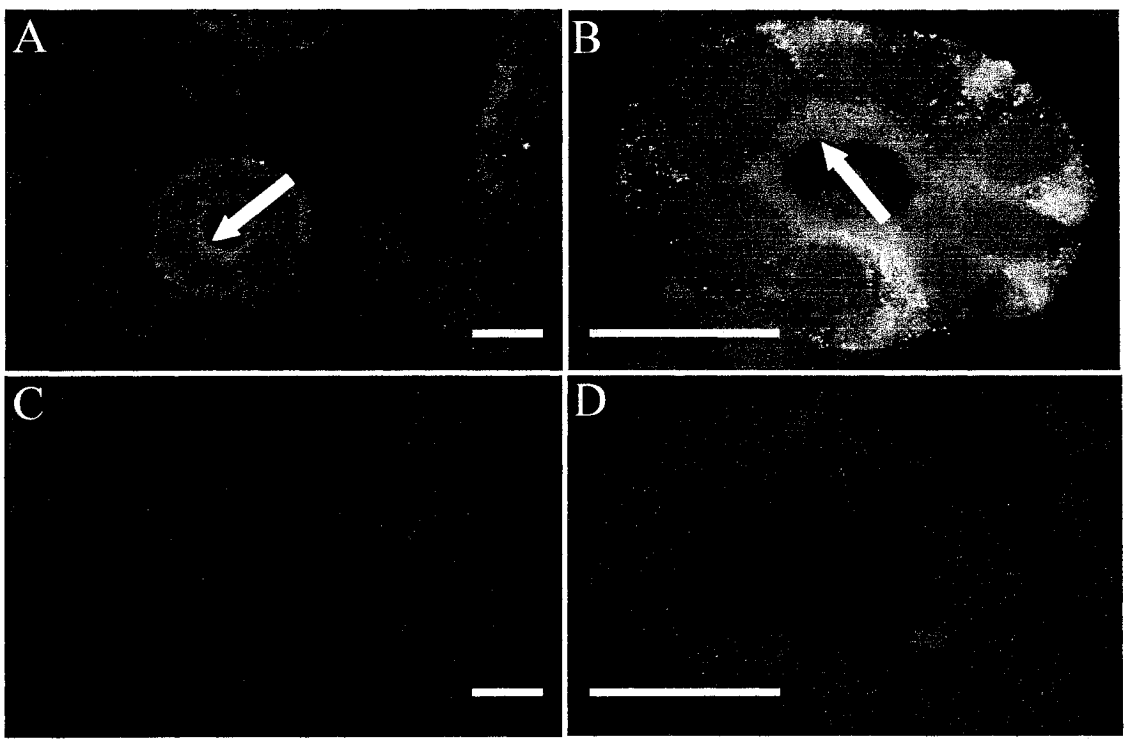

A
B
C
D

Figure 2-7. Representative light micrographs revealing the presence of trout CA IV and Na^+/K^+-ATPase proteins in renal tubules by fluorescence immunocytochemistry. Green colour indicates CA IV, red colour indicates Na^+/K^+-ATPase and nuclei are stained blue (DAPI). Panels A - C show different tubules viewed at 40, 63 and 100X magnification, respectively. In cells co-expressing the two proteins, CA IV was localized to the apical regions of tubules (arrows) whereas Na^+/K^+-ATPase was confined to basolateral regions. In some tubules not expressing CA IV, Na^+/K^+-ATPase was broadly distributed. Specific fluorescence was prevented by (D) preabsorption of the CA IV antibody with immunizing peptide or (E) omission of both primary antibodies. Scale = 25 μm.

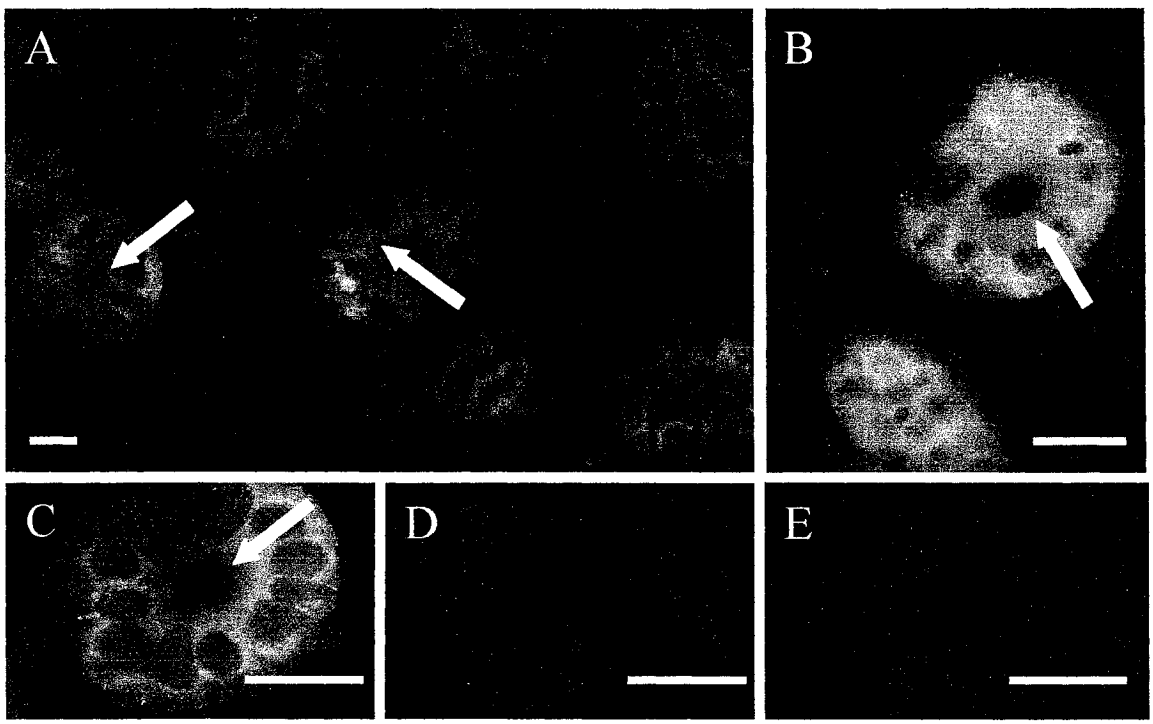

A
B
C
D
E

Figure 2-8. Representative light micrographs showing the localization of (A, B) trout cytoplasmic CA (tCAc) and (C, D) trout CA IV to proximal (white or blue arrows) or distal (red arrows) tubules. 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 (A, B). In contrast, tCA IV appeared to be exclusively localized to proximal tubule cells (C, D). Scale = 25 μm.

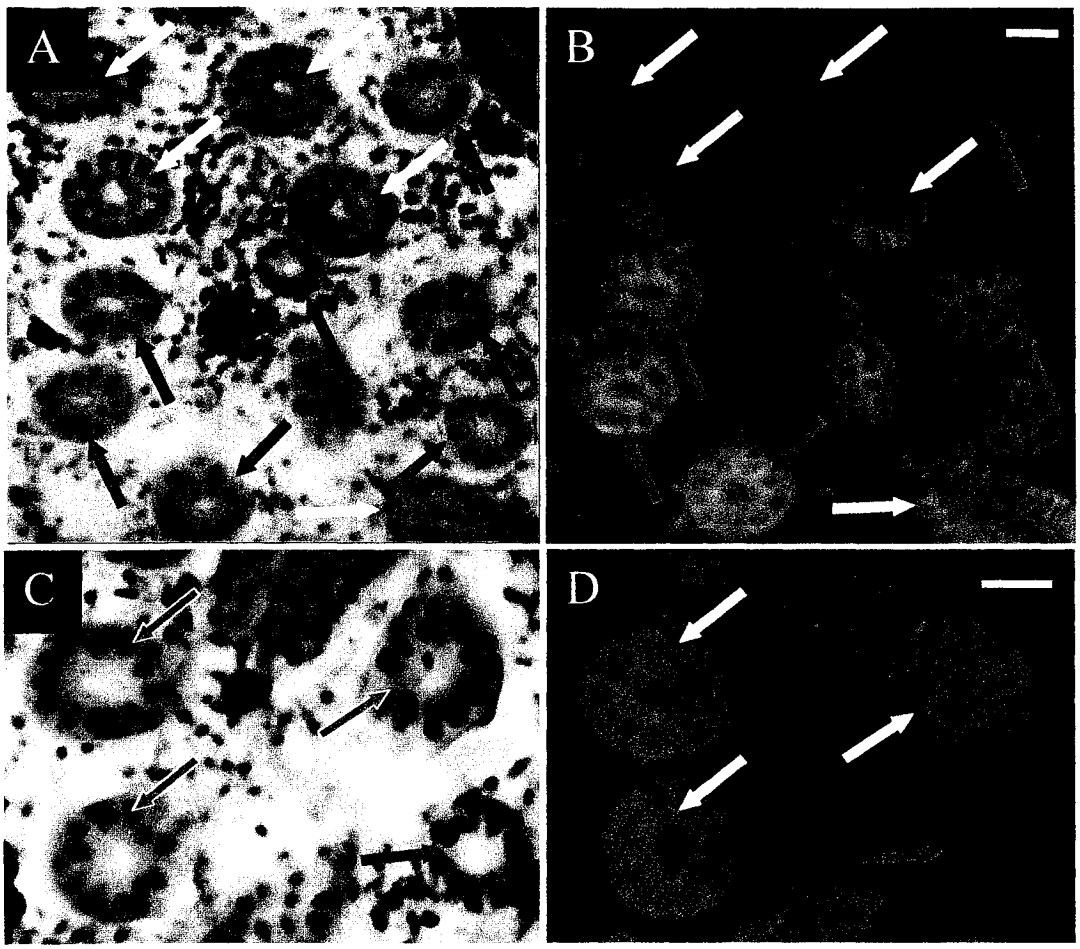

A
B
C
D

Figure 2-9. The effects of exposure to hypercapnia (6.0 mm Hg) on relative levels of (A) trout cytoplasmic CA (tCAc) or (B) trout CA IV mRNA levels in posterior kidney. Control (cross-hatched) data (taken at 3, 6, 12 and 24 h) were pooled and assigned a value of 1 and statistically compared to the corresponding hypercapnia periods (only the 3 h control data, however, were used to statistically analyse the 1, 2 and 3 h hypercapnia groups); statistical differences (two-tailed one sample Student's t-test; $P < 0.05$) from the corresponding control group are denoted by asterisks.

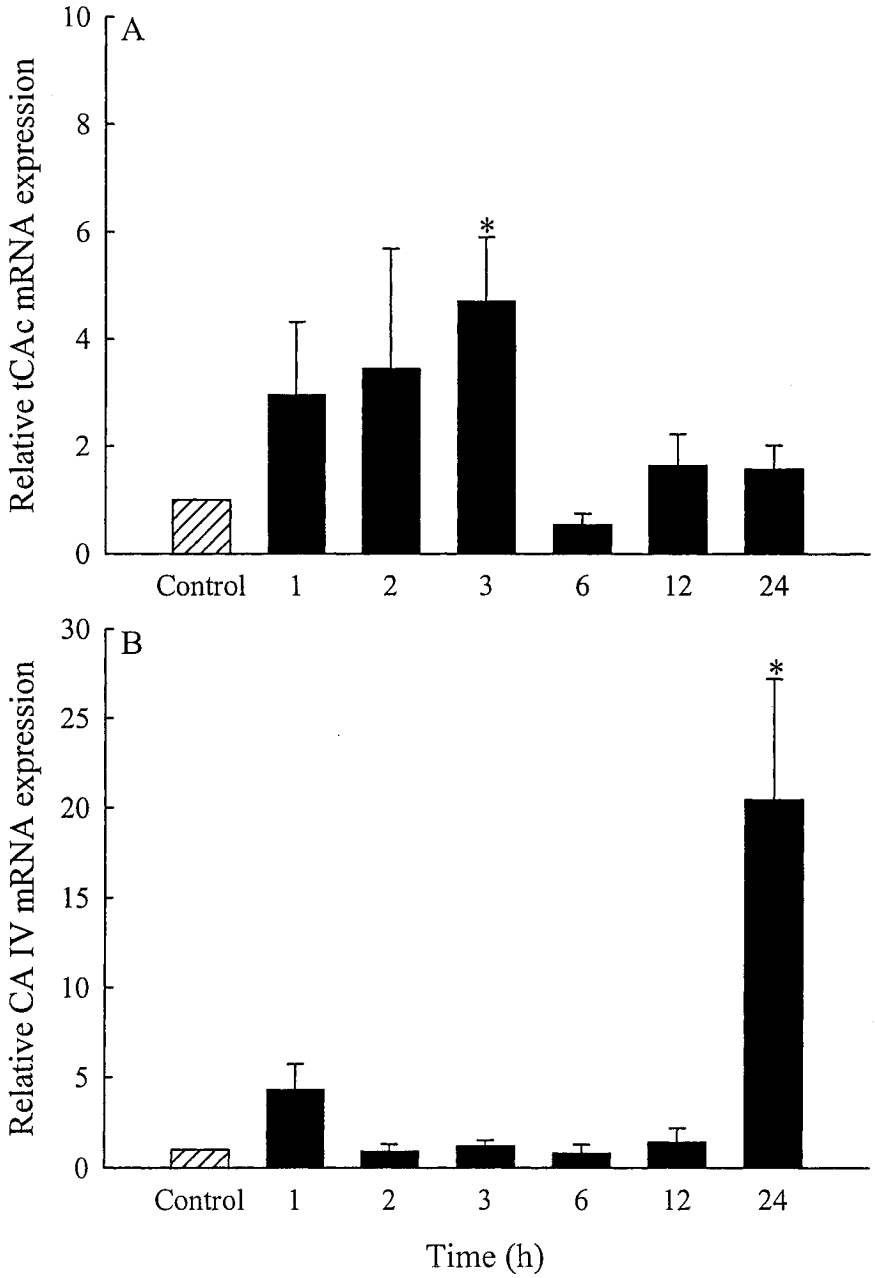

A
Relative tCAc mRNA expression
10
8
6
4
2
0
*
Control
1
2
3
6
12
24
B
Relative CA IV mRNA expression
30
25
20
15
10
5
0
*
Control
1
2
3
6
12
24
Time (h)

Figure 2-10. Changes in trout cytoplasmic CA (tCAc) protein levels in posterior kidney after 24 h of hypercapnia as determined quantitatively by (A, B) western blots (N = 5 each for normocarbia and hypercapnia) or qualitatively by (C, D) fluorescence immunocytochemistry. Using a human CA II antibody, western blots revealed an immunoreactive band in kidney tissue at 29 kDa; the antibody also cross-reacted with bovine CA II, a component of the protein marker ladder. Densitometric analysis of blots from (A) normocarbic or (B) hypercarbic tissues revealed a significant increase in tCAc protein levels during hypercapnia. This result was consistent with examination of immunofluorescence micrographs derived from (C) normocarbic and (D) hypercarbic tissues. See Figure 6 for further details. Scale = 25 μm.

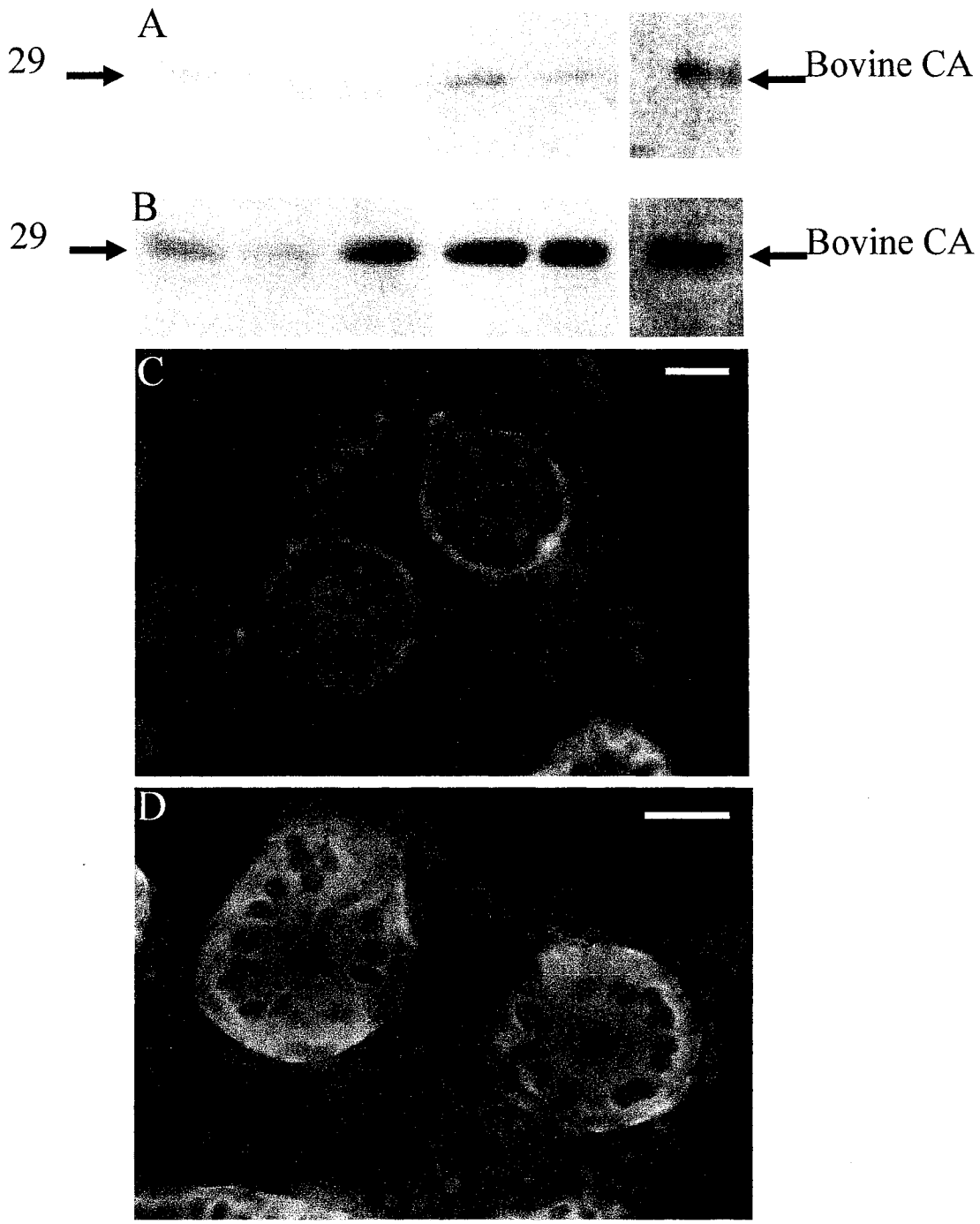

A
29
Bovine CA
B
29
Bovine CA
C
D

Figure 2-11. Changes in trout CA IV protein levels in posterior kidney after 24 h of hypercapnia as determined quantitatively by (A, B) western blots (N = 6 each for normocarbia and hypercapnia) or qualitatively by (C, D) fluorescence immunocytochemistry. Using a homologous CA IV antibody (see Figure 3), densitometric analysis of blots from (A) normocarbic or (B) hypercarbic tissues revealed a significant increase in CA IV protein levels during hypercapnia. This result was consistent with examination of immunofluorescence micrographs derived from (C) normocarbic and (D) hypercarbic tissues. See Figure 2-7 for further details. Scale = 25 μm.

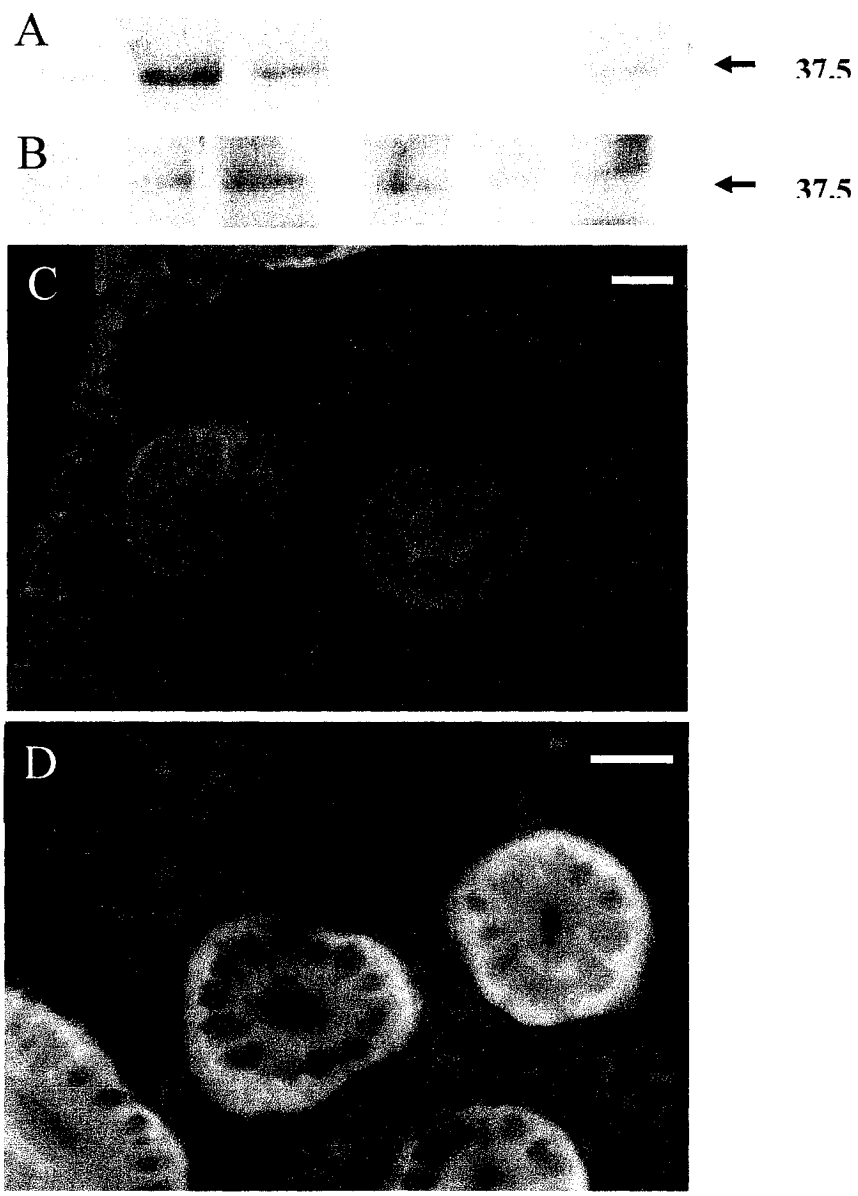

A
37.5
B
37.5
C
D

Figure 2-12. The effects of total CA inhibition using acetazolamide (A – C) or specific inhibition of extracellular CA using F3500 (D – E) on arterial blood acid base status when administered under control conditions or during hypercapnia. Filled bars represent values prior to administration of CA inhibitors; unfilled bars represent values 3 h after administration of inhibitors. All values are represented as means ± SEM; values not sharing common letters are statistically significant ($P < 0.05$).

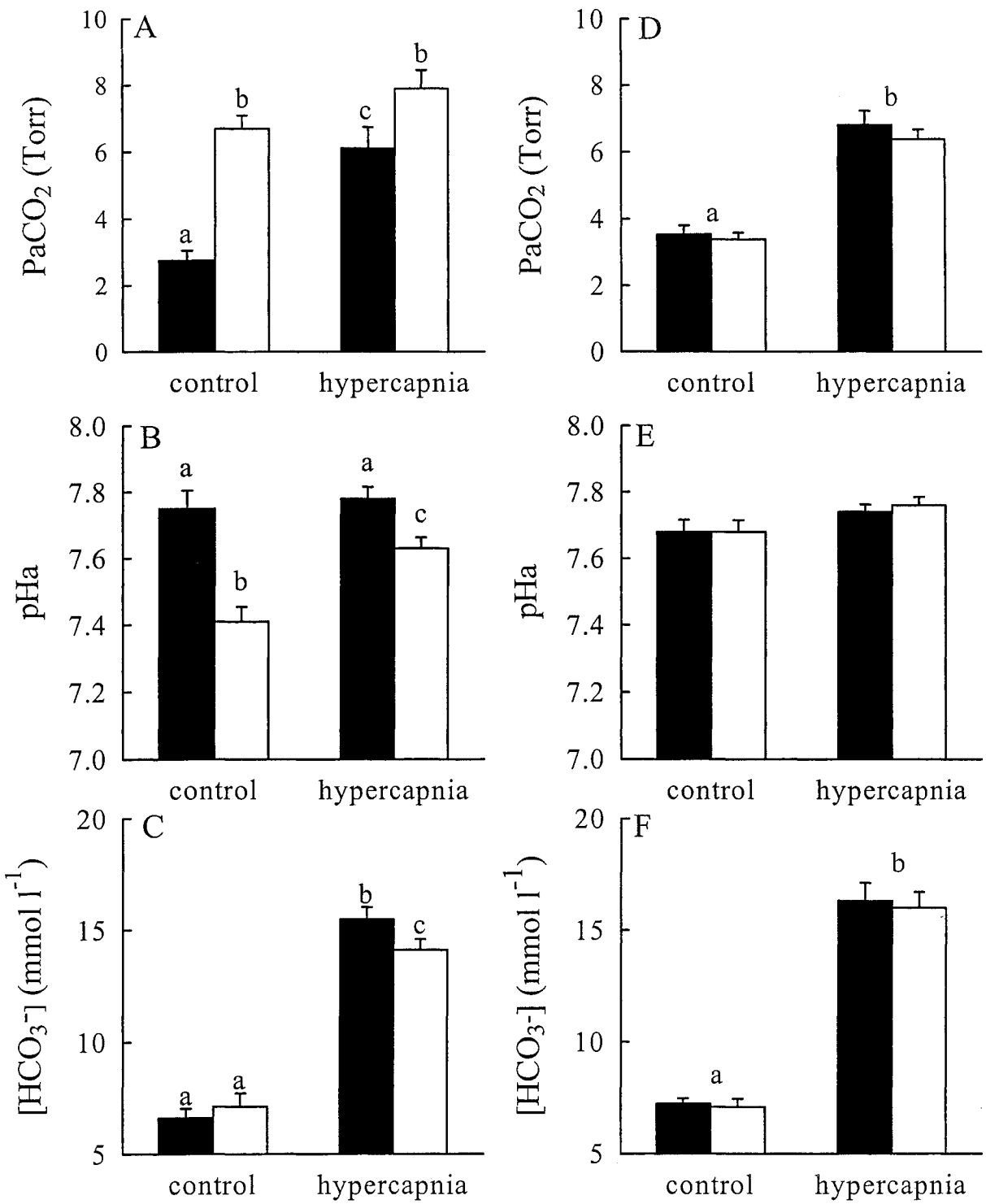
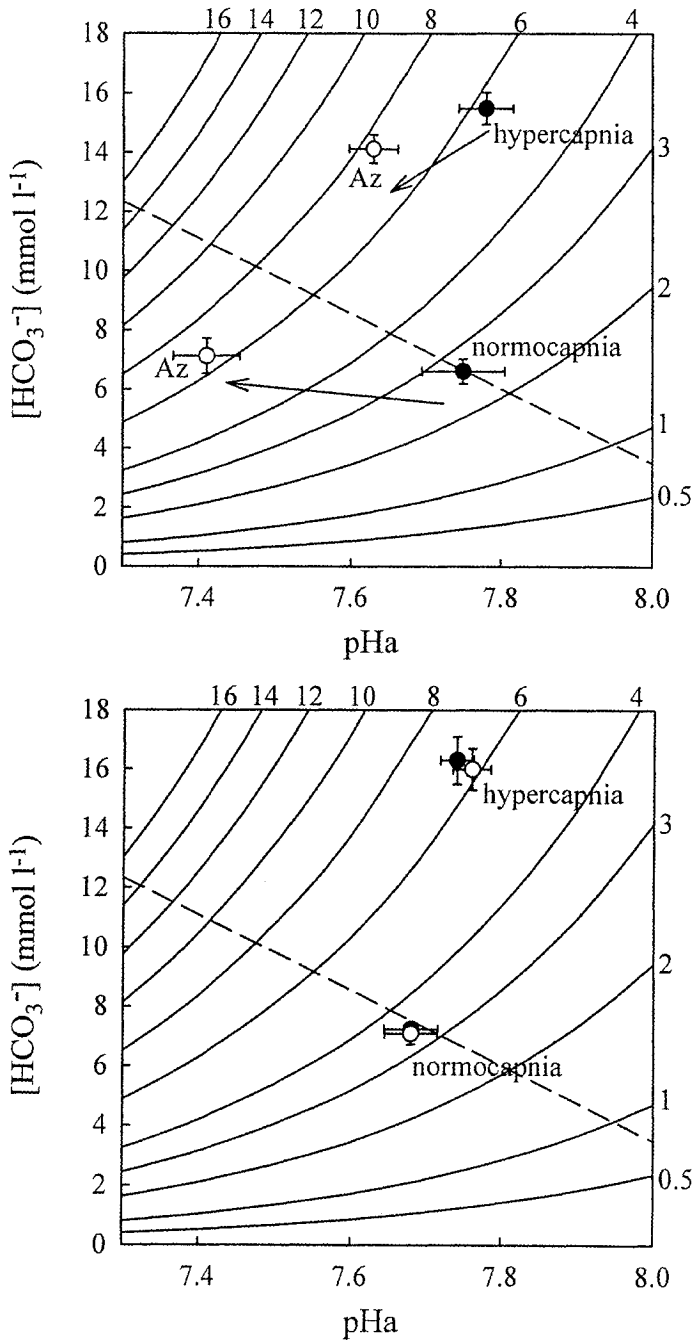

A
B
C
D
E
F
PaCO2 (Torr)
pHa
[HCO3-] (mmol l-1)
control
hypercapnia
a
b
c

Figure 2-13. pH-HCO_3^- diagrams illustrating the effects of intra-arterial injections of A) acetazolamide or B) F3500 on arterial blood acid-base status in rainbow trout during normocarbia or hypercapnia. Solid lines represent PCO_2 isobars and the dashed lines represent the *in vitro* non-HCO_3^- buffer line (-10 mmol l^{-1} pH $unit^{-1}$; Perry and Vermette, 1987). Pre-injection values are represented by filled symbols; post-injection values are represented by unfilled symbols. Data are presented as means ± SEM.



● Pre-injection
○ Post-injection

Figure 2-14. The effects of total CA inhibition using acetazolamide (A – C) or specific inhibition of extracellular CA using F3500 (D – E) on (A, B) renal net acid flux, (C, D) renal titratable acidity flux ($J_{TA-HCO3}$) and (E, F) renal ammonia excretion (J_{NH4+}) when administered under control conditions or during hypercapnia. Filled bars represent values prior to administration of CA inhibitors; unfilled bars represent values 3 h after administration of inhibitors. All values are represented as means ± SEM; values not sharing common letters are statistically significant ($P < 0.05$).

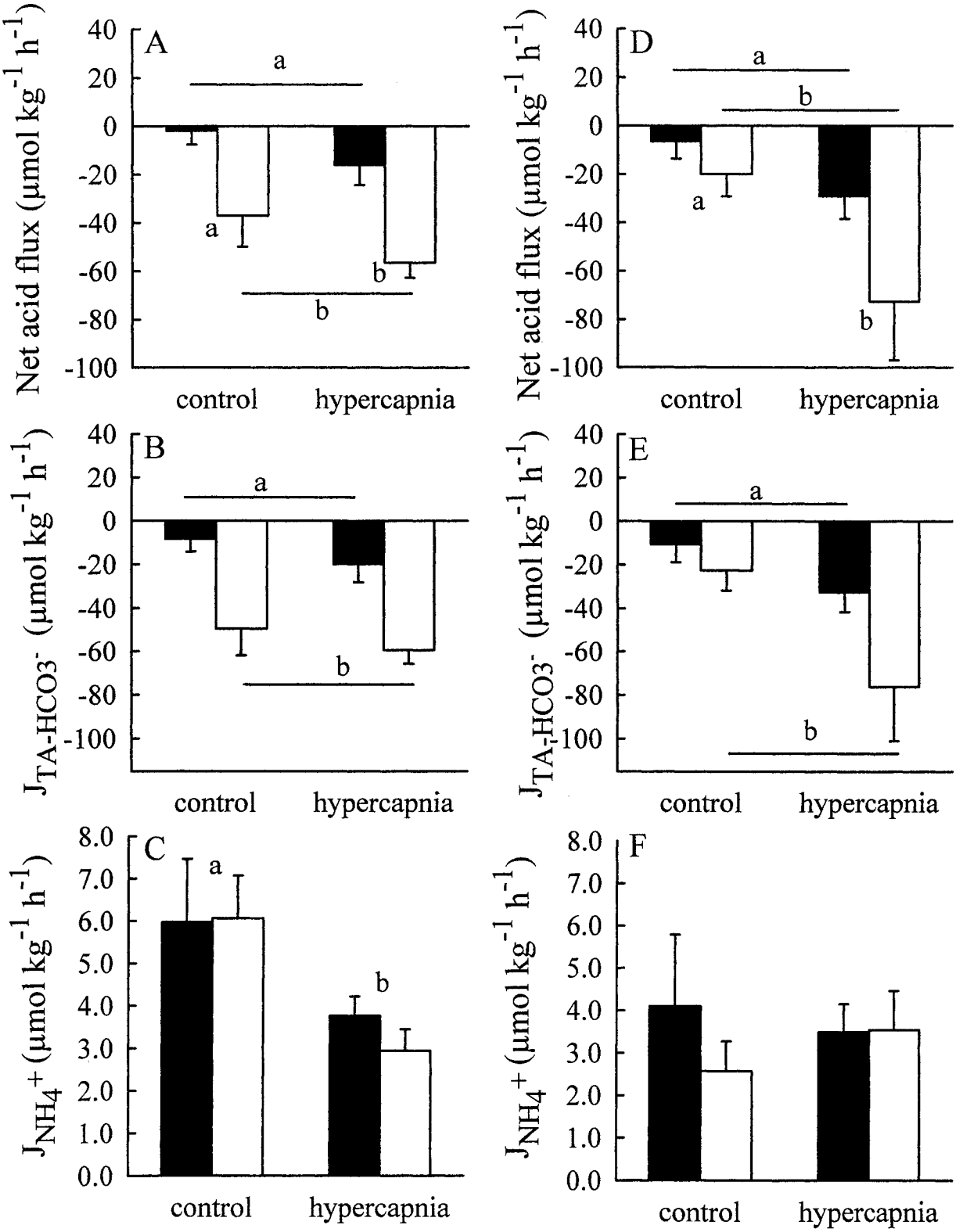

A
B
C
D
E
F
Net acid flux (µmol kg-1 h-1)
JTA-HCO3- (µmol kg-1 h-1)
JNH4+ (µmol kg-1 h-1)
40
20
0
-20
-40
-60
-80
-100
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
b
control
hypercapnia

Figure 2-15. The effects of total CA inhibition using acetazolamide (A – C) or specific inhibition of extracellular CA using F3500 (D – E) on (A, B) urine pH, (C, D) urine HCO_3^- levels and (E, F) urine sodium levels when administered under control conditions or during hypercapnia. Filled bars represent values prior to administration of CA inhibitors; unfilled bars represent values 3 h after administration of inhibitors. All values are represented as means ± SEM; values not sharing common letters are statistically significant ($P < 0.05$).

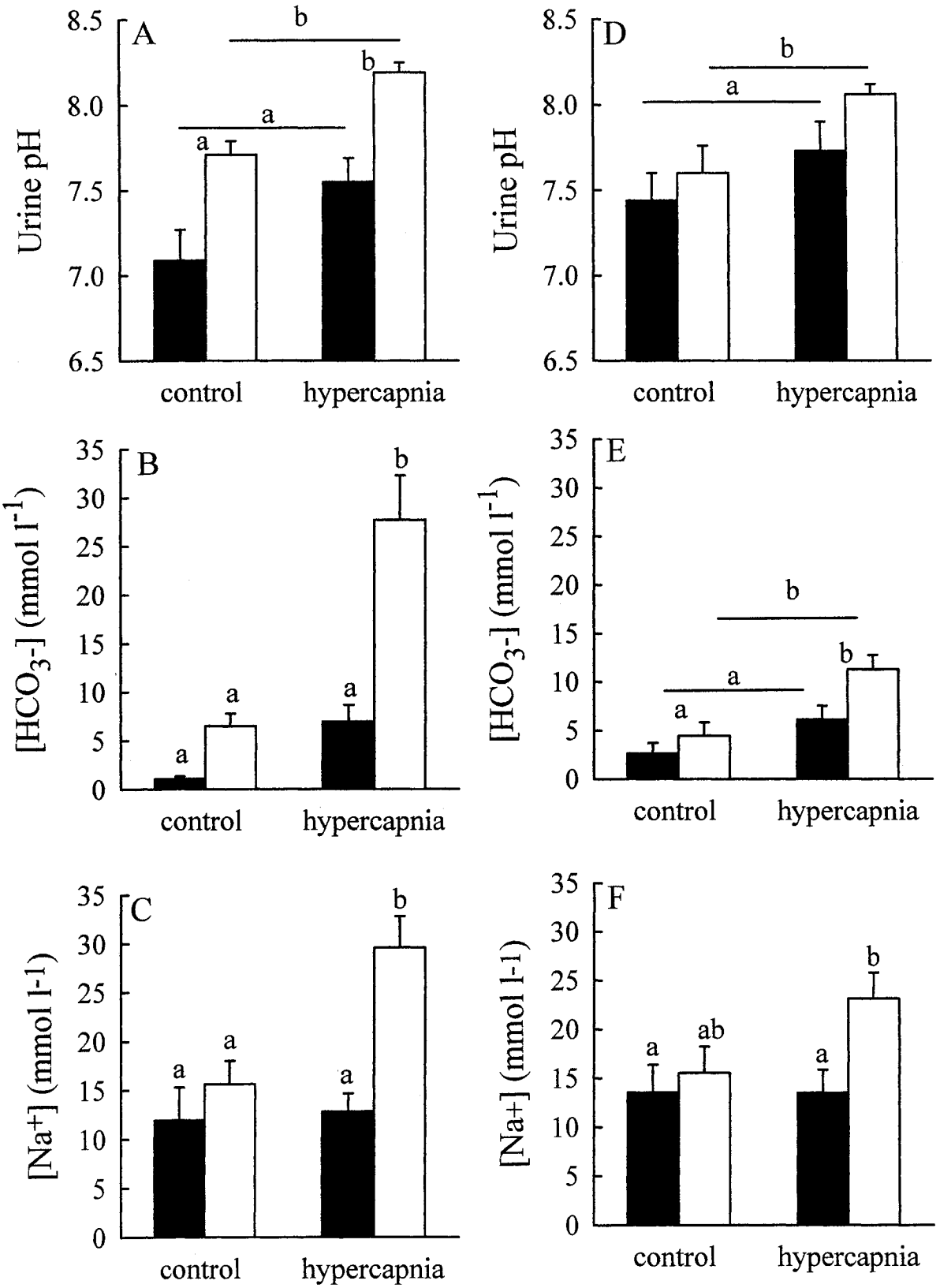

A
B
C
D
E
F
Urine pH
[HCO3-] (mmol l-1)
[Na+] (mmol l-1)
8.5
8.0
7.5
7.0
6.5
35
30
25
20
15
10
5
0
a
ab
b
control
hypercapnia

Table 2-1: The interactive effects of hypercapnia and carbonic anhydrase (CA) inhibition on urine flow rate (UFR, ml kg^{-1} h^{-1}) and urine concentrations (in mmol l^{-1}) of K^+, Ca^{2+},and Cl^- in rainbow trout (*Oncorhynchus mykiss*). Acetazolamide was used to inhibit total CA activity whereas F3500 was used to selectively inhibit apical membrane-associated CA. Data are presented as means ± 1 SEM; N values are indicated in parentheses. Statistical differences from the pre-injection values are indicated with asterisks.

	Acetazolamide				F3500			
	Control (8)		Hypercapnia (8)		Control (8)		Hypercapnia (8)	
	pre-injection	post-injection	pre-injection	post-injection	pre-injection	post-injection	pre-injection	post-injection
UFR	5.68 ± 1.18	7.76 ± 1.00*	3.78 ± 0.61	3.31 ± 0.24	4.59 ± 0.71	3.75 ± 0.70	4.6 ± 0.48	5.27 ± 1.53
$[K^+]$	1.01 ± 0.13	1.33 ± 0.18	1.16 ± 0.13	1.60 ± 0.44	0.97 ± 0.27	1.15 ± 0.34	1.39 ± 0.52	1.85 ± 0.62
$[Ca^{2+}]$	1.72 ± 0.24	1.69 ± 0.19	1.66 ± 0.12	1.76 ± 0.22	1.55 ± 0.15	1.66 ± 0.08	1.75 ± 0.18	1.95 ± 0.17
$[Cl^-]$	10.06 ± 3.32	8.35 ± 3.28	5.7 ± 1.61	6.86 ± 2.73	8.91 ± 3.16	9.64 ± 3.77	9.59 ± 5.36	9.41 ± 4.42

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 HCO_3^- reabsorption (Wood and Jackson 1980; Wheatly et al. 1984; Perry et al. 1987). Such increases in tubular HCO_3^- reabsorption are reliant on a stimulation of tubular H^+ secretion via increased Na^+/H^+ exchange (NHE3) activity (Wu et al. 1996). Based on the results of numerous studies, urinary acidification and HCO_3^- reabsorption in mammals are known to depend on the activities of cytosolic (CA II) and membrane-associated (CA IV) isoforms of CA (Schwartz 2002; Swenson 2000). In contrast, only a single study (Nishimura 1977) has implicated renal CA in the promotion of HCO_3^- reabsorption in freshwater fishes and that study did not attempt to distinguish between the roles of cytosolic and membrane-associated CA's. Thus, the results of the present study are significant because they provide compelling evidence for the involvement of both cytosolic and membrane-associated CA in urinary acidification and HCO_3^- reabsorption in a freshwater fish, the rainbow trout. To our knowledge, this is the first study to implicate CA IV in acid-base regulation in a non-mammalian vertebrate.

CA isoforms in rainbow trout

Prior to this study, two cytosolic CA isoforms were cloned, sequenced and partially characterised in rainbow trout (Esbaugh et al. 2005), a broadly distributed isoform termed tCAc and a rbc specific form termed (tCAb). Although these trout

isoforms closely resemble the tetrapod CA II isoform, phylogenetic analysis clearly demonstrated that the trout genes for cytosolic CA's originated prior to a gene duplication event that gave rise to CA's I, II and III in tetrapods (Esbaugh et al. 2005). 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 CA's of teleost fish.

In this study we have cloned an additional CA isoform from rainbow trout kidney that most closely resembles a mammalian membrane bound isoform, CA IV. Although no functional characterisations 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 IV's (Okuyama et al. 1992; Winkler et al. 1997; Fleming et al. 1993), 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) (Okuyama et al. 1992). It is noteworthy that the two trout cytosolic isoforms of CA lack disulfide bridges and consequently exhibit sensitivity to SDS treatment (Esbaugh et al. 2005). 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 C-terminus that serves to tether the enzyme to the apical plasma membrane (Schwartz 2002). A probable anchor site in

the trout sequence is a serine residue at position 277 just prior to a hydrophobic domain (AA's 282 – 292) (Eisenhaber et al. 1999).

Using the recently acquired tCAIV sequence (this study) and the previously published sequence of tCAc (Esbaugh et al. 2005), various tools were developed to examine the expression of these genes in trout kidney and their responses to respiratory acidosis.

Localisation 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 using the human antibody. This same human antibody was used successfully in a previous study to localize cytosolic CA in the inner ear of salmon (Tohse et al. 2004).

tCAc and tCA IV were both expressed in the posterior kidney. Interestingly, for tCA IV, slightly different results were obtained when evaluating mRNA *versus* protein levels. When assessing mRNA either by northern blot or real time PCR, the posterior kidney was the predominant site of tCA IV expression. However, when assessing protein, the highest levels of tCA IV were found in brain with the heart, kidney and white muscle also expressing detectable quantities. Although we are currently unable to explain the discrepant results, the important finding for the purposes of this study, was that tCA IV is present in kidney and thus potentially able to participate in renal HCO_3^- reabsorption.

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* (Gilmour et al. 2001) and two Antarctic species (*Chaenocephalus aceratus* and *Notothenia coriiceps* (Tufts et al. 2002)), the heart of lamprey (*Petromyzon marinus*; (Esbaugh and Tufts 2004) and the kidney of the marine winter flounder (*Pleuronectes americanus* (Pelis et al. 2003). Additionally, we have recently cloned and sequenced CA IV from the gill of dogfish (S.F. Perry et al., GenBank accession number DQ092628) where it is believed to play an important role in CO_2 excretion (Gilmour and Perry 2004). Current models for CO_2 excretion in trout and other teleosts contend that CO_2 excretion depends exclusively on cytosolic CA (Perry and Gilmour 2002). 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 (Gilmour et al. 2002; Henry et al. 1997; reviewed by Gilmour 1998a, b).

The results of *in situ* hybridization, immunocytochemistry and conventional histology demonstrated that tCAc and tCA IV were localised to a subset of renal tubules. tCA IV appeared to be exclusively localised to the apical membrane/brush border of proximal tubule cells whereas tCAc was associated with the apical regions of proximal and distal tubules. Although it has been shown that the proximal tubule of trout, like in mammals, is composed of discrete sections (e.g. P1 and P2 in fish) (Anderson and Loewen 1975), 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 membrane of S2 and S1 proximal tubules

(Schwartz et al. 2000). 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 (see below) suggest that the apical membrane CA IV is externally oriented and thus able to catalyse reactions within the lumen of the proximal tubule.

Cytosolic and membrane-associated CA's 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 to a permeant CA inhibitor, acetazolamide (Maren 1967), and a non-permeant inhibitor, F3500 (Conroy et al. 1996). F3500 is a polymer of aminobenzolamide that is small enough to be filtered by the kidney yet too large to enter cells (Conroy et al. 1996). Previous research on mammals (Maren et al. 1997) and fish (Gilmour and Perry 2004) have shown that it does not inhibit red blood cell CA activity. Thus, in this study we have 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 empirical data to support the view (Perry et al. 2003) that both cytosolic and membrane associated CA's are involved in renal acid secretion and HCO_3^- reabsorption in freshwater rainbow trout. The membrane-associated CA IV, because of its exposure to the tubular lumen, is able to catalyse the dehydration of filtered HCO_3^-. The CO_2 thus formed diffuses into the tubule cells where cytosolic CA catalyses its hydration to HCO_3^- and H^+. The HCO_3^- is moved across the basolateral membrane via a Na^+/HCO_3^- co-transporter (NBC1) (reviewed by Soleimani 2002; Romero and Boron 1999) whereas the H^+ enters the lumen via a Na^+/H^+ exchanger and V-type ATPase (Swenson 2000).

The relative involvement of CA IV and tCAc in renal acidification and HCO_3^- reabsorption appears to be dependent on the existing acid-base status of the blood. Under normocapnic conditions, the selective inhibition of CA IV using F3500 was without significant effect on renal acid excretion or urine HCO_3^- levels. However, under hypercapnic conditions, CA IV was shown to play a significant role in renal HCO_3^- reabsorption as indicated by reduced acid excretion and elevated levels of urinary HCO_3^- after treatment with F3500. The increased importance of CA IV in renal HCO_3^- reabsorption during hypercapnia is consistent with its increased expression after 24 h coupled with a greater filtered HCO_3^- load. The larger effect of acetazolamide on renal acid excretion and urine HCO_3^- levels demonstrated that the cytosolic CA isoform (tCAc) plays a more important role than the membrane associated isoform (tCA IV) in renal HCO_3^- reabsorption. This is in agreement with the results of a previous study showing that the effect of CA inhibition on renal HCO_3^- reabsorption using F3500 was

approximately 40% of that after total CA inhibition using acetazolamide (Maren et al. 1997).

In summary, under resting conditions, renal HCO_3^- reabsorption relies on H^+ extrusion into the filtrate within the proximal tubule, a process that is dependent upon the catalysed hydration of CO_2 by cytosolic CA. During hypercapnia, acid-base regulation is achieved by the accumulation of HCO_3^- within the plasma as a result of large increases in branchial acid output. The kidney prevents the additional filtered HCO_3^- from being excreted by increasing acid secretion and HCO_3^- reabsorption. The increase in the expression of tCAc during hypercapnia presumably facilitates additional H^+ secretion while the greater expression of tCA IV likely reflects the increasing need to dehydrate the additional filtered HCO_3^-.

CHAPTER 3

THE ROLE OF BRANCHIAL CARBONIC ANHYDRASE IN ACID-BASE REGULATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Chapter 3 will be submitted as a manuscript to *Journal of Experimental Biology*, authors Georgalis, Perry and Gilmour.

Abstract

The objective of the present study was to examine the branchial distribution of the recently identified rainbow trout cytoplasmic carbonic anhydrase isoform (tCAc; Esbaugh et al. 2005), and to investigate its role in the regulation of acid-base disturbances in rainbow trout (*Oncorhynchus mykiss*). *In situ* hybridization using a oligonucleotide probe specific to tCAc revealed tCAc mRNA expression in both pavement cells and mitochondria-rich cells. Similarly, tCAc immunoreactivity was localized to pavement cells and mitochondria-rich cells in the interlamellar region and along the lamellae of the gills of rainbow trout using a polyclonal antibody raised against a 20 amino acid region specific to tCAc. Exposure of rainbow trout to hypercapnia (~ 0.8% CO_2) for 24 h resulted in significant increases in both tCAc mRNA expression (quantified by real-time PCR) and protein levels (quantified by western analysis). This hypercapnia-induced up-regulation of tCAc expression was of benefit *in vivo* in correcting the acid-base disturbance associated with hypercapnia, as *in vivo* inhibition of branchial CA activity resulted in a significantly greater reduction in net acid excretion in hypercapnia-exposed trout than in trout held in normocapnic water. The results of the present study support the widely-held premise that branchial intracellular carbonic anhydrase activity plays a key role in regulating acid-base balance in freshwater teleost fish.

Introduction

A plethora of evidence has clearly established the gills of freshwater fish as the predominant site of acid-base relevant ion transfer, i.e. the transfer of H^+ and/or HCO_3^-, for the maintenance of systemic pH (reviewed by Perry and Laurent 1990; Evans et al. 2005; Claiborne et al. 2002). Carbon dioxide entering gill epithelial cells may be hydrated to HCO_3^- and H^+ in the presence of the enzyme carbonic anhydrase (CA). Regulation of extracellular pH is then achieved by adjusting the rate of either acid excretion (via coupled H^+ excretion and Na^+ uptake) or base excretion (via Cl^-/HCO_3^- exchange) (Cameron et al. 1976; Wood. et al 1984; Claiborne 1984; Perry et al. 2003). For example, compensation for extracellular acidosis is accomplished in rainbow trout primarily by HCO_3^- accumulation via the gills (Goss et al. 1992). The accumulated HCO_3^- must be retained during urine formation, but in contrast to the key role played by the kidney in regulating acid-base disturbances in tetrapod vertebrates (Levine et al. 1986), in teleost fish less than 5% of the net acid excretion required to re-establish acid-base homeostasis is carried out by the kidney (Heisler 1988). Morphological adjustments of the gill epithelium contribute to the regulation of systemic acid-base disturbances in freshwater teleosts (Goss et al. 1995). Extracellular acidosis elicits a reduction in the surface area of chloride cells that is exposed to the water, whereas an increase in surface area occurs under alkalotic conditions. These responses aid in the restoration of acid-base homeostasis because chloride cells are thought to be the site of branchial Cl^-/HCO_3-exchange; thus, for example, the reduction in exposed chloride cell area during acidosis

lowers HCO_3^- excretion, contributing to the accumulation of HCO_3^- that serves to compensate for the extracellular acidosis (Goss et al. 1995).

One component of the model for branchial acid-base regulation that has received little attention to date is the role played by branchial intracellular CA activity. High levels of CA activity have been found in the gills of all fish species examined to date (Henry and Swenson 2000). In teleost fish, this branchial CA activity is restricted to the cytoplasm (Gilmour 1998; Henry and Swenson 2000), although in elasmobranch and chimaeran fish, membrane-associated branchial CA activity is also present (Gilmour et al. 2002). Immunohistochemical evidence indicates that branchial cytoplasmic CA is present in both pavement and chloride cells, with a generally apical location (Rahim 1988; Sender et al. 1999; Wilson et al. 2000). Rahim et al. (1988) also provided immunological evidence for the presence of distinct branchial and blood CA isoforms in rainbow trout and carp, a finding that was recently confirmed by the cloning of rainbow trout blood (tCAb) and cytosolic (tCAc) CA isoforms (Esbaugh et al. 2005). Northern and real-time PCR analyses identified the gills as a site of considerable tCAc mRNA expression (Esbaugh et al. 2005).

With this background in mind, the present study was conceived with three objectives. The first objective was to provide further support for the presence of a distinct CA isoform, tCAc, in rainbow trout gills by localizing tCAc mRNA expression and immunoreactivity using *in situ* hybridization and immunohistochemistry. A second goal was to provide experimental support for the hypothesis that tCAc plays a significant role in the regulation of systemic acid-base balance. This goal was accomplished by estimating acid excretion during inhibition of branchial CA activity. The third and main

objective of the present study was to test the hypothesis that upregulation of branchial CA activity (specifically tCAc) is one component of the response to an acid-base challenge. Relatively little information exists on the impact of environmental factors, particularly those imposing an acid-base challenge, on branchial CA activity. Dimberg and Hoglund (1987) reported significant increases in branchial CA activity in rainbow trout at 20 to 80 days of exposure to hypercapnia. However, physiological compensation for the acidosis elicited by exposure to hypercapnia is typically accomplished in a relatively short time frame, e.g. 24 to 72 h (Goss and Perry 1993). Thus, in the present study the impact of acute (24 h or less) hypercapnic exposure on tCAc mRNA expression and immunoreactivity were assessed using real-time PCR and by western analysis in conjunction with immunohistochemistry, respectively. To test the hypothesis that hypercapnia-induced changes in tCAc expression contribute to the compensatory responses that restore acid-base balance during hypercapnia, acid efflux and blood acid-base variables were assessed during inhibition of branchial CA activity by acetazolamide.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*; approximate mass 250 g, $N = 65$), were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish were maintained on a 12L:12D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechloraminated city of Ottawa tapwater at 13°C. Fish were allowed to acclimate to the holding conditions for at least 2 weeks prior to any experimentation, and were fed to satiation on commercial trout pellets every second day.

Series I: Molecular analysis of rainbow trout gill cytoplasmic CA (tCAc)

Experimental protocol and tissue collection

To expose rainbow trout to the acid-base challenge imposed by hypercapnia, fish were placed in individual opaque acrylic boxes for a 24 h acclimation period and were then exposed to external hypercapnia (final nominal water CO_2 tension, $PwCO_2$ = 6.5 mm Hg) for up to 24 h. To achieve the final $PwCO_2$, a water equilibrium column supplying the experimental chambers was gassed with 2% CO_2 in air ($PwCO_2$ = 14 mm Hg) to achieve ~0.8% in the water in the holding boxes. Mixed gases were provided using gas mixing flowmeters (Smart-track, Sierra Intruments, C100 L-LE DD 20V1SV1 PV2 V1 S1 CO). The PCO_2 of water exiting the equilibration column to feed the experimental chambers was monitored using a CO_2 electrode (Cameron Instruments) housed in a thermostatted cuvette and linked to a meter (Cameron Instruments) and data acquisition system (Biopac, version 3.7.3). Fish allocated to control groups were treated

as described above, except that the equilibration column supplying the experimental chambers was gassed with air rather than CO_2-enriched air.

Gill tissue was sampled from trout exposed to hypercapnia or control conditions for analysis of tCAc mRNA expression by real-time PCR or *in situ* hybridization, for immunohistochemical localization of tCAc, or for measurement of tCAc protein abundance by western analysis. In addition, blood, brain, heart, spleen, liver, white muscle and anterior and posterior kidney tissue were sampled from trout exposed to control conditions for analysis of tCAc protein distribution by western analysis. In all cases, trout were killed by a blow to the head. Where tissue was used for western analysis or immunohistochemistry, saline perfusion was carried out prior to tissue collection to clear the tissues of blood. Perfusions were performed by exposing and cannulating the bulbus arteriosus with polyethylene tubing (PE 160; Clay-Adams). Approximately 50 ml of ice-cold, heparinized (100 IU ml^{-1} heparin) Cortland's saline (Wolf, 1963) containing 10^{-5} mol l^{-1} isoproterenol was then infused via the cannula; the ventricle was severed during this infusion to allow fluid in the circulatory system to drain from the body. In most cases, the collected tissues were immediately frozen in liquid N_2 and stored at -80° C until analysis. Tissues used for *in situ* hybridization or immunohistochemistry were immersion fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH = 7.4) overnight, then transferred to 15% sucrose followed by 30% sucrose for 2 h in each case; tissues were kept at 4°C throughout the fixation procedure and were then stored at -20°C until use.

Analysis of tCAc mRNA expression

Real-time PCR was used to quantify branchial tCAc mRNA expression in control versus hypercapnia-exposed trout, while *in situ* hybridization was used to localize tCAc mRNA within the branchial epithelium.

For the quantification of tCAc mRNA expression by real-time PCR, gill tissue was sampled from hypercapnia-exposed trout at 1, 2, 3, 6, 12 and 24 h ($N = 6$ at each time), and from control trout at 3 h (representing a control point for 1-3 h), 6, 12 and 24 h. Total RNA was extracted from 30 mg aliquots of powdered tissue samples using the 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) for 15 min at 37°C. The RNA was eluted in 70 µl of nuclease-free H_2O 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).

tCAc mRNA levels were assessed by real-time PCR on samples of cDNA (0.5 µl) 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 primer = 300 nmol l^{-1}; 2x Master Mix = 12 µl; ROX = 1:30000 final dilution. The annealing and extension temperatures over 40 cycles were 58° C (30 sec) and 72° C (30 sec), respectively. The primer pairs used were those designed and reported by Esbaugh et al. (2005; see Appendix 1): β-actin forward (5'-CCA ACA GAT GTG GAT CAG CAA-3'), β-actin reverse (5'-GGT GGC ACA GAG CTG AAG TGG TA-3'), tCAc forward (5'-CAG TCT CCC ATT GAC ATC GTA-3') and tCAc

reverse (5'-CGT TGT CGT CGG TGT AGG T-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 non-specific amplicons during the real time PCR runs, the PCR products were analysed by gel electrophoresis in initial experiments. Single bands of the expected size were obtained in all times. 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 a modification of the delta-delta Ct method (Pfaffl, 2001). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

For the localization of tCAc mRNA by *in situ* hybridization (which was carried out with the aid of Brian McNeill), a 48-mer oligonucleotide probe (5'-CCAGGTACGATGTCAATGGGAGACTGGCGGGGTCCGTTGGCAACCC-3') complementary to 48 nucleotides of the mRNA encoding tCAc was synthesized and DIG-labeled by Genedetect.com. Sections (8 – 10 μm) were cut using a cryostat (Leica) at -25°C, collected onto either poly-L-lysine-coated slides (Sigma) or electrostatically charged slides (SuperFrost plus, Fisher Scientific), allowed to dry for 30 min, and then stored at -20°C.

Sections were rehydrated in 1X PBS containing 0.1% Tween 20 (PBST) two times for 15 min each time, then digested for 10 – 20 min at room temperature in

proteinase K (10 – 20 μg mL^{-1} in PBST; Gibco BRL, Orand Island, NY) and rinsed in 1X PBST for 2 x 10 min. After digestion, tissues were re-fixed in 4% formaldehyde in PBS for 5 min, rinsed (2 X 10 min) in 1X PBST and air-dried at 58°C for 15 min. Probes (approximately 900 pg) were denatured for 3 min at 94° C in a solution containing 250 μg ml^{-1} of salmon sperm DNA and 250 μg of Poly A, topped to 12.5μl with DEPC H_2O. Probes were then quickly chilled on ice and centrifuged for 1 min at 7,500*g*. 100 μl of hybridization buffer (50 % deionized formamide, 1X Denhardt's, 0.2% SDS, 5% dextran sulphate, 0.75mol l^{-1} NaCl, 25 mmol l^{-1} EDTA and 25 mmmol l^{-1} PIPES) was added to each probe, the solution was then vortexed and placed on the sections. Sections were hybridized overnight at 37° C in a humid chamber. Subsequently, sections were thoroughly washed at 37° C in 2X SSC (2 X 15 min) and 0.2X SSC (2 X 15 min), followed by one wash in 0.1X SSC for 10 min at room temperature and twice in 0.1M PBS for 10 min at room temperature. For detection of hybridization, sections were incubated first with 1% goat serum, 2 mg ml^{-1} BSA in 0.1M PBS with 0.3% Triton-X at room temperature for 1 h, followed by an incubation in anti-digoxigenin conjugated to alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:1000 in the above solution overnight at 4° C. After 2 washes in 0.1 mol l^{-1} PB at room temperature for 15 min and a brief rinse in water, the slides were washed twice for 5 min in coloration buffer (100 mmol l^{-1} Tris pH 9.5, 50 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 0.1% Tween-20). Nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolyl phosphate (BCIP) tablets (Sigma) were dissolved in 10 ml of H_20 and layered over the sections. Colour was allowed to develop in a humid chamber for at least 4 h at room temperature in the dark until satisfactory coloration was achieved. The slides were then washed (2 X 15 min)

with 0.1 mol l^{-1} PBS. The slides were mounted with 60% glycerol and cover slipped. Sections were viewed using a Zeiss Axiphot light microscope and a Hamamatsu C5985 chilled CCD camera. Images were captured using Metamorph imaging system (Version 4.01).

Two types of negative control experiments were performed to assess the specificity of hybridization. In one case, probe was omitted from the hybrization protocol and in the other sections were pre-treated with excess unlabelled probe. For the latter, sections were first incubated with 5X more unlabeled probe in hybridization buffer for 3 h at 37° C. This solution was then removed from the sections and replaced with hybridization buffer containing 5X unlabeled probe and approximately 900 pg of probe, and sections were incubated overnight at 37° C and then subjected to the usual protocol.

Quantification and localization of tCAc proteins

Western analysis was used to quantify tCAc protein expression across different tissues, and in gill tissue from control versus hypercapnia-exposed trout, while immunohistochemistry was used to localize tCAc protein within the branchial epithelium. For use in western analysis and immunohistochemistry, an antiserum was raised in rabbits against a synthetic 20 amino acid peptide corresponding to a sequence unique to tCAc (peptide sequence CWNTKYPSFGDAASKSDGLA representing amino acids 122 to 141; accession number AAR99329). This peptide sequence exhibited 3 amino acid differences from the corresponding sequence for the blood-specific CA cytoplasmic isoform (tCAb; accession number AAP73748 with corresponding sequence 260 amino acids). The antiserum was purified by ELISA to yield a polyclonal antibody (Abgent, San Diego, CA).

For western analysis, tissue samples were homogenized on ice in10 ml Tris-SO_4 buffer (25 mmol l^{-1} Tris-SO_4, 0.9% NaCl, pH 7.4) containing protease inhibitors (complete^{TM} Mini protease inhibitor cocktail tablets, Roche) and 2 μg ml^{-1} pepstatin A (Sigma). Samples were held on ice for 15 min and then centrifuged at 7500 *g* for 10 min at 4° C. The supernatant was collected and stored in 100 μl samples at -80° C. Total protein concentrations in the tissue homogenates were measured by the bicinchonic acid method (micro BCA protein assay; Pierce) using BSA as the standard. Electrophoresis was carried out on a vertical mini-gel apparatus (Bio-Rad) consisting of a reducing SDS-PAGE gel, a 14% polyacrylamide separating gel, and a 4% polyacrylamide stacking gel. Appropriate volumes of sample were loaded into each well to yield protein concentrations of 100 - 150 μg per well. Gels were run for 60 min at constant voltage (120 V) and then equilibrated in transfer buffer for 30 min. Proteins were transferred to nitrocellulose membranes using a Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA) at a constant voltage (30 mA) overnight. After transfer, the membranes were blocked for 1 h in Tris-buffered Tween 20 (TBS-T) containing 5% milk powder. Membranes were then incubated with a 1:100 dilution of primary tCAc antiserum in TBS-T containing 5% milk powder for 1.5 h at 37° C. Membranes were then washed three times in TBS-T (5 min per wash) and incubated for an additional 1 h at room temperature with a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) in TBS-T containing 5% milk powder. Finally, membranes were washed three times (5 min per wash) in TBS-T and immunoreactive bands were visualized via the enhanced chemiluminescence reaction (ECL; Pierce) using a gel

documentation system (BIORAD Chemi Doc) and digital image processing software (Quantity One, version 4.1.1).

For the localization of tCAc by immunohistochemistry, sections (8 – 10 μm) were cut using a cryostat (Leica) at -25°C, collected onto either poly-L-lysine-coated (Sigma) slides or electrostatically charged slides (SuperFrost plus, Fisher Scientific), and allowed to air dry. Where appropriate, a hydrophobic barrier was created around each section with a PAP pen (Electron microscopy suppliers, Fort Washington) to allow sections to be treated individually. Mounted sections were rehydrated (3 times; 5 min each time) in a blocking buffer consisting of 0.1 X PB containing 2% normal goat serum, 0.9% Triton-X, 1% gelatin and 2% BSA, and were then incubated with one of two primary antibodies. Sections were incubated for 2 h at room temperature in a humidified chamber with a 1:200 dilution (in blocking buffer) of the polyclonal antibody raised against the 20-amino acid tCAc peptide, with a 1:100 dilution (in blocking buffer) of a monoclonal antibody (α5) specific for the α subunit of chicken Na^+,K^+-ATPase (Takeyasu et al. 1988), or with both antibodies together. The α5 hybridoma antibody developed by Douglas M Fanbrough was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA 52242, USA). This antibody has been used to describe the distribution of Na^+,K^+-ATPase in a wide range of organisms, including both elasmobranch and teleost fish (Wilson et al. 2000, 2002; Piermarini et al. 2002, Choe et al. 2004). To demonstrate specificity of the tCAc antibody, sections were incubated with the tCAc antibody in the presence of an excess (20 μg) of the peptide against which the antibody was raised. Negative control sections were incubated with blocking buffer

lacking antibodies or with pre-immune serum. Following incubation, slides were washed three times for 5 min each time in 0.1 M PB and incubated with a 1:400 dilution (in 0.1 mol l^{-1} PB) of Alexa 488-coupled goat anti-rabbit IgG (Fisher), or Alexa 546-coupled goat anti-mouse IgG (Fisher), or both, as appropriate, for 2 h at room temperature in a humidified chamber. The slides were then washed again (3 x 5 min in 0.1 x PB) and mounted with a mounting medium (Vector Laboratory) containing 4',6'-diamidino-2-phenylindole (DAPI) for the visualization of nuclei. Sections were viewed using a Zeiss Axiophot light microscope and a Hamamatsu C5985 chilled CCD camera. Images were captured using Metamorph imaging system (Version 4.01).

Series II: Determination of the role of branchial CA activity in acid-base regulation

Experimental protocol

Rainbow trout were anaesthetized by immersion in an oxygenated solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g l^{-1}), weighed, and then placed on a surgical table that allowed continuous irrigation of the gills with the same anaesthetic solution. All trout were fitted with indwelling dorsal aortic catheters (Clay-Adams PE50 polyethylene tubing) according to the basic method of Soivio et al. (1975). At the same time, an external urinary catheter was sutured around the vent according to the procedure of Curtis and Wood (1991). Urinary catheters were modified Bard all-purpose urethral catheters (size 12 French, elastic rubber, Bard). Trout were then placed in darkness in experimental chambers served with flowing, aerated, dechloraminated tap water at 13° C to recover for 24 h. Cannulae were flushed with heparinised (100 IU ml^{-1} ammonium heparin; Sigma)

modified (4.5 mmol l^{-1} $NaHCO_3$) Cortland saline (Wolf 1963). In addition, urinary catheters were flushed with water.

A preliminary experiment was carried out to determine the time period of maximum acid excretion elicited by exposure of rainbow trout to hypercapnic conditions (nominal water PCO_2 = 6.5 mm Hg). For this experiment, fish were neither cannulated nor catheterized, and overall net acid-base fluxes were estimated for control (N = 9) and hypercapnia-exposed (N = 9) trout prior to the initiation of hypercapnia (-3 to 0 h), and 1 to 4 h, 4 to 7 h, 10 to 13 h, and 22 to 25 h after the onset of exposure to hypercapnia. On the basis of this preliminary trial, 8 to 12 h was selected as the period of maximum acid excretion induced by hypercapnia, and the subsequent experiment focused on this time period. As the focus of this experiment was branchial acid excretion, the purpose of the urinary catheter was simply to prevent urine elimination into the water, and therefore urine was allowed to drain by gravity into a vial placed outside the holding chamber and about 3 – 5 cm below the surface of the water. Branchial net acid-base fluxes were estimated for control (N = 11) and hypercapnia-exposed (N = 11) trout prior to the initiation of hypercapnia (-2 to 0 h) and 8 to 10 h after the onset of hypercapnia. Trout were then treated with the CA inhibitor acetazolamide (Az; 30 mg kg^{-1}; delivered as a bolus injection in 1 ml of saline via the dorsal aortic cannula) and branchial net acid-base fluxes were estimated for a further 2 h period, i.e. 10 to 12 h after the onset of hypercapnia. An arterial blood sample (600 µl) was withdrawn via the dorsal aortic cannula at the beginning of each measurement period; any red cells not used for analysis were resuspended in Cortland saline and returned to the fish.

Hypercapnia was achieved by equilibrating the water feeding the experimental chambers with 2% CO_2 in air using a gas equilibration column as described above. Water PCO_2 was monitored using a CO_2 electrode (Cameron Instruments) housed in a thermostatted cuvette and linked to a meter (Cameron Instruments) and data acquisition system (Biopac). The gas equilibration column feeding the experimental chambers holding fish in the control group was supplied with air. Water flow from each column was sufficient to supply inflowing water to 3 experimental chambers simultaneously, and therefore experiments were carried out simultaneously on groups of 3 hypercapnia-exposed and 3 control fish. Net acid-base fluxes were estimated for periods (2 or 3 h) in which water flow to the experimental chambers was halted; the volume of water in the boxes was set to a known value. During flux periods, the water in the boxes was aerated directly with either air (control group) or 0.8% CO_2 in air (hypercapnia-exposed group). The PCO_2 of water in the experimental chambers was monitored during flux periods using a CO_2 electrode as described above and any deviations from the desired $PwCO_2$ of 6.5 mm Hg were corrected by adjusting the CO_2 composition of the gas mixture used to aerate the boxes. After each flux period, the experimental chambers were flushed with flowing water from the gas equilibration column for 10 to 15 min; experimental chambers were maintained on flowing water between flux periods. At the beginning and end of each flux period, 10 ml of water were collected from each experimental chamber. Net acid base flux was determined from the measurement of titratable alkalinity and ammonia concentration in these water samples. Water samples were analyzed within 6 h of sampling.

Analytical techniques

Following withdrawal, blood samples were centrifuged (~10000 *g* for 1 min) to obtain plasma. Plasma total CO_2 concentrations were determined in duplicate on 50 µl samples using a Capnicon total CO_2 analyser (CC501; Cameron Instruments), while pH was assessed using a pH electrode and calomel reference (Cameron Instruments – E301 Glass pH electrode) housed in a thermostatted low-volume pH chamber (Cameron Instruments) and connected to a PHM 72 acid-base analyzer (Radiometer). The arterial blood PCO_2 and bicarbonate concentration ($[HCO_3^-]$) were then calculated from the Henderson-Hasselbach equation using appropriate values for αCO_2 and pK' (Boutilier et al. 1984).

The change in titratable alkalinity in water samples from the beginning to the end of each flux period was determined by titrating 5 ml water samples to pH 4.00 with 0.02 N HCl. Samples were continuously aerated during titration to ensure mixing and removal of CO_2 (see McDonald and Wood 1981). Total ammonia in the water samples was analyzed using a micro-modification of the salicylate-hypochlorite colorimetric assay of Verdouw et al. (1978). The net acid-base flux was then calculated as the sum of the titratable alkalinity flux and the ammonia flux, signs considered, as described by McDonald and Wood (1981).

Statistical analyses

Data are reported as mean values ± 1 standard error of the mean (SEM). The statistical significance of effects of exposure to hypercapnia on gill tCAc mRNA expression as determined by real time PCR was analyzed using one-sample Student's *t*-

tests. The statistical significance of differences in net acid-base flux between control and hypercapnia-exposed fish in the preliminary time-course experiment, as well as the impact of hypercapnia on gill tCAc protein levels as determined by western analysis, were assessed by Student's *t*-tests. A two-way repeated measures (RM) analysis of variance (ANOVA) with sampling period (pre exposure, pre-Az and post-Az) and treatment group (control or hypercapnia-exposed) as factors was used to analyse the effects of acetazolamide injection and treatment group (control vs hypercapnia-exposed) on blood acid-base variables and net acid-base fluxes. The 2-way RM ANOVA was followed by Bonferroni tests for *post hoc* multiple comparisons as appropriate. The fiducial limit of significance in all tests was 0.05, and all statistical analyses were carried out using SigmaStat v3.0 (SPSS) software.

Results

Localization of tCAc mRNA and proteins

In situ hybridization was used to examine the distribution of tCAc mRNA expression within the gills of rainbow trout ($N = 3$ control or hypercapnic fish; one slide per fish). Positive hybridization signals were observed in epithelial cells of the lamellae, as well as within the interlamellar region (Figure 3-1). No hybridization was evident in the negative control sections from which probe was omitted. In addition, sections treated with excess unlabeled probe failed to exhibit positive hybridization signals, indicating that the signal was specific.

The distribution of tCAc proteins was assessed with a western analysis of a range of tissues, followed by a more specific examination of the gills by immunohistochemistry. Western analysis (Figure 3-2) revealed that tCAc was widely distributed among tissues within rainbow trout, with particular abundance in gill. The distribution of tCAc in gill tissue was examined in conjunction with that of Na^+/K^+-ATPase (α5) using immunohistochemical techniques. Immunoreactivity against both proteins was detected within the lamellar epithelia as well as the interlamellar region of the gill (Figure 3-3). Double-labelling allowed the relative distributions of the two proteins to be compared. Cells exhibiting only tCAc immunoreactivity or only α5 immunoreactivity were present in both the lamellar epithelia and interlamellar regions, as were cells that displayed co-localization of tCAc and α5. Whereas tCAc immunoreactivity tended to be localized most intensely at the apical membrane of the lamellar epithelial cells, Na^+/K^+-ATPase immunoreactivity was localized principally in the basolateral regions of epithelial cells

that did not appear to be directly adjacent to each other (Figure 3-3A). Immunoreactivity for tCAc was eliminated by preincubation with excess peptide against which the antibody was raised (Figure 3-3D), and all immunofluorescence was eliminated when primary antibodies were omitted (Figure 3-3E).

Effects of hypercapnia on tCAc expression

Exposure of rainbow trout to hypercapnia (nominal $PwCO_2$ = 6.5 mm Hg) for periods ranging from 1 h to 24 h generally resulted in increases in relative tCAc mRNA expression in the gills as assessed by real-time PCR (Figure 3-4). Branchial tCAc mRNA expression relative to that of ß-actin appeared to increase during the initial stages of hypercarbic exposure, peaking at 3 h, the only time point at which the change in relative mRNA expression was significantly different from the control value of 1 (one-sample Student's *t*-test, $P < 0.05$) before declining again over the subsequent 21 h. Even at 24 h, however, relative tCAc expression in the gills of hypercapnia-exposed trout appeared to be higher, although the difference was not statistically significantly, than that in normocapnic fish, a trend that was supported qualitatively by examination of tissue sections from the gills of fish exposed to normocapnia and hypercapnia for 24 h by *in situ* hybridization (Figure 3-4 inset).

The abundance of tCAc proteins in the gills was also significantly increased by exposure to hypercapnia. Quantification of immunoblots of gill tissue from trout exposed to normocapnia or hypercapnia for 24 h indicated that branchial tCAc protein levels were significantly higher (one-tailed Student's *t*-test, $P = 0.063$) in hypercapnic over normocapnic fish (Figure 3-5A). Qualitative support for this finding was provided by

scrutiny of tCAc immunofluorescence in gill sections from normocapnic (Figure 3-5B) and hypercapnic (Figure 3-5C) trout. Interestingly, α5 immunofluorescence also appeared to be enhanced in sections from hypercapnic trout, although this effect was not quantified.

The role of branchial CA in acid-base regulation

Measurement of the overall (i.e. branchial and renal) net excretion of acid equivalents ($J_{net}H^+$) over a 24 h period revealed that, on average, net acid excretion occurred, and that at both 4 – 7 h and 9 – 12 h, net acid excretion was significantly higher in trout exposed to hypercapnia than in those held under normocapnic conditions (Figure 3-6). On this basis, the time frame in which to examine the role of branchial CA activity in regulation of acid-base status was selected as 8 – 12 h after the onset of hypercapnic exposure.

As expected on the basis of the preliminary experiment, branchial $J_{net}H^+$ was significantly higher in trout exposed to hypercapnia than in those held under normocapnic conditions (Figure 3-7A; 2-way RM ANOVA with treatment group and sampling time as factors, $P = 0.12$ for treatment group, $P < 0.001$ for sampling time and $P = 0.021$ for the interaction of these two terms). Branchial $J_{net}H^+$ was decreased significantly by acetazolamide treatment in both the control ($P = 0.015$) and hypercapnic ($P < 0.001$) groups (Figure. 3-7A), and the extent of the decrease in branchial $J_{net}H^+$ was significantly greater in hypercapnic (281.7 ± 52.03 N = 11) over control (121.0 ± 37.2, N = 11) fish (Student's *t*-test, $P = 0.021$). These differences were the result primarily of changes in branchial $J_{net}TA$ (Figure 3-7B; 2-way RM ANOVA, $P = 0.001$ for treatment group, $P =$

0.002 for sampling time and $P = 0.788$ for the interaction of these two terms), as branchial $J_{net}NH_3$ was not significantly affected by either exposure to hypercapnia or injection of acetazolamide (Figure 3-7C; 2-way RM ANOVA, $P = 0.13$ for treatment group, $P = 0.078$ for sampling time and $P = 0.061$ for the interaction of these two terms). Branchial $J_{net}TA$ was significantly higher in hypercapnic than control fish ($P = 0.001$), and was reduced significantly by acetazolamide injection ($P = 0.002$).

Analysis of the acid-base status of arterial blood (Table 1), indicated that there were no significant differences between the control and hypercapnia groups prior to the onset of hypercapnia (2-way RM ANOVA with treatment group and sample time as factors, $P < 0.001$ for both factors and the interaction of these two factors for all three variables tested, pHa, $PaCO_2$ and $[HCO_3^-]$; see Table 1). Within the control group, arterial pH (pHa) fell significantly as a result of acetazolamide injection ($P < 0.001$), while arterial PCO_2 ($PaCO_2$) rose ($P < 0.001$). Within the hypercapnia treatment group, exposure to hypercapnia and acetazolamide injection both elicited significant falls in pHa together with increases in $PaCO_2$ ($P < 0.001$ in all cases). The HCO_3^- ion concentration of the arterial blood ($[HCO_3^-]$) was significantly elevated by exposure to hypercapnia ($P < 0.001$), but no further increase occurred following acetazolamide injection. Consequently, pHa values were significantly lower, and $PaCO_2$ and $[HCO_3^-]$ values significantly higher, in hypercapnic fish in comparison to those in control fish at both the pre-Az and post-Az sampling times ($P < 0.001$ in all cases).

Figure 3-1: Trout cytoplasmic CA (tCAc) mRNA localization in the gills of rainbow trout (*Oncorhynchus mykiss*) by *in situ* hybridization. The images present two sets (A and B, C and D) of *in situ* hybridization results from serial sections of the gill incubated with probe (A, C), no probe (B), or probe in the presence of excess unlabeled probe (D), and are typical of the sections examined. Strong hybridization signals for tCAc are evident in epithelial cells of the lamellae at both low (A) and high (C) magnification. Scale = 40 μm (A, B) and 20 μm (C, D).

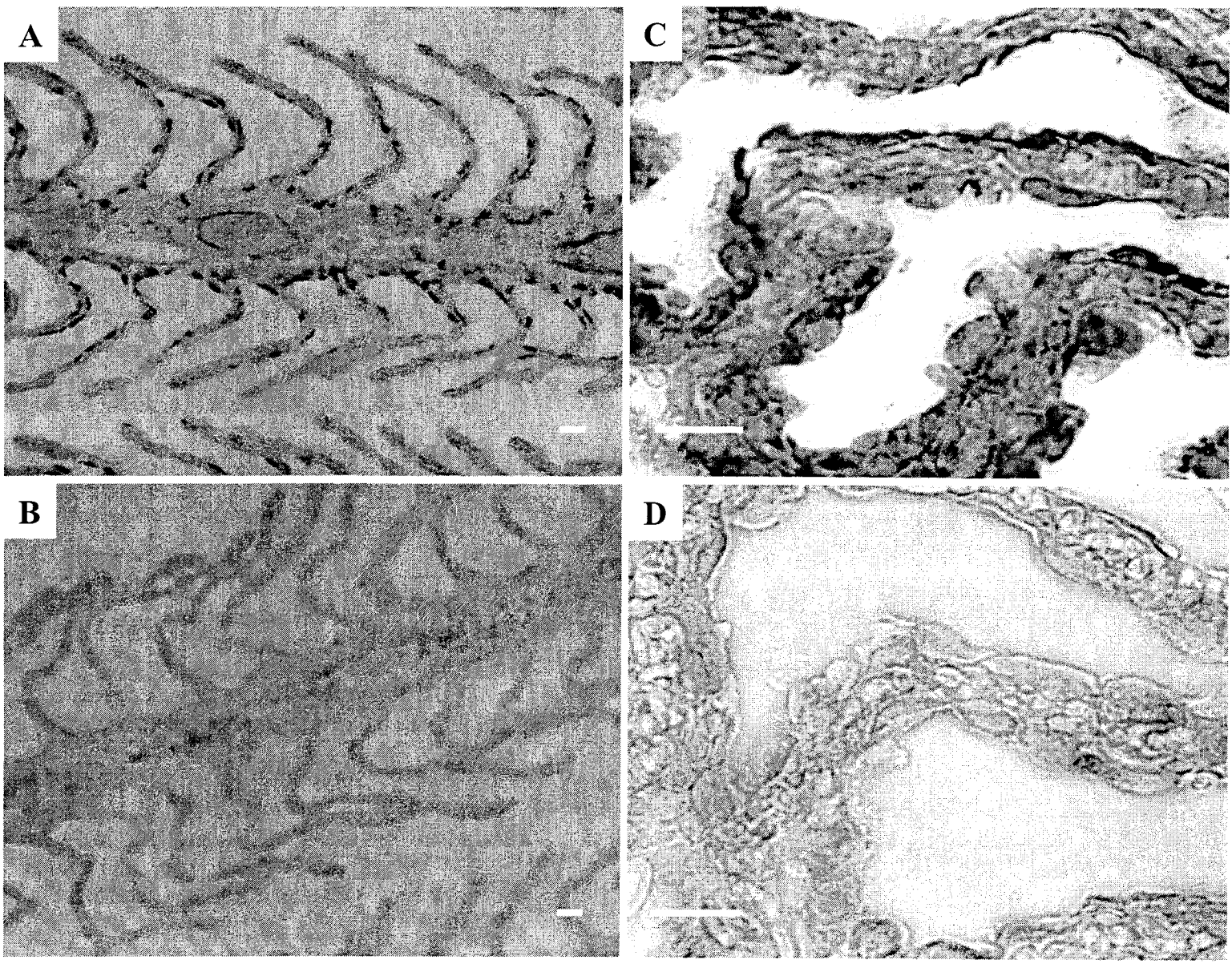

A
C
B
D

Figure 3-2: Western analysis of the expression of tCAc in different tissues of rainbow trout (*Oncorhynchus mykiss*). An antibody raised against a peptide sequence specific to tCAc was used to detect a ~30 kDa protein in all tissues examined; the protein was identified as tCAc on the basis of size and elimination of bands by pre-absorption of the antibody with the peptide against which it was raised (see Chapter 2). Expression of tCAc was particularly high in brain and gill, and low in kidney.

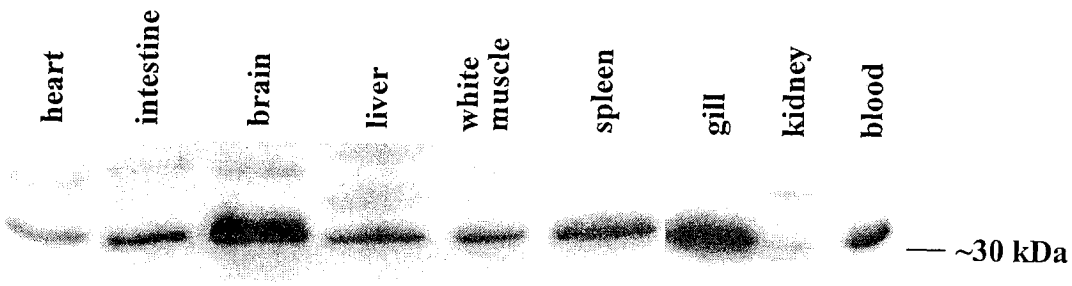

heart
intestine
brain
liver
white muscle
spleen
gill
kidney
blood
~30 kDa

Figure 3-3: The localization of trout cytoplasmic CA (tCAc) protein in the gills of rainbow trout (*Oncorhynchus mykiss*) by immunohistochemistry. The images are overlays of three images collected individually for tCAc immunoreactivity (green), α5 (Na^+/K^+ APTase) immunoreactivity (red), and nuclei visualization (blue). Areas of overlap of tCAc and α5 immunoreactivity are indicated in yellow. Nuclei were visualized using 4',6'-diamidino-2-phenylindole. Low (A), medium (B) and high (C) magnification images representative of the sections examined indicate that cells displaying only tCAc immunoreactivity, only α5 immunoreactivity, or co-localization of tCAc and α5 immunoreactivities were present in the lamellar epithelia as well as in the interlamellar regions. Pre-absorption of the tCAc antibody using the peptide against which the antibody was raised eliminated tCAc immunoreactivity without affecting α5 immunoreactivity (D). Omission of primary antibodies eliminated all immunofluorescence (E). Scale = 20 μm.

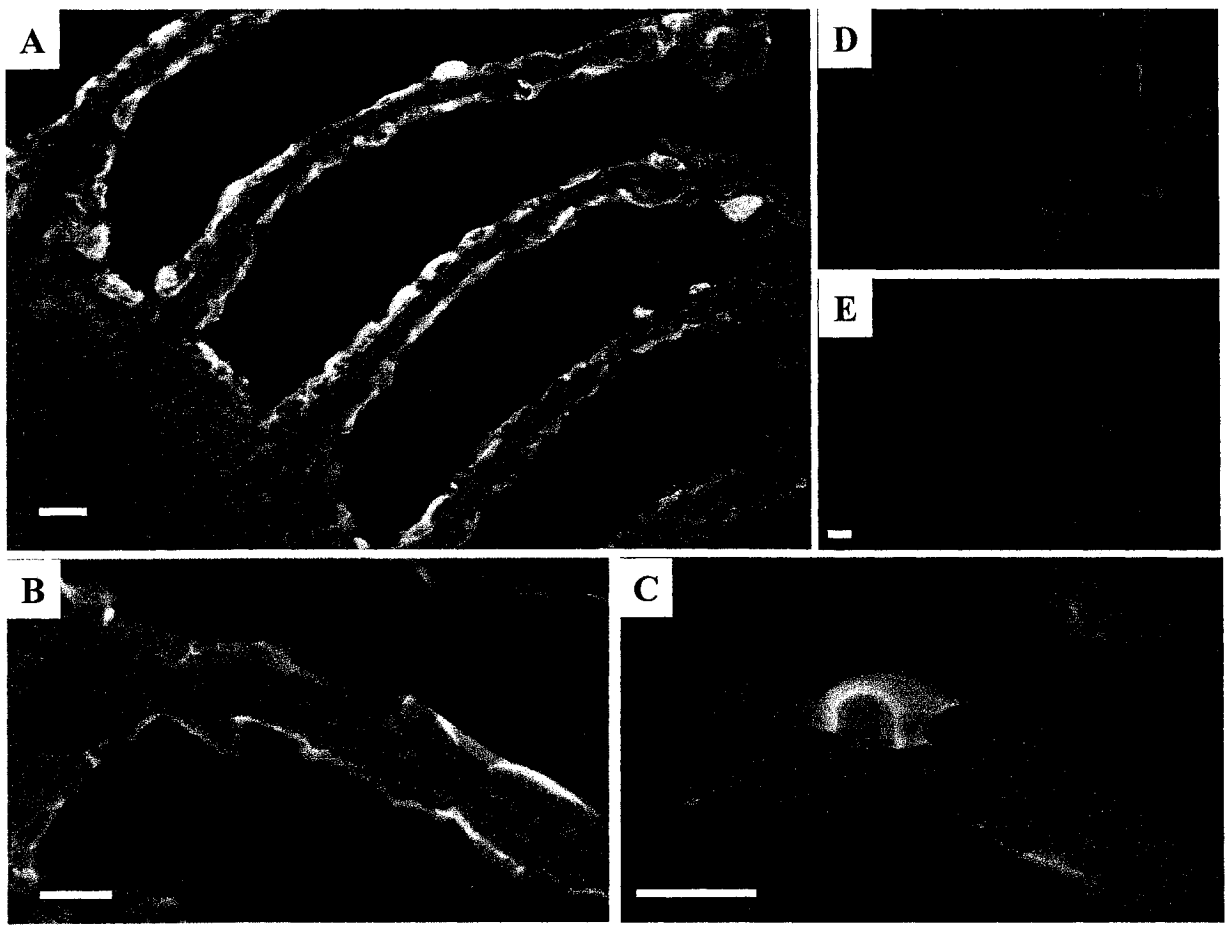

A
D
E
B
C

Figure 3-4: The effect of exposure to hypercapnia (filled bars), for periods ranging from 1 to 24 h, on trout cytoplasmic CA (tCAc) mRNA expression in the gills of rainbow trout (*Oncorhynchus mykiss*) relative to the expression of ß-actin mRNA (relative tCAc mRNA expression), as determined by real-time PCR. Values are means ± 1 SEM and $N = 6$ for each period of exposure to hypercapnia. For statistical analysis, values were compared to corresponding relative tCAc mRNA expression values for fish ($N = 6$ in each case) held under normocapnic conditions for the corresponding period of time; a single group of control fish sampled at 3 h served as the control for 1, 2 and 3 h of exposure to hypercapnia. In each case, mRNA expression in the control groups was given a relative value of 1 (unfilled bar), and an asterisk therefore indicates relative tCAc mRNA expression in the hypercapnic fish that was significantly different from 1 (one-sample Student's *t*-test, $P < 0.05$). The inset panel presents representative images of tCAc mRNA localization by *in situ* hybridization in the gills of (A) normocapnic and (B) hypercapnic (24 h at a nominal $PwCO_2$ of 6.5 mm Hg) trout. Scale = 20 μm.

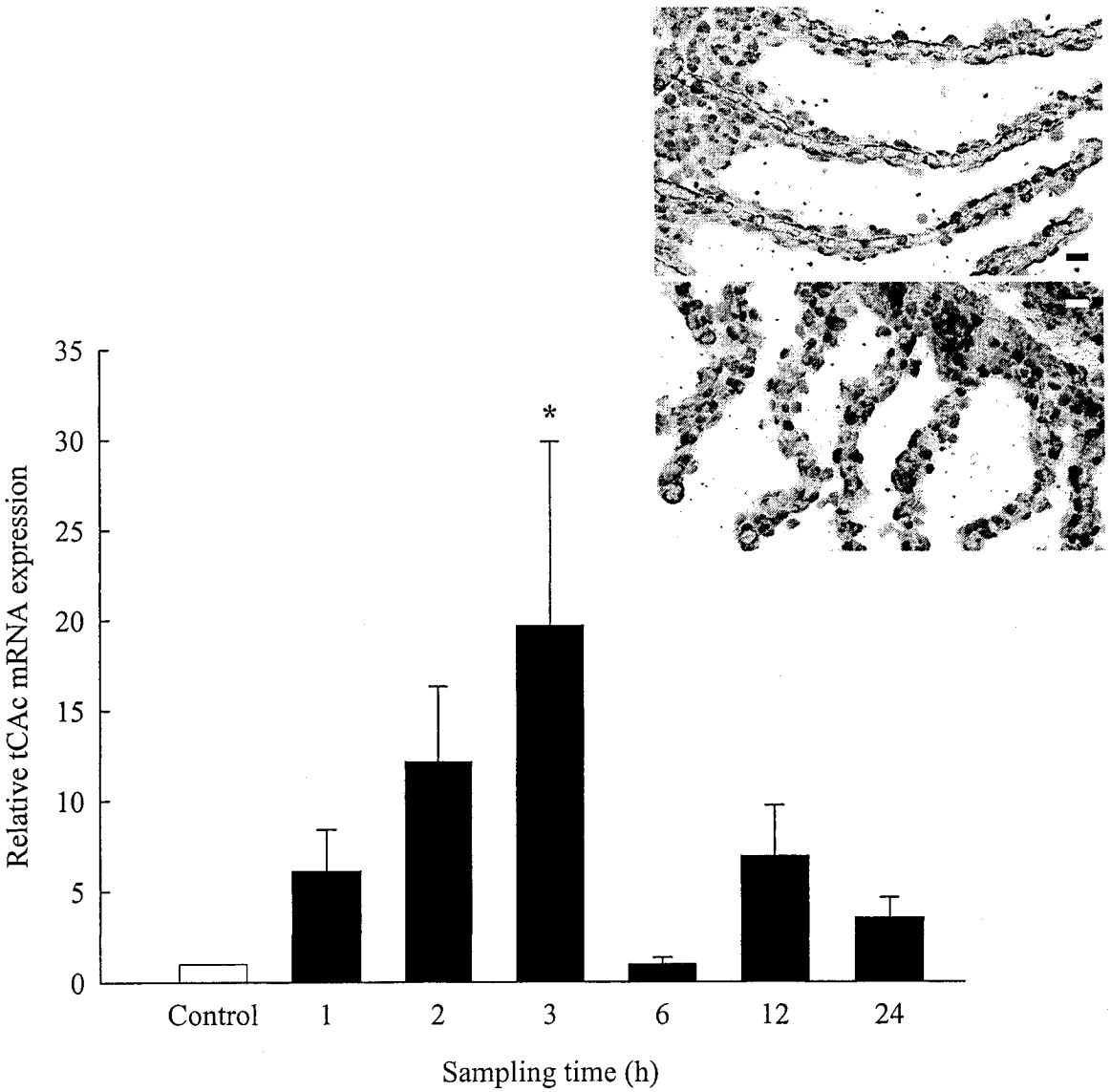
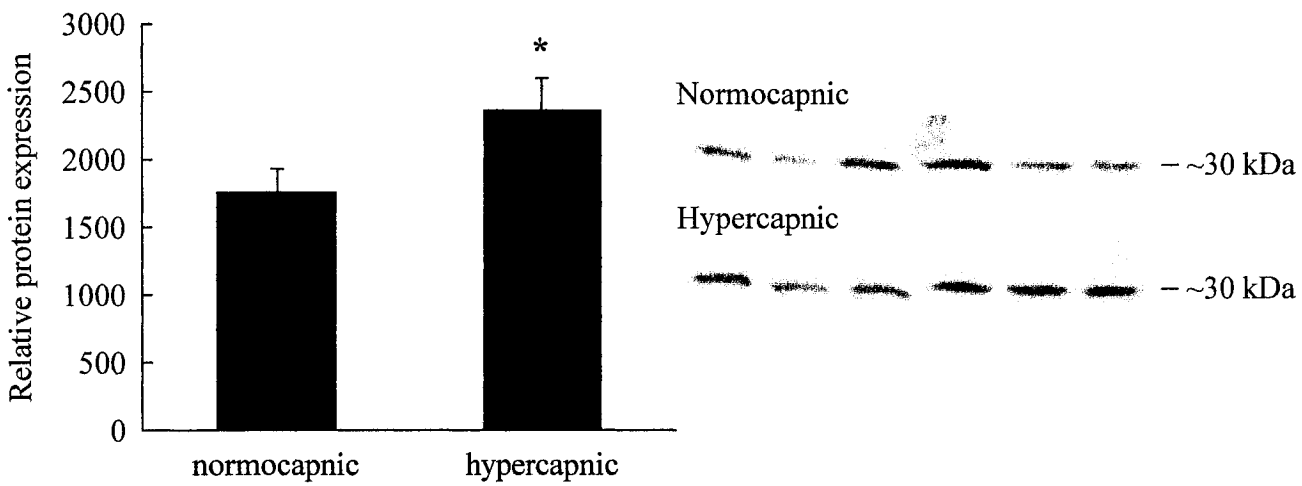

35
30
25
20
15
10
5
0
Relative tCAc mRNA expression
*
Control
1
2
3
6
12
24
Sampling time (h)

Figure 3-5: The effects of exposure to hypercapnia on the expression of trout cytoplasmic CA (tCAc) protein in the gills of rainbow trout (*Oncorhynchus mykiss*). An antibody raised against a peptide sequence specific to tCAc was used to detect tCAc protein expression by (A) western analysis and (B, C) immunohistochemistry in perfused gills from fish held under normocapnic conditions or exposed to hypercapnia (nominal $PwCO_2$ = 6.5 torr) for 24 h. In (A), expression of a ~29 kDa protein was significantly greater (one-tailed Student's *t*-test, $P = 0.063$) in gills extracted from hypercapnic trout (N = 6) than in those extracted from normocapnic fish (N = 6). Protein expression from the immunoblots presented on the right was quantified by digital image processing software (Quantity One, version 4.1.1) and depicted in the figure on the left. In (B) and (C), representative overlay images visualizing tCAc immunoreactivity (green), α5 immunoreactivity (red), and nuclei (4',6'-diamidino-2-phenylindole; blue) are presented for gill sections from trout exposed to (B) normocapnia and (C) hypercapnia for 24 h. Co-localization of tCAc and α5 is indicated in yellow. Scale = 20 μm.


3000
2500
2000
1500
1000
500
0
Relative protein expression
*
normocapnic
hypercapnic
Normocapnic
~30 kDa
Hypercapnic
~30 kDa

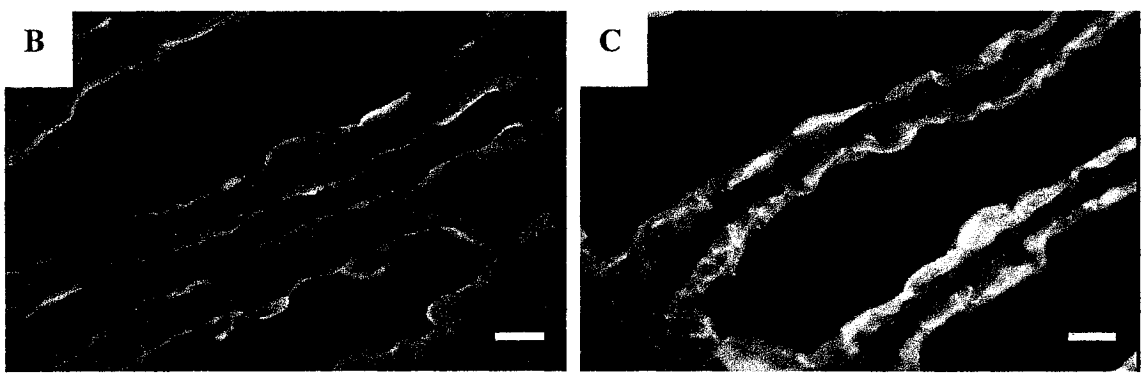

B
C

Figure 3-6: Net excretion of acid equivalents ($J_{net}H^+$) in rainbow trout exposed to normocapnia (unfilled bars; N = 9) or hypercapnia (nominal $PwCO_2$ = 6.5 mm Hg; filled bars; N = 9) over a 24 h period. Data are mean values ± 1 SEM. An asterisk indicates a significant difference between control and hypercapnia-exposed trout within a given flux period (Student's *t*-test, $P = 0.048$ for 4 – 7 h; $P = 0.049$ for 9 – 12 h).

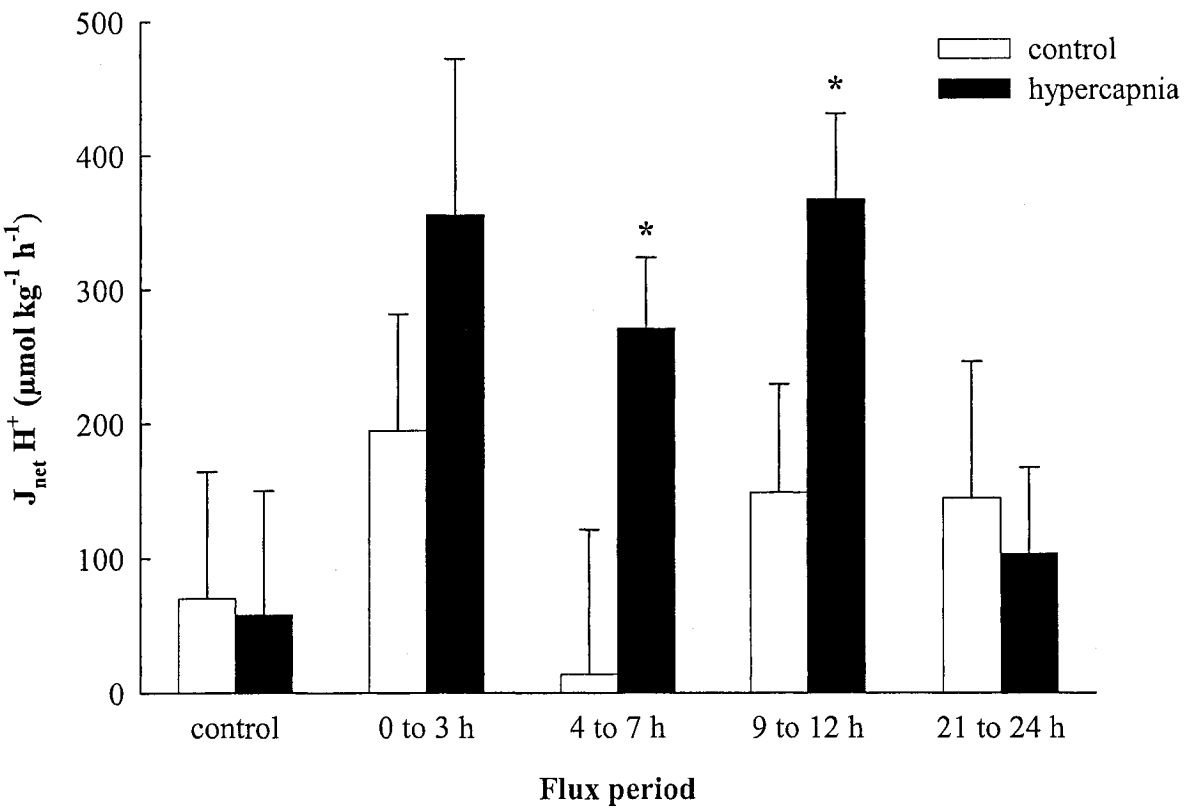

500
400
300
200
100
0
J_{net} H^+ (µmol kg^{-1} h^{-1})
control
hypercapnia
*
*
control
0 to 3 h
4 to 7 h
9 to 12 h
21 to 24 h
Flux period

Figure 3-7: The effects of exposure to hypercapnia (nominal $PwCO_2$ = 6.5 mm Hg) and acetazolamide treatment (30 mg kg^{-1}) on branchial excretion of (A) acidic equivalents ($J_{net}H^+$), (B) titratable alkalinity ($J_{net}TA$) and (C) ammonia ($J_{net}NH_3$) in rainbow trout (*Oncorhynchus mykiss*). $J_{net}H^+$ was calculated as the sum of $J_{net}TA$ and $J_{net}NH_3$, signs considered. Data are mean values ± 1 SEM: N = 11 for both control (normocapnic) and hypercapnic treatment groups. Data were analyzed by two-way repeated measures ANOVA with treatment group (normocapnic versus hypercapnic) and sample time (pre-exposure, pre-Az injection and post-Az injection) as factors; *P* values are indicated on the figure. An asterisk (*) indicates a significant difference as a result of Az injection within a treatment group, while a dagger (†) indicates a significant difference between control and hypercapnic treatment groups within a given sampling time. Where significant interactions did not occur, only the *P* values are indicated on the figure.

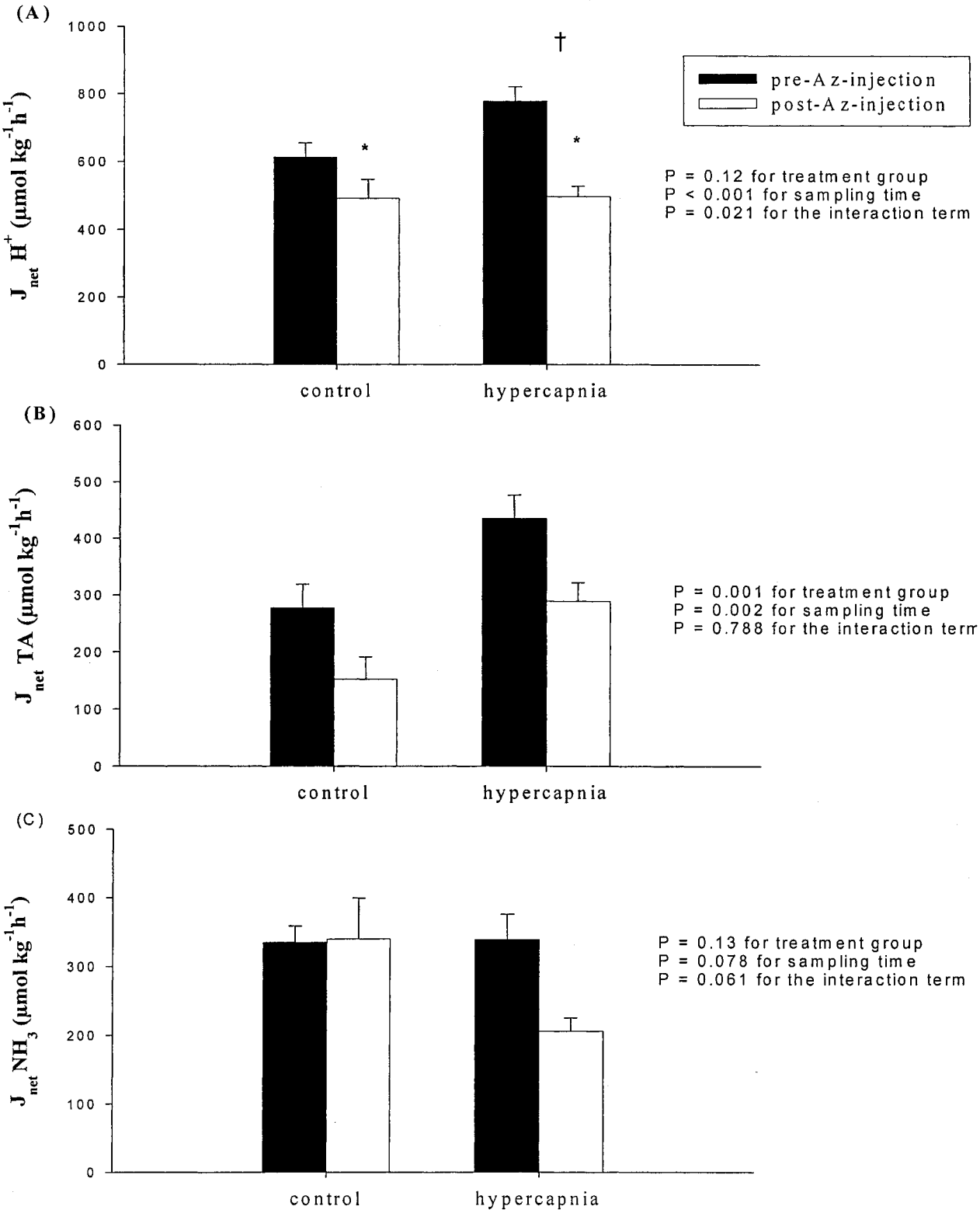

(A)
1000
800
600
400
200
0
J_{net} H^+ (µmol $kg^{-1}h^{-1}$)
†
*
*
pre-Az-injection
post-Az-injection
P = 0.12 for treatment group
P < 0.001 for sampling time
P = 0.021 for the interaction term
control
hypercapnia
(B)
600
500
400
300
200
100
0
J_{net} TA (µmol $kg^{-1}h^{-1}$)
P = 0.001 for treatment group
P = 0.002 for sampling time
P = 0.788 for the interaction term
control
hypercapnia
(C)
500
400
300
200
100
0
J_{net} NH_3 (µmol $kg^{-1}h^{-1}$)
P = 0.13 for treatment group
P = 0.078 for sampling time
P = 0.061 for the interaction term
control
hypercapnia

Table 3-1: The effects of exposure to hypercapnia (nominal $PwCO_2$ = 6.5 mm Hg) and acetazolamide treatment (30 mg kg^{-1}) on arterial blood pH (pHa), partial pressure of CO_2 ($PaCO_2$) and HCO_3^- concentration ($[HCO_3^-]$).

	Control			**Hypercapnia**		
	pre-exposure	pre-Az	post-Az	pre-exposure	pre-Az	post-Az
pHa	7.78 ± 0.02^a	7.75 ± 0.02^a	7.57 ± 0.01^b	7.76 ± 0.02^a	$7.58 \pm 0.01^{b*}$	$7.40 \pm 0.01^{c*}$
$PaCO_2$ (mm Hg)	2.97 ± 0.24^a	3.36 ± 0.30^a	4.96 ± 0.22^b	3.35 ± 0.29^a	$7.73 \pm 0.15^{b*}$	$12.15 \pm 0.17^{c*}$
$[HCO_3^-]$ (mmol l^{-1})	7.68 ± 0.20^a	7.94 ± 0.28^a	7.89 ± 0.26^a	8.07 ± 0.27^a	$12.3 \pm 0.07^{b*}$	$12.6 \pm 0.43^{b*}$

Data are presented as mean values ± 1 SEM; N = 8 for both control and hypercarbic fish. The data were analyzed by 2-way RM ANOVA with treatment group (control vs hypercapnia) and sample time (pre-exposure, pre-Az injection, or post-Az injection) as factors. For all three variables (pHa, $PaCO_2$ and $[HCO_3^-]$), $P < 0.001$ for treatment group, sample time and interactions between these factors. An asterix (*) indicates a significant difference at a given sampling time from the corresponding value for the control group, while within a treatment group (control or hypercapnia), groups that do not share a letter are significantly different from one another.

Discussion

The results of the present study provide compelling evidence that branchial CA plays an important role in acid-base regulation in rainbow trout, a role that is enhanced under conditions of acid-base challenge perhaps owing to increased CA expression. It has long been postulated that branchial CA contributes to ionic and acid-base regulation in freshwater teleost fish by catalyzing the hydration of CO_2 within the branchial epithelium to the acid-base equivalents, H^+ and HCO_3^-, that are used as counter-ions for Na^+ and Cl^- uptake, respectively (reviewed by Perry and Laurent 1990; Goss et al. 1992, 1995; Henry and Swenson 2000; Claiborne et al. 2002; Perry et al. 2003; Hirose et al. 2003; Evans et al. 2005). The contribution of branchial CA to ion uptake is supported by the significant reductions in Na^+ or Cl^- influx that have been documented in most, although not all (Kerstetter and Kirschner 1972), studies following inhibition of branchial CA by acetazolamide *in vivo* (Maetz and Garcia-Romeu 1964; Boisen et al. 2003; Chang and Hwang 2004), *in situ* (Kerstetter et al. 1970), or in perfused gill preparations (Payan et al. 1975). Fewer studies have focused on the role of branchial CA in acid-base regulation. Henry et al. (1988) attributed the lack of pH compensation in response to a respiratory acidosis induced by red blood cell CA inhibition to the concomitant inhibition of branchial CA activity, while Lin and Randall (1991) demonstrated that net proton excretion across the gills was reduced by acetazolamide addition to the external water. Additional evidence was obtained from studies on marine dogfish, which utilize branchial Na^+/H^+ and Cl^-/HCO_3^- exchanges for acid-base regulation, although not ionic regulation (e.g. Tresguerres et al. 2005; see reviews by Perry and Laurent 1990; Claiborne et al.

2002; Evans et al. 2005). The branchial clearance of an infused HCO_3^- load was impaired in dogfish treated with benzolamide to selectively inhibit gill CA activity (Swenson and Maren 1987); in addition, a preliminary report suggested that compensation for a respiratory acidosis induced by exposure to hypercapnia was similarly impaired in benzolamide-treated dogfish (Swenson and Claiborne 1985).

In the present study, branchial acid excretion and blood acid-base status were monitored directly and simultaneously to provide a comprehensive picture of the role of branchial CA activity in acid-base regulation. The significant reduction in branchial net acid excretion following acetazolamide treatment (Figure 3-7) provides the clearest evidence to date that branchial CA functions in acid-base regulation. Branchial net acid excretion was reduced despite the significant respiratory acidosis induced by inhibition of red blood cell CA activity, and was accounted for by decreases in the titratable alkalinity flux rather than changes in branchial ammonia excretion. Moreover, branchial net acid excretion was inhibited by acetazolamide treatment to a significantly greater extent in trout exposed to environmental hypercapnia (Figure 3-7). Exposure to hypercarbic water elicits an initial respiratory acidosis that is regulated at constant $PaCO_2$ through the accumulation of HCO_3^- via the gills and its retention at the kidney (Claiborne and Heisler 1984, 1986; Perry et al. 1987a,b; Goss and Perry 1993; Larsen and Jensen 1997; Choe and Evans 2003, see reviews by Goss et al. 1995; Perry et al. 2003). The changes in net acid excretion and blood acid-base status detected in the present study were consistent with this pattern. Significantly higher branchial net acid excretion in hypercapnia-exposed trout (Figure 3-7) resulted in HCO_3^- accumulation (Table 1), such that at 8 – 10 h of hypercarbic exposure, a metabolic alkalosis was superposed on the respiratory

acidosis induced by hypercapnia, yielding partial pH compensation (Figure 3-8). Injection of acetazolamide at this point not only exacerbated the respiratory acidosis, but added to it a component of metabolic acidosis (base deficit of ~1.4 mmol L^{-1}) that likely reflected the inhibition of branchial net acid excretion as well as the inhibition of the renal CA-dependent HCO_3^- retention mechanism (Chapter 2). While adjustments of acid or base excretion at the gill constitute the main mechanism for acid-base regulation in fish (Cameron and Kormanik 1982; Wheatly et al. 1984; Perry et al. 1987a,b; Wood 1988; Goss and Perry 1993; Choe and Evans 2003), accounting for ~90% of the net movement of acid-base equivalents (Claiborne et al. 2002), the kidney plays an important complementary role in acid-base regulation by reabsorbing accumulated HCO_3^- ions from the filtrate (Perry et al. 2003). Inhibition of this CA-dependent process results in HCO_3^- loss in the urine (Chapter 2), contributing to the base deficit of acetazolamide-treated hypercapnic trout. In normocapnic situation, the inhibition of this CA-dependent process results in an increase in urine pH which is more related to the large deficit of net acid efflux than the loss of HCO_3^- in the urine (Chapter 2).

The greater impact of acetazolamide treatment on branchial net acid excretion in hypercapnia-exposed trout reflects the increased requirement for net acid excretion to compensate for the hypercapnia-induced respiratory acidosis. Assessment of Na^+ and Cl^- fluxes during hypercapnia in rainbow trout suggests that the necessary net acid excretion (or HCO_3^- accumulation) is achieved primarily through the reduction of Cl^-/HCO_3^- exchange, which is coupled with a quantitatively less important enhancement of Na^+-linked proton extrusion (Perry et al. 1987a; Goss and Perry 1993, reviewed by Goss et al. 1992, 1995). Morphological adjustment of the branchial epithelium, specifically a

reduction in the exposed surface area of mitochondria-rich (chloride) cells, the presumed site of branchial Cl^-/HCO_3^- exchangers (see below), constitutes one mechanism through which this reduction in Cl^-/HCO_3^- exchange is achieved (Goss and Perry 1993, reviewed by Goss et al. 1992, 1995; Perry 1997; Perry et al. 2003). The modest enhancement of proton extrusion during hypercapnia is supported by increased branchial H^+-ATPase activity (Lin and Randall 1993; Sullivan et al. 1995; Galvez et al. 2002) that reflects, at least to some degree, greater gene expression of the vacuolar H^+-ATPase (Sullivan et al. 1996; Perry et al. 2000a,b). The results of the present study indicate that the abundance of the trout gill cytoplasmic CA isoform (tCAc) is also increased during hypercapnia (Figure 3-5), at least in part owing to enhanced gene transcription (Figure 3-4). Increased branchial CA expression presumably serves to ensure that H^+-ATPase activity is not limited by proton availability. Dimberg and Höglund (1987) also detected increased gill CA activity in rainbow trout exposed to hypercapnia, but as the higher activity was measured at 20 and 80 days of hypercapnia, it likely was not involved in acute acid-base regulation. The significant induction of gill CA activity in response to hypercapnic exposure indicates, however, that branchial CA expression is sensitive to acid-base challenges. A similar conclusion was reached by Hirata et al. (2003) for the Osorezan dace, which exhibited increased branchial CA mRNA expression following exposure to acidic (pH 3.5) water. Differences in branchial CA expression have also been reported in response to salinity challenges (Girard and Istin 1975; Dimberg et al. 1981; Zbanyszek and Smith 1984; Flügel et al. 1991; Kültz et al. 1992; Martinez-Tabche et al. 1992), although not in all cases (Mashiter and Morgan 1975; Sender et al. 1999), and in response to differences in acclimation temperature (Houston and McCarty 1978; Houston and

Mearow 1979).

Although the cellular location and molecular nature of acid and base translocating proteins in the gills of freshwater teleosts remain topics of considerable debate, current models (see reviews by Claiborne et al. 2002; Perry et al. 2003; Hirose et al. 2003; Evans et al. 2005) suggest that acid excretion is accomplished by a vacuolar H^+-ATPase that is located in the apical membrane of mitochondria-rich cells that do not bind peanut lectin agglutinin (PNA; Galvez et al. 2002); these cells are also termed mitochondria-rich (MR) pavement cells. Proton secretion is linked to Na^+ uptake that may occur through apically-located Na^+-selective channels. Base excretion, on the other hand, is linked to Cl^- uptake, likely through a Cl^-/HCO_3^- exchanger that is apically located in PNA^+ MR (chloride) cells. Given the role played by CA in providing H^+ and HCO_3^- for acid and base excretion, a broad distribution of tCAc expression would be expected to occur in the gills of rainbow trout. Examination of tCAc mRNA and protein distribution in the present study revealed patterns of expression that were consistent with current models. Positive hybridization signals for tCAc mRNA were visible both along the lamellae and in the interlamellar regions (Fig. 1). Because MR (chloride) cells in freshwater rainbow trout are typically concentrated in the interlamellar regions and only sparsely distributed along the lamellae (Perry 1997), the distribution of positive hybridization signal suggests that both pavement cells and MR (chloride) cells expressed tCAc mRNA. Similarly, immunoreactivity for tCAc was present essentially equally in Na^+/K^+-ATPase immunopositive and immunonegative cells (Fig. 3-3). The abundant tubular system that characterizes MR (chloride) cells houses ion-transporting enzymes, such as Na^+/K^+-ATPase (Jürss and Bastrop 1995; Perry 1997), and therefore high levels of this enzyme,

assessed biochemically or through fluorescence microscopy, are frequently used as a tool to identify MR (chloride) cells (e.g. Li et al. 1995; Wilson et al. 2000a). Co-localization of tCAc and Na^+/K^+-ATPase immunoreactivity was therefore indicative of CA-containing MR (chloride) cells, whereas cells that were immunopositive for tCAc alone were likely pavement cells (Figure 3-3). Interestingly, not all putative MR (chloride) cells exhibited tCAc immunofluorescence. Equally interesting was the apparent, although not quantified, increase in Na^+/K^+-ATPase immunofluorescence in the gills of hypercapnia-exposed trout (Fig. 3-5B,C). In view of the reduction in exposed MR (chloride) cell area that typically occurs during hypercapnic exposure (see above), further investigation of the apparent increase in Na^+/K^+-ATPase abundance is clearly warranted.

The branchial distribution of tCAc observed in the present investigation is in agreement with that observed previously, and complements the results of earlier studies by focusing on the specific CA isoform that is present in the gills (Esbaugh et al. 2005), and by examining the impact of hypercapnic exposure on this isoform. CA was localized to both pavement and MR (chloride) cells in rainbow trout using Hansson's technique, a histochemical approach based on a cobalt phosphate/cobalt sulphate vital stain (Conley and Mallatt 1988). Immunocytochemical localization of branchial CA using an antibody raised against purified trout gill CA confirmed the broad distribution reported by Conley and Mallatt (1988), and additionally indicated that CA was concentrated in the apical region of both pavement cells and MR (chloride) cells (Rahim et al. 1988). However, these patterns are not true of all species examined to date; in some species CA appears to be confined to either pavement cells or MR (chloride) cells, while in others it is found in both (Dimberg et al. 1981; Conley and Mallatt 1988; Flügel et al. 1991; Sender et al.

1999; Wilson et al. 2000a,b; Hirata et al. 2003; Choe et al. 2004).

In summary, the findings of the present study provide three significant contributions to our knowledge of the distribution and function of CA in the gills of freshwater fish. First, evidence for expression of the trout general cytoplasmic CA isoform (tCAc) in both pavement cells and MR (chloride cells) has been obtained. The results confirm and extend the findings of Esbaugh et al. (2005), who identified the tCAc isoform but focused exclusively on its mRNA expression, by examining the cellular localization of the protein as well as mRNA. Second, trout branchial CA (tCAc) has been demonstrated to play an important role in the branchial regulation of acid-base balance, as CA inhibition significantly decreased branchial net acid excretion. Finally, the larger impact of CA inhibition on branchial net acid excretion in trout exposed to hypercapnia, coupled with the significantly higher tCAc mRNA and protein expression in hypercapnic trout, provide evidence that branchial CA expression is regulated in response to acid-base challenges, further supporting the conclusion that this enzyme is a key contributor to acid-base regulation in freshwater rainbow trout.

CHAPTER 4

GENERAL DISCUSSION

Discussion

Freshwater fish are able to regulate their internal acid-base status via a combination of branchial, renal, and metabolic processes (Heisler 1986; Goss et al. 1995; Claiborne et al. 2002; Perry et al 2003; Evans et al. 2005). The gills are the primary site for acid-base regulation, with significant contributions being made also by renal acid excretion (Perry et al. 1987) and intracellular buffering (Cameron et al. 1980; Wood et al. 1999; Perry et al. 2003). During the regulation of respiratory acidosis, the teleost kidney plays an important role in the reabsorption of HCO_3^-; for each H^+ secreted, a HCO_3^- must be reabsorbed (Wood and Jackson 1982; Perry et al. 1987b; Wood et al. 1999; Swenson 2000, Perry and Fryer 1997; Perry et al 2003; Wood et al. 1999). Little is known about the specific mechanisms of acid secretion and HCO_3^- reabsorption by the fish kidney. By analogy to other vertebrate kidneys, H^+ secretion presumably occurs via Na^+/H^+ exchange and H^+ pumping (via V-ATPase) and HCO_3^- reabsorption likely occurs via the Na^+-HCO_3^- co-transporter (NBC1). The renal expression of both the V-ATPase and NBC1 increase during acidosis (Perry and Fryer 1997; Perry et al. 2003). It is, however, the gills of freshwater fish that play the major role in acid-base regulation through the exchange of ions and acid-base equivalents between the body and the external environment. In particular, H^+ excretion at the gill is linked to Na^+ uptake, while HCO_3^- loss is linked to Cl^- uptake.

The acid-base challenge utilized in the experiments reported in this thesis was exposure to hypercapnia. Exposure to hypercapnia induces a respiratory acidosis owing to elevation of internal CO_2 tensions in response to the elevated water PCO_2 (e.g.

Cameron and Randall 1972; Eddy et al. 1977; Perry et al. 1987a; Hyde and Perry 1989; Crocker and Cech 1998). The respiratory acidosis, in turn, results in complex changes in ion fluxes through physiological and morphological adjustments at the gill. These changes include a decrease of Cl^-/HCO_3^- exchange rate and an increase of Na^+/H^+ exchange rate or H^+ pumping (Claiborne et al. 1986, 2002; Dimberg et al. 1987; Perry et al. 1987a, 1997; Swenson 2000; Piermarini et al. 2001). These changes are thought to be accomplished by a reduction in the apical exposure of the chloride cells (or mitochondrial rich cells), thereby reducing the number of Cl^-/HCO_3^- exchanges exposed to the water This is caused by a covering of the chloride cells by adjacent pavement cells where H^+ excretion through the Na^+/H^+ exchange or V-ATPase-mediated H^+ pumping is thought to occur (Goss et al. 1992, 1993, 1995). Meanwhile, at the kidney level, HCO_3^- levels must be maintained to regulate blood pH (Heisler 1984; Perry et al. 2003). The increase in HCO_3^- reabsorption and acid secretion are driven by an increase of NBC1 and V-ATPase expression (reviewed in Perry et al. 2003). In brief, a respiratory acidosis is characterized by increases in extracellular $[H^+]$ that accompany elevated PCO_2. Acid-base regulation is achieved by decreasing the rate and/or extent of Cl^-/HCO_3^- exchange, and by increasing the rate and/or extent of Na^+/H^+ exchange or H^+ pumping; the latter is less important, at least in rainbow trout, than the former (Goss et al. 1992b; Perry et al 2003; Perry et al 2000b; Sullivan et al 1996; Wilson et al 2000a). During metabolic alkalosis, there is an increase in HCO_3^- levels and a decrease in H^+ levels. There is a combination of an increase in chloride cells (mitochondrial rich cells), corresponding in an increase of the Cl^-/HCO_3^- exchange rate (hence increased Cl^-/HCO_3^- exchangers) and a reduction in the

Na^+/H^+ exchange rate (H^+ pump/Na^+ channel). Metabolic alkalosis is compensated more rapidly than the metabolic acidosis (reviewed in Goss et al. 1992).

The focus of this thesis was the role of carbonic anhydrase (CA) in the renal and branchial responses to a respiratory acidosis induced by hypercapnia. Although renal and branchial responses to the acid-base challenge of hypercapnic acidosis have been examined previously in rainbow trout (Perry et al. 1987a; Perry et al. 1987b; Goss and Perry 1993) as well as other freshwater teleost fish (e.g. Claiborne and Heisler 1984; Claiborne and Heisler 1986; Goss et al. 1992; Piermarini et al. 2002; Tufts et al. 1996), the work reported in this thesis represents the first comprehensive attempt to demonstrate the role of branchial and renal CA isoforms in acid-base regulation, and to characterize the impact of hypercapnic acidosis on branchial and renal CA. Renal and branchial responses will be discussed separately.

The kidney

In this thesis, we provide the first direct evidence for the presence of a membrane bound CA IV in renal tubules in a teleost fish. Pelis et al (2003) provided some indirect evidence for membrane-associated CA activity in flounder nephrons. Using the rainbow trout (*Oncorhynchus mykiss*) as a model, a type IV-like CA (tCA IV) was cloned and characterized from kidney, a polyclonal antibody for tCA IV was generated, the potential for up-regulation of tCA IV during hypercapnia was evaluated, and the critical role of tCA IV in urinary acidification under normal conditions and during hypercapnia was evaluated using specific inhibitors for cytosolic CA versus membrane bound CA.

Trout CA IV was localized on the apical membrane of the tubule lumen in the proximal segments of the nephron. Similarly, CA IV has been localized to the apical membranes of the proximal segment (S1 and S2) tubules of mammalian nephrons (Carter et al. 1990; Brown et al. 1990; Schwartz et al. 2000; Winkler et al. 2001; Nicoleta et al. 2004). In the lumen of the mammalian proximal tubule, membrane-associated CA facilitates the dehydration of carbonic acid that is generated from the combination of secreted protons and filtered HCO_3^- (Schwartz 2002). This reaction keeps free H^+ at low levels. Proton secretion into the lumen results in the rapid formation of CO_2 which diffuses into the tubule cells where it is hydrated in the presence of CA II with the resultant protons secreted into the lumen while the HCO_3^- exits the cell via the basolateral membrane (Gilmour 1998; Swenson 2000; Perry et al. 2003). Overall, HCO_3^- reabsorption is favored. Further investigations should focus on whether other membrane-associated forms, such as CA XIV or CA XII are also present, as is the case in mammals (Schwartz 2002; Schwartz et al. 2003; Purkerson et al. 2005).

Given this localization, and by analogy with the function of CA IV in the mammalian nephron (reviewed in: Schwartz 2002; Innocenti et al. 2005), trout kidney CA IV is postulated to play a role in urinary acidification. Although the kidney in freshwater fish is quantitatively less important than the gill in regulating acid-base disturbances (Cameron and Kormanik 1982; Wheatly et al. 1984; Perry et al. 1987a; Perry et al. 1987b; Wood 1988; Choe and Evans 2003), it does function to regulate blood pH by producing urine that is more or less acidic, and in addition it plays an important role in the reabsorption of HCO_3^- ions, particularly during periods of HCO_3^- accumulation (Perry et al. 1997). How do the different isoforms of CA, CA IV and

cytosolic CA (tCAc), contribute to this mechanism? Under normal conditions, H^+ required to titrate the filtrated HCO_3^- is provided by an apical membrane V- ATPase and a Na^+/H^+ exchanger that has yet not been identified in renal tubule of trout (Perry et al. 2003). The apical CA IV catalyses the dehydration of filtrated HCO_3^- into H_2O and CO_2 that diffuses into the cell to be converted to H^+ and HCO_3^-. Accumulating HCO_3^- exits the cell via a basolateral Na^+/HCO_3^- co-transporter (NBC1; Perry et al 2003) and the excess H^+ is secreted into the urine. In mammals, renal HCO_3^- reabsorption depends on a basolateral NBC1 operating in efflux mode and this reabsorption takes place in the proximal tubules (Romero et al. 1999; Schwartz 2002). The results of experiments in which blood and urine acid-base status were measured in rainbow trout prior to and following CA inhibition support this model. Indeed, the administration of acetazolamide, either during normocapnia or hypercapnia, caused a rapid increase in $PaCO_2$ and a reduction of pHa, suggesting a mixed respiratory/metabolic acidosis. The urinary net acid efflux was also significantly reduced by acetazolamide to a similar extent during normocapnia or hypercapnia owing to changes in TA-HCO_3^- flux. Urine pH, HCO_3^- and Na^+ levels were markedly increased by acetazolamide although for HCO_3^- and Na^+, the changes were statistically different only during hypercapnia.

Because acetazolamide is a highly permeable inhibitor that will affect both cytoplasmic and membrane-associated CA activities, it cannot be used to distinguish between the roles of cytoplasmic and luminal membrane-associated CAs in mediating HCO_3^- reabsorption. Moreover, analysis of the effects of acetazolamide inhibition is complicated to some extent by the fact that acetazolamide also inhibits red blood cell CA activity, leading to a respiratory acidosis (e.g. Hoffert and Fromm 1973; Henry et al.

1988). For these reasons, experiments were also carried out using the inhibitor F3500, a selective inhibitor of extracellular CA activity owing to its high molecular weight (Conroy et al. 1996; Maren et al. 1997). Similar approaches were adopted in mammalian systems to differentiate between the roles of CA II and CA IV in filtrate acidification (Karlmark et al. 1979). With F3500 treatment, no effect on blood pH occurred but urine pH increased after inhibition, as did the levels of HCO_3^- ions in the urine, resulting in a decrease in net acid efflux.

Based on quantitative analysis of either the mRNA or protein levels, I reported an up regulation of both tCAc and tCA IV mRNA after 3 and 24 h, respectively, following exposure to hypercapnia, and I also observed a slight increase in the expression of the tCAc and the tCA IV proteins. During hypercapnia, the kidney plays a greater role in urinary acidification which indicates the sensitivity of the system to acid-base challenges. In mammals, the proximal tubule reabsorption of HCO_3^- is enhanced under chronic respiratory acidosis (Krapf 1989; Santella et al. 1991).

Finally, some unanswered questions remain: what are the mechanisms through which urine pH is adjusted in the freshwater fish kidney and to which specific kidney segments are the particular ion transport mechanisms localized?

The gill

The regulation of acid-base balance by the gills of fish has been studied for over 70 years (Smith 1930). Although numerous models have been proposed for different species and conditions (Wilson et al. 2000; Clairborne et al. 2002; Choe et al. 2004; Piermarini et al 2002; Clairborne et al 2002; Perry et al. 2003), they all support the

concept that the gills of fish are an important site of ionic and acid-base regulation because of the coupling of Na^+ uptake from the external environment to acid excretion, and Cl^- uptake to base excretion. Considerable debate remains as to the specific mechanisms through which these exchanges are accomplished, and indeed, the mechanisms may vary among species (e.g. Wilson et al. 2000). In general, coupled Na^+ and H^+ movements are thought to occur via Na^+/H^+ exchangers (NHE) and/or Na^+ channels linked to proton extrusion via the vacuolar H^+-ATPase (Claiborne et al. 2002; Perry et al. 2003; Evans et al. 2005), while Cl^- uptake and base excretion occur via Cl^-/HCO_3^- exchangers such as members of the AE family (Perry et al. 1987a, 1999; Goss et al. 1990a and b) or pendrin (Piermarini et al. 2002; Claiborne et al. 2002; Hirose et al. 2003). Moreover, the cell types housing the various exchange mechanisms remain a source of considerable debate (see reviews by Claiborne et al. 2002; Perry et al. 2003; Hirose et al. 2003; Evans et al. 2005). It has been suggested that the Na^+ channels, the H^+-ATPase pumps and the Cl^-/HCO_3^- exchangers are mainly localized in the apical membrane of mitochondria-rich cells, whereas the Na^+,K^+-ATPase and Na^+/HCO_3^- co-transporters are mainly localized on the basolateral membrane of the mitochondria-rich cells (see reviews by Claiborne et al. 2002; Perry et al. 2003; Evans et al. 2005).

Acid-base regulation is accomplished by appropriate modulation of these ion/acid-base equivalent exchanges. In addition to regulation of the exchange mechanisms themselves, morphological adjustments of the branchial epithelium may contribute to acid-base regulation by increasing or decreasing the availability of particular exchange mechanisms. For example, the availability of Cl^-/HCO_3^- exchangers can be altered morphologically at the gill through adjustments of the exposed surface area

of MR (chloride) cells (Goss et al. 1992a and b; Clairborne et al 2002; Perry et al 2003; Goss et al. 1992; Goss et al. 1995). During acidosis, chloride uptake is reduced by a decrease in the exposed surface area of MR (chloride) cells, while the opposite effect has been noted during alkalosis (Goss et al. 1992b). The need to generate H^+ and HCO_3^- ions for use by these exchange mechanisms means that cytoplasmic CA takes on a crucial role. tCAc provides H^+ and HCO_3^- for these exchange mechanisms by catalyzing the hydration of CO_2 that enters the branchial epithelium by diffusion (Goss et al. 1998, Perry, 2003; Perry and Laurent, 1990).

Given this mechanism, situations that place an increased demand on Cl^-/HCO_3^- and/or Na^+/H^+ exchange might be expected to increase the requirement for CA and lead to increased CA expression. Several examples of such effects have been documented in the literature. For example, environmental and internal conditions, such as changing salinity (Dimberg et al. 1981; Klutz et al. 1992), temperature (Houston et al. 1978), and acid-base status (Dimberg et al. 1987; Hirata et al. 2003) can affect gill CA expression.

Swenson and Maren (1987) used benzolamide, a relatively impermeant CA inhibitor (Maren 1967), to selectively inhibit branchial cytoplasmic CA activity without affecting RBC activity. Thus, benzolamide might have been a better inhibitor to use in this thesis to more specifically delineate the role of the branchial CA. However, benzolamide does slowly permeate the RBC (reviewed by Gilmour 1997, 2001) and therefore it is possible that RBC CA activity would have been inhibited over the relatively long time course of the experiments in Chapter 3.

The freshwater fish gill is a useful model for the examination of acid-base regulating epithelia, however a number of questions remained unanswered. We still don't

know the exact cellular location of the various exchange mechanisms. Not all Na^+/K^+-ATPase immunopositive cells in the gills were tCAc immunopositive; are there MR cells that lack CA? We noticed an increase of Na^+/K^+-ATPase immunopositive cells in the gills during hypercapnia (results not shown); is there a proliferation of MR cells or do MR pavement cells express Na^+/K^+-ATPase activity under these conditions? And finally, a better understanding of the characteristics and the roles of the different exchangers, pumps and transporters in various acid-base challenges is needed.

Conclusion

The results of this study provide the first direct evidence for the existence of a membrane-bound type IV CA isoform in the kidney of a freshwater teleost fish. The CA IV is localized specifically to the proximal segment of the nephron and plays an important role in renal bicarbonate reabsorption, thereby contributing to whole body acid-base regulation. A cytoplasmic CA isoform (tCAc) is also found in the kidney, where it contributes to HCO_3^- reabsorption. More importantly, however, tCAc is present in the gill where it plays a key role in acid and/or base excretion. Because of the more important role of the gill than the kidney in whole body acid-base regulation in freshwater fish, tCAc is probably the more important CA isoform in terms of acid-base regulation. The fact that both isoforms are up-regulated during the acid-base challenge of hypercapnia indicates that CA expression is sensitive to the acid-base requirements of the animal.

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APPENDIX

is present in both rbcs and gill tissue. Rahim et al. (1988) provided immunological evidence of a gill CA isozyme in rainbow trout *Oncorhynchus mykiss* and carp *Cyprinus carpio* that was distinct from the rbc CA isozyme. By contrast, Sender et al. (1999) found that the gill and rbc CA enzymes in the flounder *Platichthys flesus* were not immunologically distinct. Recently, however, Esbaugh et al. (2004) provided further evidence of CA activity in the gill cytoplasm of rainbow trout that was not that of the rbc CA isozyme. It is therefore unclear what isozyme is responsible for the gill cytoplasmic CA activity in teleosts.

Blood and branchial CA activities serve different functions. The primary physiological role of rbc CA is to catalyse the hydration of CO_2 to HCO_3^- at the tissue site of production, and dehydration of HCO_3^- to CO_2 at the respiratory surface, to facilitate the transport and excretion of CO_2 from the body (Perry, 1986; Perry and Laurent, 1990; Henry and Heming, 1998; Tufts and Perry, 1998; Henry and Swenson, 2000). Selective pressures preventing the rate of these reactions from limiting CO_2 transport and excretion are believed to be the primary forces driving the increase in rbc CA catalytic rate that is apparent through the fish lineage (Henry et al., 1993; Tufts et al., 2003). In contrast, the primary purpose of cytoplasmic gill CA is to catalyse the hydration/dehydration reactions of CO_2 within the branchial epithelium to provide counter ions for ion exchange processes that regulate acid–base balance and ionic homeostasis (Henry and Heming, 1998; Henry and Swenson, 2000; Marshall, 2002). A notable exception, however, is the membrane-associated CA in the gills of dogfish, which contributes to CO_2 excretion (Gilmour et al., 2001).

The main objective of this study was to determine if branchial CA activity in rainbow trout was the result of a general cytoplasmic CA isozyme, with kinetic properties, tissue distribution and physiological functions distinct from those of the rbc-specific CA isozyme. In particular, the trend from agnathans to teleosts towards a faster rbc CA isozyme (Henry et al., 1993; Tufts et al., 2003), leads to the prediction that a trout general cytoplasmic CA isozyme with a wide tissue distribution will exhibit a lower turnover number (slower catalytic rate) than the rbc-specific isozyme. In addition, a second series of experiments tested the differential regulation of the two cytoplasmic CA isozymes in response to a physiological disturbance, anaemia.

Materials and methods

Experimental animals and tissue collection

Rainbow trout *Oncorhynchus mykiss* Walbaum used at Queen's University were obtained from Pure Springs Trout Farm (Belleville, Ontario, Canada); rainbow trout used at the University of Ottawa were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Prior to experiments, the fish were maintained in large fibreglass tanks supplied with flowing, aerated, dechloraminated (Ottawa) or dechlorinated (Kingston) freshwater at 13°C, and were fed to satiation with commercial trout pellets on alternate days. The photoperiod was 12 h:12 h L:D, and fish were acclimated to the holding facility for at least 2 weeks prior to use.

To induce anaemia, rainbow trout (61.5±1.5 g, N=23; mean ± S.E.M.) were lightly anaesthetised in a solution of ethyl-*p*-aminobenzoate (0.1 g l^{-1}) and 1 ml of blood was removed by caudal puncture. Fish were then placed in a single 0.5 m diameter holding tank. At each of 12 h, 72 h, and 15 days, six fish were removed from the holding tank, and blood and gill tissues were sampled as described below. Haematocrit was measured in duplicate at the time of sampling, using microcapillary tubes centrifuged at 6000 ***g*** for 6 min.

Tissues were collected from individual rainbow trout that were anaesthetized in either CO_2-saturated water (kinetic analyses), or 0.1 g l^{-1} of ethyl-*p*-aminobenzoate (molecular analyses). Blood was collected into a heparinized syringe by caudal puncture and transferred to a microfuge tube. The rbcs and plasma were then separated by centrifugation and immediately frozen in liquid nitrogen. The rbc pellets used in kinetic analyses were washed three times with saline prior to being frozen, to ensure that no other blood components were present. The gills, along with other tissues (heart, brain, gut, liver, spleen, anterior and posterior kidney), were removed after perfusing the body with saline to clear it of blood. Perfusions were performed by exposing and cannulating the bulbus arteriosus with polyethylene tubing (PE 160; Clay-Adams, Missaussauga, ON, Canada), and using a peristaltic pump to pump 100 ml of heparinized (50 i.u. ml^{-1} sodium heparin) Cortland's saline (Wolf, 1963) into the body, followed by 1 l of non-heparinized Cortland's saline. Immediately after cannulating the bulbus arteriosus, the ventricle was severed to allow fluid in the circulatory system to drain from the body. Upon sampling, all tissues were carefully examined for blood clots; any observed were removed. Tissue samples were then frozen in liquid nitrogen and stored at –80°C.

Series I: kinetic analysis of rainbow trout gill and rbc cytoplasmic CA

Tissue homogenization and fractionation

To facilitate homogenization, adult trout tissues (1–2 g; N=4) were cut into fine pieces using scissors and a scalpel. The tissue was then added to 8 volumes of refrigerated Tris buffer (in mmol l^{-1}: 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.4 using 10% phosphoric acid) per gram tissue and homogenized using a motor-driven Teflon–glass homogenizer until no pieces of tissue remained (approximately 5 passes). Next, the crude homogenate was centrifuged (100 000 ***g*** for 90 min; Beckman L8-55M ultracentrifuge; Henry et al., 1993) at 4°C to remove cellular debris, mitochondria and membrane fractions from the tissue cytoplasmic fraction. The cytoplasmic fractions were then examined to determine the relative levels of CA and haemoglobin.

Measurement of carbonic anhydrase activity and haemoglobin concentration

Carbonic anhydrase activity was measured using the electrometric ΔpH method (Henry, 1991; Henry et al., 1993).

The reaction medium consisted of 10 ml of Tris buffer kept at 4°C. After the enzyme source was added, the reaction was started by the addition of 400 µl of CO_2-saturated distilled water from a 1000 µl gas-tight Hamilton syringe. The reaction velocity was measured over a pH change of 0.15 units. To obtain the true catalysed reaction rate, the uncatalyzed rate was subtracted from the observed rate, and the buffer capacity was taken into account to convert the rate from pH units $time^{-1}$ to mol H^+ $time^{-1}$. The pH was measured using a Radiometer GK2401 C combined pH electrode connected to a Radiometer PHM64 research pH meter. Haemoglobin concentration was measured using Drabkin's method (Sigma, Oakville, CA, USA) with cyanomethaemoglobin (Sigma) as a standard.

Kinetic analysis

To determine the kinetic properties of the rbc and gill cytoplasmic CAs, experiments were conducted to examine the velocity of CO_2 hydration at increasing concentrations of CO_2. The reciprocals of these values were plotted on a Lineweaver-Burke plot (Maren et al., 1980; Henry et al., 1993), from which the V_{max} and K_m values were obtained. The enzyme units (eu) were kept between 1 and 2 (Maren et al., 1960), and these values were recorded for each trial.

The enzyme concentration was obtained by measuring CA activity in the presence of different concentrations of acetazolamide (Az), a potent CA inhibitor. These data were then plotted on an Easson-Stedman plot (Easson-Stedman, 1937), using the equation:

$$I_o/i = 1/(1-i)K_i + E_o ,$$

where I_o is the inhibitor concentration, i is the fractional inhibition at a given inhibitor concentration, K_i is the inhibition constant, and E_o is the concentration of enzyme (Maren et al., 1960, 1980; Henry et al., 1993). For each inhibitor concentration, assays were performed in duplicate and the mean activity was plotted. E_o and K_i Az values were calculated for each sample. For each trial, the eu value was determined and a ratio of E_o/eu was then calculated; the E_o of further samples could then easily be determined based on the calculated eu (Maren et al., 1980, 1993).

The catalytic rate constant (k_{cat}) was then calculated using the formula:

$$k_{cat} = V_{max}/E_o ,$$

as described by Maren et al. (1980). The inhibition constant for chloride was also calculated, using the method of Dixon (1953). Mean values of k_{cat}, K_m, K_i Az and K_i Cl^- were obtained for the four samples utilised. To examine the sensitivity of each CA isozyme to the rainbow trout plasma inhibitor of CA (pICA), CA activity in the cytoplasm of the rbcs and gills was assayed in the presence of increasing volumes of separated trout plasma.

Series II: molecular analysis of rainbow trout gill cytoplasmic CA

Determination of cDNA sequence

The following procedures were performed independently by groups at Queen's University (using gill tissue), and the University of Ottawa (using whole blood). Total RNA was extracted from rainbow trout gills and whole blood by the acid/phenol method of Chromczynski and Sacchi (1987), as modified for fish blood by Currie et al. (1999), or using Trizol (Invitrogen, Burlington, ON, Canada).

First strand cDNA was synthesized from purified rainbow trout RNA from gill and whole blood using AMV reverse transcriptase (RT) and random primers. A 333 bp internal segment of rainbow trout CA coding region was amplified by PCR at an annealing temperature of 50°C, using a forward primer (CA-F; 5′-CAG TTC CAT TTC CAT TGG GG-3′) and reverse primer (CA-R; 5′-CAG AGG AGG GGT GGT CAG-3′). All PCR reactions involved an initial denaturation at 94°C for 30 s followed by 30 cycles of: 94°C for 30 s; annealing temperature for 60 s; 72°C for 90 s, and ending with a final extension for 10 min at 72°C. Both the forward and reverse primers were designed on the basis of high sequence identity among zebrafish CA (GenBank, U55177), gar *Lepisosteus osseus* rbc CA (GenBank, AY125007), human CA I (GenBank, X05014), and human CA II (GenBank, J03037). The resulting PCR product was ligated into a pDrive vector (Qiagen, Missaussauga, ON, Canada) and sequenced. This sequence information was used to perform 3′ rapid amplification of cDNA ends (RACE). The cDNA for 3′ RACE was amplified using the 3′ RACE adapter primer (Invitrogen) and Superscript II (Invitrogen). The 3′ sequence was amplified with nested PCR using the Abridged Universal Amplification primer (Invitrogen), and CA forward primers (1st round) (5′-CCT TGC TGT TGT AGG AGT CTT C-3′) and (2nd round) (5′-GGT CCT TGA TGC TTT TGA TG-3′). The 3′ RACE product was ligated into a PCR 2.1 vector (Invitrogen) and sequenced.

The 3′ cDNA sequence and a GenBank 5′ cDNA sequence for a rainbow trout CA homologue (CB94032) were combined to yield a 780 bp coding region (TCAc). The complete coding region sequence was entered in GenBank (AY514870).

Northern blot analysis

For northern blots, 10 µg of total RNA was fractionated by glyoxal/dimethyl sulphoxide (DMSO) denaturing electrophoresis on a 1% agarose gel and transferred to a Duralon nylon membrane (Stratagene, Missaussauga, ON, Canada) using 20× standard saline citrate (SSC). Membranes were ultraviolet-crosslinked (Fisher UV crosslinker) twice at optimal setting prior to hybridization.

Probes for rbc CA (TCAb) and β-globin (a haemoglobin subunit) were generated from first strand cDNA from rainbow trout rbc mRNA. A 446 base pair probe for β-globin was amplified as described by Lund et al. (2000). Both the TCAb and TCAc probes were 333 base pair fragments that were amplified using the CA-F and CA-R primers, as previously described. Probes were labelled using [α-^{32}P]dCTP (specific activity 10^9 cts min^{-1} μg^{-1} DNA) and the Ready-To-Go labelling system (Pharmacia, Piscataway, NJ, USA). Membranes were prehybridized at 60°C for 3 h in Church's

buffer. Blots were then hybridized overnight in the same solution at 60°C, with approximately 10^9 cts min^{-1} of denatured probe. The blots were then washed twice using 1×SSC/0.1% SDS solution (20 min, 60°C) and once using 0.25×SSC/0.1% SDS (20 min, 60°C). Finally, blots were exposed to a phosphor screen (Kodak, Rochester, NY, USA) and visualized and quantified using a phosphoimager (Molecular Devices, Sunnyvale, CA, USA) driven by ImageQuant software. All membranes were also probed with a human 18S rRNA probe (Battersby and Moyes, 1998) to correct blots for loading differences, and were expressed relative to the band with the greatest density.

Real-time PCR

Total RNA was extracted from 30 mg aliquots of powdered tissue samples using the 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) for 15 min at 37°C. The RNA was eluted in 70 μl of nuclease-free H_2O and its quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf, Missaussauga, ON, Canada). cDNA was synthesized from 2 μg of RNA using random hexamer primers and Stratascript reverse transcriptase (Stratagene).

TCAb, TCAc and haemoglobin mRNA levels were assessed by real time PCR on samples of cDNA (0.5 μl) using a Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) and a Stratagene MX-4000 multiplex quantitative PCR system. ROX (Stratagene) was used as a reference dye. The PCR conditions (final reaction volume, 25 μl) were as follows: 0.5 μl cDNA template, 300 nmol l^{-1} forward and reverse primer, 12 μl 2×Master Mix, 1:30000 ROX final dilution. The annealing and extension temperatures over 40 cycles were 58°C (30 s) and 72°C (30 s), respectively. The following primer pairs were designed using Primer3 software: β-actin forward (5′-CCA ACA GAT GTG GAT CAG CAA-3′), β-actin reverse (5′-GGT GGC ACA GAG CTG AAG TGG TA-3′), TCAc forward (5′-CAG TCT CCC ATT GAC ATC GTA-3′), TCAc reverse (5′-CGT TGT CGT CGG TGT AGG T-3′), TCAb forward (5′-TTG GCT TTG TGG ATG ATG TT-3′), TCAb reverse (5′-AGG GGA ACT TGA TTC CAT TG-3′), haemoglobin forward (5′-ATG GTC GAC TGG ACA GAT CC-3′), haemoglobin reverse (5′-CTG AGT CCA TGG AGA CAC GA-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 non-specific amplicons during the real-time PCR runs, the PCR products were analysed by gel electrophoresis in initial experiments. Single bands of the expected size were obtained in all instances. Furthermore, 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 mRNAs was determined (using actin as an endogenous standard) by a modification of the delta-delta Ct method (Pfaffl, 2001). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

Sequence analysis

The TCAc sequence was compared with TCAb (GenBank, AY307082), gar rbc CA, zebrafish retina CA, and dace (*Tribolodon hakonensis*) gill CA (GenBank, AB055617) sequences, as well as human CA I, CA II and CA VII (GenBank, AY075019). Alignment of the amino acid sequences was performed using ClustalW (version 1.8) multiple sequence alignment. In addition, a comparative analysis of the active sites was performed between TCAc, TCAb, gar rbc CA and dace gill CA, as well as human CA VII and consensus CA I and CA II sequences, as reported by Tashian et al. (2000).

A phylogenetic analysis of amino acid sequences was also carried out, which included rainbow trout TCAb and TCAc, gar rbc CA, dace gill CA and zebrafish retina CA. This analysis also included: mouse CA I (GenBank, NM_009799), CA II (GenBank, BC055291), CA III (GenBank, NM_007606), CA Vb (GenBank, NM_019513) and CA VII (GenBank, NM_053070); human CA I, CA II, CA III (GenBank, NM_005181), CA Va (GenBank, NM_001739), CA Vb (GenBank, NM_007220) and CA VII; rat CA I (GenBank, XM_226922), CA II (GenBank, NM_019291), CA III (GenBank, NM_019292), CA V (GenBank, NM_019293) and CA VII (GenBank, XM_226204), and chicken CA II (GenBank, X12639), *Xenopus* CA II (GenBank, BC041213) and zebrafish CA VII (BC049309). Alignment used for the phylogenetic analysis was performed by ClustalX (version 1.81). Phylogenetic hypotheses were constructed using both neighbour joining (NJ; Saitou and Nei, 1987) and maximum parsimony (MP) as performed by PAUP* (beta test version 4.0b10; Swofford, 2000). MP analysis consisted of a heuristic search with TBR branch swapping and ACCTRAN character state optimization enforced, and with random stepwise addition and 1000 random addition replicates. NJ was performed on a matrix of mean character distances. Support for nodes for both analytical procedures was performed using the bootstrap analysis with 1000 pseudoreplicates. All analyses were performed using mouse and human CA VII as outgroups, as previously described by Hewitt-Emmett and Tashian (1996).

Gaps in sequence alignment were accounted for in three distinct series of analyses. In the first analysis, all possibly informative gaps were included and treated as missing data. In the second analysis, all gaps were removed, and in the third analysis, all gaps were treated as a distinct character state. The final analysis could only be performed using MP analysis. All subsequent trees were compared qualitatively for differences, with no major differences arising.

Statistical analysis

Values are expressed as means ± S.E.M. Statistical

differences in the kinetic properties and inhibitor sensitivities of TCAb and TCAc were analysed using unpaired Student's *t*-tests. One-way analysis of variance (ANOVA) followed by *post hoc* multiple comparisons using the Bonferroni test, as appropriate, were used to statistically analyse the effect of anaemia or acid infusion on relative rbc or gill mRNA expression of TCAb and TCAc. In all analyses, the fiducial level of significance was 5%.

Results

Sequence analysis

Isolation and sequencing of the final cDNA product for the rainbow trout gill CA (TCAc) yielded a complete coding sequence of 780 base pairs, or 259 amino acids (Fig. 1). The coding region of TCAc was aligned with and compared to gar *Lepisosteus osseus* rbc, zebrafish *Danio rerio* retina, dace *Tribolodon hakonensis* gill, and rainbow trout rbc (TCAb) CA sequences, as well as to human CA I, II and VII. The TCAc sequence closely resembled all of the fish CA sequences, with amino acid percentage identities ranging from 74 to 76%. Comparisons with human CA sequences revealed that TCAc most closely resembled CA II, with 62% amino acid identity.

NJ and MP analyses of vertebrate cytoplasmic CAs produced generally well supported phylogenetic trees of similar topology (Fig. 2). These analyses suggested that CAs I, II and III constitute a single monophyletic clade, while the fish cytoplasmic CAs (with the exception of zebrafish CA VII) constitute a separate clade. The fish cytoplasmic CA clade is basal to that of other vertebrate CAs (CA I, II, and III), but appears after the divergence of CA V and VII. Within the fish CA group, slight differences in topology were obtained with the two different approaches. NJ analysis revealed that TCAb and zebrafish retina CA were closely grouped, and TCAc and dace gill CA grouped together. The gar rbc CA was the most ancestral sequence. The tree formed by MP analysis showed a similar topology (tree not shown), with the exception that dace gill CA and TCAc did not group together, but diverged after gar rbc CA and prior to the TCAb/zebrafish retina CA group. It should also be noted that zebrafish CA VII grouped most closely with mammalian CA VII rather than with the fish cytoplasmic CA clade.

```
1   atgtctcatgcatggggatacgcaccggacaatggacccgacaaatggtgtgaaggcttc
      M  S  H  A  W  G  Y  A  P  D  N  G  P  D  K  W  C  E  G  F
61  ccaattgccaacggaccccgccagtctcccattgacatcgtacctggggaggctgccttc
      P  I  A  N  G  P  R  Q  S  P  I  D  I  V  P  G  E  A  A  F
121 gacgcagccttgaaggcgctcactttgaagtacgaccctccacctccattgacattctc
      D  A  A  L  K  A  L  T  L  K  Y  D  P  S  T  S  I  D  I  L
181 aacaacggacattcctttcaagtgacctacaccgacgacaacgacaactcaactctgaca
      N  N  G  H  S  F  Q  V  T  Y  T  D  D  N  D  N  S  T  L  T
241 ggggggcccatttcagggacgtacaggctaaagcagttccacttccactggggcgccagc
      G  G  P  I  S  G  T  Y  R  L  K  Q  F  H  F  H  W  G  A  S
301 gacgacaggggttctgagcataccgtggccgggaccaagtatgctgccgagctccacctg
      D  D  R  G  S  E  H  T  V  A  G  T  K  Y  A  A  E  L  H  L
361 gtacactggaacaccaagtaccccagctttggtgatgctgctagcaagtctgatggcctt
      V  H  W  N  T  K  Y  P  S  F  G  D  A  A  S  K  S  D  G  L
421 gctgttgtaggagtcttcctccaggttggaaatgaaaatgccaatcttcagaaggtcctt
      A  V  V  G  V  F  L  Q  V  G  N  E  N  A  N  L  Q  K  V  L
481 gatgcttttgatgccattaaagccaagggcaagcagacctctttcgagaattttgacccc
      D  A  F  D  A  I  K  A  K  G  K  Q  T  S  F  E  N  F  D  P
541 accatcctgctccccaagtccctagactactggacttacgacggctccctgaccacacct
      T  I  L  L  P  K  S  L  D  Y  W  T  Y  D  G  S  L  T  T  P
601 cctctgctggagagtgtcacctggatcgtctgcaaggagtcaatcagcgtcagccctgcc
      P  L  L  E  S  V  T  W  I  V  C  K  E  S  I  S  V  S  P  A
661 cagatgggcaaattccggagcctgcttttctctggagagggcgaggccgcctgctgcatg
      Q  M  G  K  F  R  S  L  L  F  S  G  E  G  E  A  A  C  C  M
721 gtggacaactaccgccccccctcagcccctcaagggccgcgctgtgcgtgcatccttcaaa
      V  D  N  Y  R  P  P  Q  P  L  K  G  R  A  V  R  A  S  F  K
781 taa
      *
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Fig. 1. Nucleotide and deduced amino acid sequence of carbonic anhydrase (CA) from the rainbow trout gill. Sequence shown is coding region only, from start codon (underlined) to stop codon (asterisk) as determined through RACE (rapid amplification of cDNA ends).

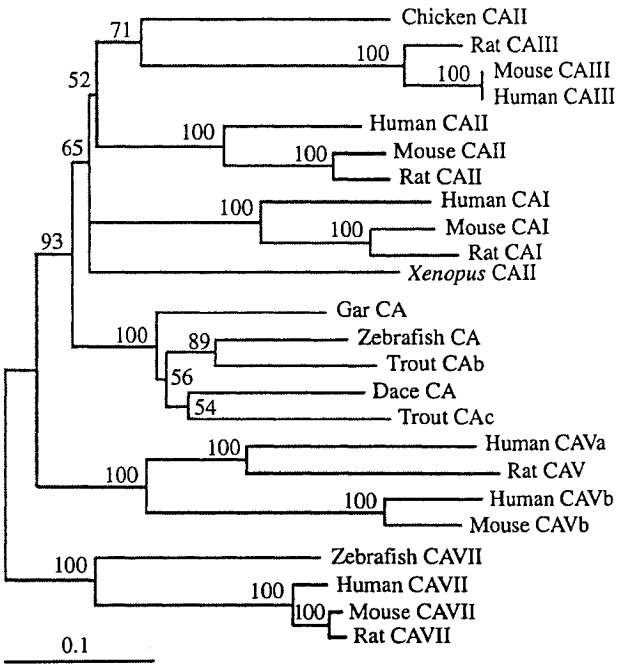

Fig. 2. Phylogenetic analysis of rainbow trout cytoplasmic carbonic anhydrase (TCAc) and other α-CA isozymes. The phylogenetic tree was constructed using neighbour joining analysis with support for nodes assessed using bootstrap analysis. The tree was ordered using mouse, rat and human CA VII as a monophyletic outgroup. Branches are drawn to scale with the length of 0.1 approximating replacement of 10% of the amino acids in the protein alignment (no Poisson correction for multiple hits).

The last aspect of the sequence analyses involved a comparison of the active site of TCAc with those of other fish CAs, as well as those from consensus CA I and II (Tashian et al., 2000) and human CA VII sequences. The active site of the TCAc sequence was most similar to the TCAb and dace gill CA sequences, differing at only one amino acid residue, while differing at two amino acid residues from the gar rbc CA sequence (Fig. 3). When compared to the mammalian CA active sites, the TCAc sequence was most similar to CA VII, differing by three amino acid residues.

Tissue distribution

The tissue distributions of both TCAb and TCAc were examined using northern blot analysis (Fig. 4) and real-time RT-PCR (Fig. 5) of perfused trout tissues. These analyses

TCAc	Y	S	N	N	H	S	F	Q	T	K	Q	H	H	E	H	E	H	V	F	L	V	G	W	Y	L	T	T	P	P	L	S	V	W	V	N	R	
	*	*						*			*	*	*	*	*	*	*							*		*	*						*		*	*	aa
				+	+	+		+				z	z				z	~		~	~				~							~	~				diff
TCAb	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
Dace	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	1
Gar	.	.	.	.	.	.	.	.	D	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
CA I	.	.	.	V	.	.	.	H	N	F	.	.	.	.	.	.	.	.	L	.	I	.	.	.	.	.	H	.	.	H	.	.	.	I	.	.	9
CA II	.	.	.	.	.	.	.	N	E	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	4
CA VII	.	.	.	.	.	.	V	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	3

Fig. 3. Comparison of the active sites of trout cytoplasmic catalytic anhydrase (CA) with those of trout red blood cell CA (TCAb), dace CA, gar CA, concensus trout CA I, CA II and human CA VII. Identical amino acids are indicated by a dot. aa diff, number of amino acid differences. *, putative active site; z, zinc binding ligand; +, proton shuttling associated ligand; ~, substrate associated pocket.

indicated that the TCAb isozyme was expressed almost exclusively in the rbc. Low levels of expression in the spleen and anterior kidney (Fig. 4), or heart and brain (Fig. 5) could be accounted for by blood that was not removed during saline perfusion. Blood contamination is, in fact, indicated by corresponding expression of haemoglobin in the spleen, anterior kidney (Fig. 4), brain and heart (Fig. 5). By contrast, the TCAc isozyme exhibited a wider tissue distribution. Although gill was the predominant site of expression of TCAc, brain tissue also displayed substantial expression, with low levels found in the kidney, gut, liver and muscle (Figs 4 and 5). Unlike northern blot analysis, real-time RT-PCR also revealed low TCAc expression in the rbcs. A comparison of the abundance of each isozyme in the rbcs, using real-time RT-PCR, indicated that TCAb was 666±183 times (N=6) more abundant than TCAc.

Kinetic analysis

The kinetic properties and inhibitor sensitivities of cytoplasmic CA isozymes from gill and rbc lysates were examined (Table 1). The K_i values for Az and chloride were similar for both the rbc and gill CA isozymes. However, when the cytoplasmic fractions of gill and rbc lysates were assayed in the presence of increasing volumes of trout plasma, which contains an endogenous CA inhibitor (Dimberg, 1994; Haswell and Randall, 1976; Henry et al., 1997), the rbc CA isozyme was found to be significantly more sensitive than the gill CA isozyme (Fig. 6). Moreover, both the turnover value (k_{cat}) and substrate affinity value (K_m) of the rbc CA isozyme were significantly higher than corresponding values for the gill CA

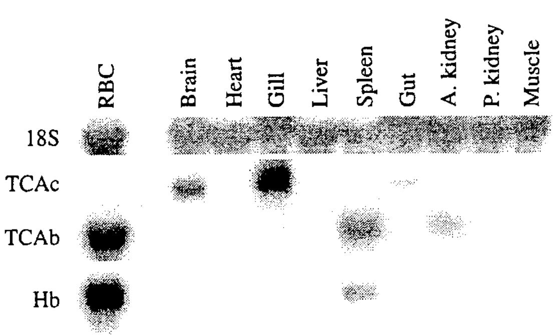

Fig. 4. Representative northern blots for rainbow trout red blood cell carbonic anhydrase (TCAb), cytoplasmic carbonic anhydrase (TCAc) and β-globin (Hb) mRNA and 18S rRNA from adult rainbow trout tissues (N=4). RBC, red blood cells; A, anterior; P, posterior.

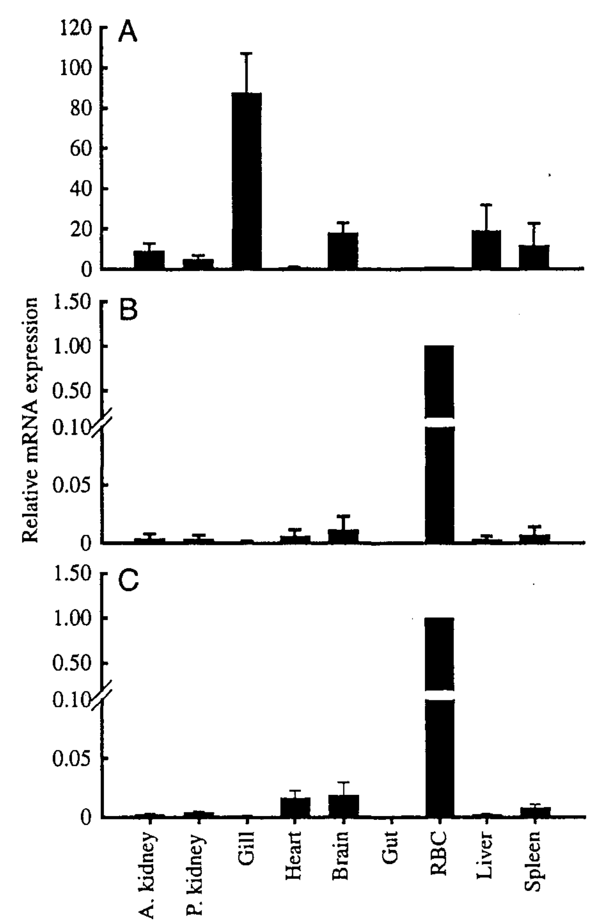

Fig. 5. Relative mRNA expression (mean ± S.E.M.) of cytoplasmic carbonic anhydrase (TCAc; A) and red blood cell carbonic anhydrase (TCAb; A) and haemoglobin (C) in rainbow trout tissues, as determined by real time RT-PCR (N=6). The red blood cell (RBC) mRNA expression was set to 1 in all cases.

Table 1. *Comparison of the catalytic and kinetic properties of cytoplasmic carbonic anhydrase isozymes from rainbow trout, using gill tissue and red blood cells as enzyme sources*

	K_m (mmol l^{-1})	k_{cat} (e^4 s^{-1})	K_i Az (nmol l^{-1})	K_i Cl^- (mmol l^{-1})
Gill	8.72±1.03	8.90±1.95	1.21±0.18	62.03±6.75
RBC	23.26±5.22*	30.28±5.83*	1.34±0.10	61.34±2.41

Values are mean ± S.E.M. (N=4), with statistically different groups indicated by an asterisk (unpaired t-test; P<0.05).
RBC, red blood cells.

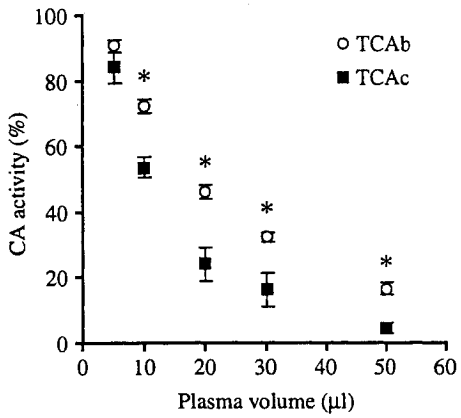

Fig. 6. The relative effect of plasma [which contains an endogenous carbonic anhydrase (CA) inhibitor] on the red blood cell (circles) and gill (squares) CA activity (mean ± S.E.M.) of rainbow trout (N=4). Significant differences are indicated by an asterisk (unpaired t-test; P<0.05).

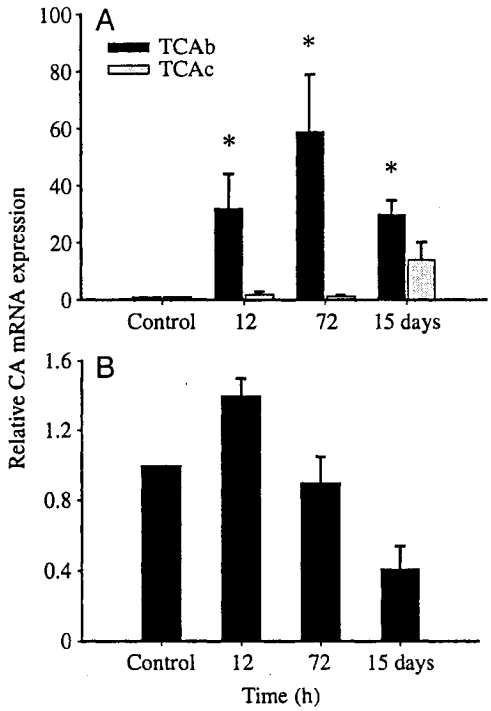

Fig. 7. The effects of anaemia on the relative mRNA expression (mean ± S.E.M.) of cytoplasmic carbonic anhydrase (TCAc) and red blood cell carbonic anhydrase (TCAb) in the red blood cells (A), and cytoplasmic carbonic anhydrase (TCAc) in gills (B) of rainbow trout, as determined by real time RT-PCR (N=6). Significant differences are indicated by an asterisk (P<0.05). Control values were set to 1 in all cases.

isozyme (Table. 1); thus, the rbc-specific isozyme appears to be a faster enzyme with a lower substrate affinity than the cytoplasmic isozyme.

Functional analysis

Changes in mRNA expression in whole blood and gill tissue for both TCAb and TCAc in response to the induction of anaemia were examined using real-time RT-PCR. Withdrawal of 1 ml of blood was sufficient to decrease haematocrit significantly (one-way ANOVA, P<0.05) by approximately 45–55%, from the control value of 47.6±3.7% (N=6) to 28.0±2.8% (N=6) at 12 h, 23.5±1.8% (N=6) at 72 h and 21.5±4.1% (N=5) at 15 days. The response to this induction of anaemia was a significant increase in rbc TCAb mRNA expression at all sample times (Fig. 7A), in the absence of any significant change in rbc or gill TCAc mRNA expression (Fig. 7A and B).

Discussion

The results of the present study show that a general cytoplasmic CA isozyme, that is distinct from the rbc-specific isozyme in structure, catalytic properties and distribution, is found in the tissues of rainbow trout. The general cytoplasmic CA isozyme (TCAc) has a lower turnover (lower catalytic activity) than the rbc-specific CA isozyme (TCAb), is found in high abundance in the gills and brain and is widely distributed in other tissues, including rbcs, in lower amounts.

The general cytoplasmic CA isozyme has a coding region of 780 base pairs (259 amino acids; Fig. 1), and high sequence identity to other known fish CA sequences (74–76%). The high sequence identity between the TCAb and TCAc isozymes (76%) may explain the results of Sender et al. (1999), who found no evidence for the presence of two distinct CA isozymes in the gill cytoplasm and rbc of flounder (*Platichthys flesus*). The gill CA activity in flounder was believed to be caused by the same isozyme found in the rbc, because antibodies produced to CA from both sources were cross-

reactive. Assuming that two isozymes do, in fact, occur in flounder, cross-reactivity between antibodies could be the result of high protein similarities.

To date, molecular analyses of the phylogeny of the α-CA gene family have been limited by the small amount of CA sequence information from non-mammalian vertebrate species (Lund et al., 2002; Tufts et al., 2003). When TCAc was included in a phylogenetic analysis of cytoplasmic α-CA isozymes, it joined a previously described fish CA monophyletic clade (Peterson et al., 1997; Lund et al., 2002; Tufts et al., 2003; Esbaugh et al., 2004). These findings suggest that the current trend of using mammalian nomenclature (slow type CA I and fast type CA II) to identify fish CA isozymes may be inappropriate (Tufts et al., 2003). In agreement with previous studies, the fish cytoplasmic CA group emerged prior to the gene duplication event that gave rise to the mammalian CA I, II and III genes, as well as the divergence of other tetrapod vertebrate isozymes, such as *Xenopus* and chicken CA II (Fig. 2). The analyses also agree with previous studies that show that the fish cytoplasmic CA group diverged after the emergence of CA V and VII in vertebrates. Unlike previous studies, however, this analysis contains two cytoplasmic CA isozymes from a single species. Interestingly, the two cytoplasmic CA isozymes from rainbow trout do not group most closely together; rather, TCAc groups closely with a gill isozyme from dace, whereas TCAb is most closely associated with a zebrafish retina sequence. There is evidence that the common ancestor of salmonid fish underwent a genome duplication event (Hinegardner and Rosen, 1972; Allendorf and Thorgaard, 1984). However, the topology of the trees formed by both NJ and MP analysis suggest that the duplication event that gave rise to the two cytoplasmic CA isozymes in trout occurred in the ancestor common to both zebrafish and trout, and possibly also dace. Either a gene duplication event that occurred at some point in the evolution of teleost fish, or a genome duplication event that occurred at the origin of modern fishes, could account for the presence of two cytoplasmic CA isozymes in trout (Amores et al., 1998; Meyer and Schartl, 1999; Robinson-Rechavi et al., 2001; Taylor et al., 2001). It is therefore necessary to examine whether multiple cytoplasmic CAs are present in more teleost families, as well as in other fish lineages, such as agnathans, dipnoans and non-teleost actinopterygians.

Examination of the amino acid residues that are specific to the active site pocket, as described by Tashian et al. (2000), can also yield insight into the functional evolution of CA isozymes. A comparison of the active site amino acid residues (Fig. 3) indicates not only that the fish cytoplasmic CA isozymes are highly similar at the amino acid level, but that the critical elements of enzyme function are almost entirely conserved, with only one amino acid difference between the teleost CA isozymes. In addition, the active site of TCAc and gar rbc CA differ by only two amino acids. It is also noteworthy that none of these amino acid differences fall within sections of the sequence that have been directly implicated in the catalytic mechanism (reviewed by Stams and Christianson, 2000).

Despite the high sequence similarity between the two trout cytoplasmic CA isozymes, significant differences were detected in their kinetic properties and inhibitor sensitivities, supporting the contention that the sequences represent discrete isozymes, with potentially physiologically distinct roles. It should be noted that because the mRNA, and presumably protein, level of TCAb in the rbc greatly exceeded that of TCAc, the kinetic properties of rbc lysates were taken to be representative of TCAb. TCAc was significantly more sensitive to the endogenous inhibitor in trout plasma than was TCAb (Fig. 6), while both isozymes exhibited similar inhibition constants when exposed to Az and chloride ions (Table 1). The inhibition constant for Az for both isozymes was within the range previously described for rainbow trout rbc CA (Henry et al., 1993; Esbaugh et al., 2004), while the value for chloride was slightly lower than that reported by Henry et al. (1993). Calculation of the turnover (k_{cat}) and substrate affinity constant (K_m) revealed that, as predicted, both were lower for the general cytoplasmic CA than for TCAb (Table 1). Indeed, the rbc-specific TCAb reaction rate was over three times faster than that of the more widely distributed TCAc. This difference in catalytic efficiency is most probably related to the different roles of the two isozymes. The primary role of the faster TCAb isozyme is to catalyse the dehydration/hydration reactions of CO_2 for the purpose of CO_2 transport and excretion (Perry, 1986; Tufts and Perry, 1998; Henry and Swenson, 2000). A single pass of blood through the gill vasculature (approximate transit time of 0.5–2.5 s; Cameron and Polhemus, 1974) can remove up to 35% of the total blood CO_2 load (Perry, 1986). Thus, an emphasis on the rapid translation of HCO_3^- from the plasma to the rbc and its conversion to molecular CO_2 is critical. Current theory contends that CO_2 excretion is rate-limited by the speed of the Cl^-/HCO_3^- exchanger (Perry, 1986; Perry and Gilmour, 1993; Desforges et al., 2001), and Henry et al. (1993) proposed that the evolution of faster rbc CA isozymes was related to the incorporation of a rapid Cl^-/HCO_3^- exchanger into the rbc membrane (see also Tufts et al., 2003). The data from the present study are certainly consistent with this hypothesis – a faster rbc isozyme related to the presence of a rapid rbc membrane anion exchanger (Cameron, 1978; Romano and Passow, 1984) and involved in CO_2 transport and excretion, *versus* a slower cytoplasmic isozyme that may play a role in numerous different functions (Henry, 1996). For example, within the gill, the general cytoplasmic isozyme probably plays a major role in acid–base balance and ionic regulation, where high activity may not be as crucial as it is to CO_2 excretion. It should also be noted that TCAb and TCAc do not display the dramatic differences in catalytic and kinetic properties associated with mammalian CA I and II, suggesting that TCAc may play a more catalytically demanding role in fish than the low activity CA I isozyme plays in mammals.

Differences between trout and mammals are also apparent in the tissue distribution of the CA isozymes. In mammals, CA I

and CA II are found in the cytoplasm of the rbcs as well as many other tissues throughout the body (Chegwidden and Carter, 2000; Parkkila, 2000; Swenson, 2000). In contrast, the major trout rbc CA isozyme, TCAb, appears to be found only in the rbc (Figs 4 and 5). Unlike TCAb, however, TCAc exhibits a widespread distribution among different tissues, with greatest expression occurring in the brain and gills, and lower expression in the liver, gut, white muscle and the anterior and posterior kidney. Presumably, TCAc has numerous physiological roles (Henry, 1996), similar to mammalian CA I and II (Chegwidden and Carter, 2000; Parkkila, 2000; Swenson, 2000). The finding that TCAc is present with TCAb in the rbcs of trout is in contrast to the prevailing belief that teleost rbcs express only one CA isozyme (Maren et al., 1980; Sanyal et al., 1982; Kim et al., 1983; reviewed by Henry and Heming, 1998), but is in accordance with a few studies that presented evidence for two isozymes (Girard and Istin, 1975; Carter et al., 1976). Whereas mammalian rbcs express two cytoplasmic CA isozymes that differ by an order of magnitude in catalytic activity, both trout rbc cytoplasmic isozymes appear to be higher activity enzymes. It is unclear why two isozymes with the potential for overlapping function would be expressed in the same tissue, although it is possible that their roles are differentiated by protein interactions. Such protein interactions – for example, with the rbc membrane Cl^-/HCO_3^- exchanger – might also explain why the slower TCAc isozyme is the more widespread isozyme in trout, while the higher activity TCAb is specific to the rbc. In mammalian rbcs, cytoplasmic CA II interacts with numerous transport proteins, while CA I is unable to do so (Vince et al., 2000; Reithmeier, 2001; Sterling et al., 2001, 2002a,b; Li et al., 2002). It remains to be determined whether an analogous system also evolved in modern fish.

Previous studies indicated that CA expression in fish is regulated in response to a variety of internal and external conditions, such as salinity (Girard and Istin, 1975; Dimberg et al., 1981; Kultz et al., 1992), temperature (Houston and McCarty, 1978; Houston and Mearow, 1979), and acid–base status (Dimberg and Hoglund, 1987). With this in mind, a preliminary study was performed to examine whether the two cytoplasmic CA isozymes in the rbc were differentially regulated in response to anaemia.

In the rbc, a dramatic increase in TCAb mRNA expression occurred in response to the induction of anaemia (Fig. 7A), whereas no significant change in TCAc mRNA expression was observed in the rbc (Fig. 7A) or gill (Fig. 7B). It should, however, be noted that young rbcs were probably released into the bloodstream over the duration of the experiment, and these young rbcs are known to have 10-fold higher levels of mRNA transcripts (Lund et al., 2000). The extent to which young rbcs may affect the results of these experiments is unclear as the haematocrit of all animals remained depressed throughout the 15-day experimental period, suggesting that few young rbcs were released into the bloodstream. In addition, if the increased expression levels of TCAb mRNA observed throughout anaemia were, in fact, due to the increased mRNA transcript levels inherent in young rbcs, then the levels of TCAc mRNA in the rbcs would also be expected to increase. The expression levels of TCAc mRNA, however, did not significantly change throughout the course of the experiment. Although these data do not unequivocally demonstrate differential regulation of the two CA isozymes, it remains plausible. The differential regulation of the two rbc CA isozymes would support the idea that they serve different functions within the rbc, which is similar to mammalian rbcs where CA II is responsible for the majority of CO_2 hydration/dehydration (Maren et al., 1976). Further experiments should be performed to more carefully quantify the effect of young rbcs, as well as the roles the two cytoplasmic isozymes may play.

In conclusion, the results of the present study demonstrate that, in rainbow trout, the CA isozyme in the rbcs is distinct in structure, tissue distribution, kinetic properties, and probably physiological role, from a second, more widely distributed or general cytoplasmic isozyme. Although both isozymes exhibited similar inhibitor sensitivities, the catalytic activity of the rbc CA isozyme was threefold higher than that of the general cytoplasmic form, likely owing to selective pressures specific to the demands of CO_2 excretion. Selective up-regulation of the rbc-specific CA isozyme within rbcs in response to anaemia supports the hypothesis of two discrete isozymes with different functions.

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