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Molecular Identification and Characterization of the Branchial Epithelial Calcium Channel in the Rainbow Trout (Oncorhynchus mykiss)

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MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF THE BRANCHIAL EPITHELIAL CALCIUM CHANNEL IN THE RAINBOW TROUT (*Oncorhynchus mykiss*)

By

Arash Shahsavarani

Thesis submitted to the
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Title: Molecular identification and characterization of the branchial epithelial calcium channel in the rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Oncorhynchus mykiss epithelial calcium channel (ECaC) is thought to regulate calcium uptake across the gill. ECaC is a member of the transient receptor potential (TRP) gene family. In mammals, ECaC is divided into two sub-families, TRPV5 and TRPV6. Rainbow trout appears to possess only a single ECaC gene similar to the mammalian TRPV5 and TRPV6. Phylogenetic analysis suggests that an ancestral form of the gene diverged from those of lower vertebrates prior to a gene duplication event that gave rise to TRPV5 and TRPV6.

In contrast to previous models of branchial calcium uptake which proposed an almost exclusive distribution of ECaC to mitochondrial rich cells (MRCs), the results of real time PCR on enriched gill cell populations as well as immunocytochemistry and *in-situ* hybridization analysis of enriched cells, cell cultures and whole gill sections suggest that ECaC is localized to a subset of both MRCs and pavement cells (PVCs).

Using real time PCR and immunocytochemistry, the response of ECaC expression to various treatments known to increase or decrease Ca^{2+} uptake was examined. A significant increase in ECaC mRNA expression was observed following 5 days exposure of rainbow trout to softwater ($[Ca^{2+}] = 20 - 30$ nmol l^{-1}). A concurrent increase in protein expression was suggested by the immunocytochemistry results (particularly in cells located at the tips of the lamellae) and confirmed by western blotting (identifying a single immunoreactive band at 90 kDa). A significant increase (almost 100-fold) in mRNA expression was also observed following exposure to 48 h of hypercapnia (~7.5 mm Hg; a treatment known to increase Ca^{2+} uptake capacity). Immunocytochemical analysis of the

hypercapnic gills suggested an increase in the apical ECaC proteins on PVCs as well as on a subset of MRCs. Treatment of fish with cortisol caused a significant increase in ECaC expression. In contrast to the above experiments, induced hypercalcemia (through infusion of $CaCl_2$, treatment known to reduce Ca^{2+} uptake) resulted in a significant reduction in ECaC gene expression.

Taken together, the results presented in this thesis demonstrate that the levels of ECaC mRNA vary in direct relation to the Ca^{2+} transporting capacity of the gill (changes in transport capacity have previously been reported by other authors). These results thus provide evidence that, in part, Ca^{2+} uptake rates are regulated by the number of apical membrane Ca^{2+} channels that in turn modulate the inward flux of Ca^{2+} into gill epithelial cells. However, the data also suggests that a more in-depth characterization of gill cells is required since the distribution of ECaC appears to be more extensive than previously proposed.

RÉSUMÉ

Les canaux calciques épithéliaux (ECaC) de *Oncorhynchus mykiss* sont soupçonnées d'être responsable de la régulation d'absorption de calcium au travers des branchies. ECaC est un membre de la famille de gènes codant pour les récepteurs de potentiel transitoire (TRP). Chez les mammifères, ECaC est divisé en deux sous-familles, TRPV5 et TRPV6. La truite arc-en-ciel semble posséder seulement qu'une sous-famille de ECaC similaire au TPRV5 et TRPV6 de mammifère. Une analyse phylogénique suggère qu'une forme ancestrale du gène a divergé de ceux des vertébrés inférieurs avant l'événement de duplication du gène qui donna lieux a TRPV5 et TRPV6.

Contrairement au modèle précédant d'absorption de calcium au niveau des branchies, qui propose une distribution de ECaC presque exclusive au cellules riches en mitochondries (MRCs), les résultats d'analyses de PCR en temps réel, d'immunocytochimie, d'hybridation *in situ* performer sur des cellules enrichies, cultures de cellules et sur des sections de branchies entières suggèrent que ECaC se trouve sur les MRCs et les cellules pavimenteuses (PVCs).

À l'aide de PCR en temps réel et à des techniques d'immunocytochimie, la réponse d'expression du gène codant pour ECaC a différents traitements visant à augmenter ou diminuer l'absorption de Ca^{2+} fut examinée. Une augmentation marquée du niveau d'expression d'ARNm ECaC fut observé après 5 jours de traitement de la truite à de l'eau douce ($[Ca^{2+}] = 20 - 30$ nmol^{-1}). De plus une augmentation du niveau d'expression de protéine ECaC fut observée (identification d'une protéine de 90kDa par Western blot), et ce tout particulièrement près du bout des lamelles branchiales. Une

augmentation prononcée (presque 100 fois) du niveau d'expression d'ARNm fut observée après 48 h d'hypercapnie (~7.5 mm Hg; un traitement visant à augmenter la capacité maximum d'absorption de Ca^{2+}). Une analyse immunocytochimique de branchies suite au traitement à l'hypercapnie suggère une augmentation de protéines ECaC du coté apical des PVCs et un groupe particulier de MRCs. Un traitement avec la cortisol (un hormone hypercalcique) a causé une augmentation significative du niveau d'expression d'ARNm. Le traitement des poissons avec l'infusion de $CaCl_2$ (connue pour causer la réduction d'absorption du Ca_{2+}) a significativement réduit l'expression du gène ECaC .

Ensemble, ces résultats démontrent que le niveau d'expression du gène ECaC est directement relier à la capacité de transport du Ca2+ dans les branchies. Ainsi, fournissant en partie des preuves que le contrôle d'absorption de calcium, a travers les branchies, peut être dirigé par le nombre de canaux ECaC. De plus, ces résultats indiquent que des recherches plus approfondies sont requises car la distribution d'ECaC est plus vaste qu'a au part avant été proposé.

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Our past is never too far behind us, and I came to realise this at some of the more challenging times when the support and encouragement of my former supervisors and colleagues were so helpful and I would like to acknowledge Drs. J.S Ballantyne and P.A. Wright and the Humphries family for their support.

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Last but not least my family. To have a great family is the most fortunate thing in ones life. I am perhaps one of the most fortunate people ever. Azar Shahsavarani, you are the kindest and most joyful person I know and I could not have asked for a better sister. To my father, Mahmood Shahsavarani, and my mother, Zohreh Ferdowsi Shahsavarani, you have sacrificed so much for your two children and you still keep on sacrificing. Everything I've accomplished and all challenges to come are dedicated to you. I love you and thank you for the unconditional love and support that you have given me. In your honour, I pledge to remain honest, proud and strong.

As a final thought, my experiences have thought a lot. I've learned to be strong, fair and not to accept unjust rulings or treatments. I'd rather face the truth even if it hurts rather than to advance in my career or life based on lies. And I would rather die poor than wealthy but cheap. Naïve as it may sound; winning the race knowing that you cheated simply means that you know that you are forever THE LOSER.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

α, Greek letter alpha

ANOVA, Analysis of variance

β, Greek letter beta

bp, base pair

CaR, Ca^{2+} sensing receptor

CaT, Ca^{2+} transport channel

cDNA, complementary DNA

DAPI, 4,6-diamidion-2-phenlidole

DSI, double seeded insert

ECaC, epithelial calcium channel

MRC, mitochondria rich cell

mRNA, messenger RNA

N, number of samples

NCX, sodium calcium exchanger

PBS, phosphate buffer saline

PBST, PBS with Tween 20

PCR, polymerase chain reaction

PMCA, plasma membrane calcium ATPase

PVC, pavement cells

RACE, rapid amplification of cDNA ends

RT-PCR, reverse transcriptase PCR

SEM, standard error of the mean

ssDNA, sheered sperm DNA

SSI, single seeded insert

STC, stanniocalcin

TRP, transient receptor potential

CHAPTER 1

GENERAL INTRODUCTION

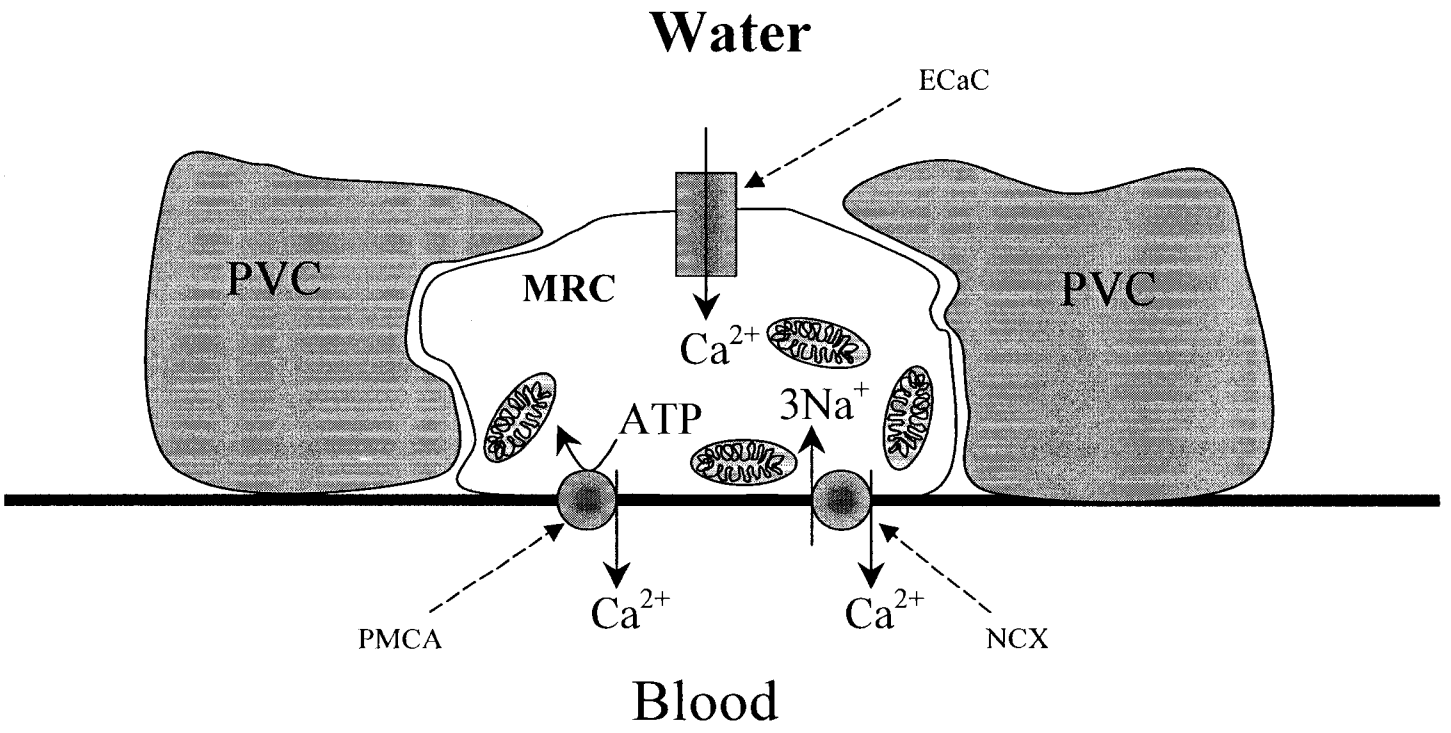
The role of calcium (Ca^{2+}) as an important element in physiological processes was first discovered by Sydney Ringer over a century ago (Rubin 1985). Ca^{2+} plays a significant role in maintaining structural and functional properties of biomembranes, influencing the cytoskeletal system by regulating microtubule assembly and disassembly (Karr et al. 1980), myosin and actin interaction (Rubin 1985), cell coupling (Loewenstein and Rose 1978) and signal transduction amongst numerous other functions.

Depending on the environment inhabited by an organism and its lifestyle, Ca^{2+} may be acquired and regulated differently. Unlike in mammals where calcium is acquired through diet (Bindels 1993), fish obtain calcium directly from their environment with the gills being the primary organ involved (Flik and Verbost 1993; Perry 1997; Perry and Flik 1988).

This thesis seeks to validate the present model for Ca^{2+} uptake in fish and to characterize the model in response to physiological and environmental challenges. The present model (Figure 1.1) has been developed through extensive physiological studies, however, questions remain as to how the various components of the model interact to both maintain uptake of Ca^{2+} from the environment and to maintain low intracellular Ca^{2+} levels within gill cells. To characterize this model several questions need to be addressed. What genes are involved in Ca^{2+} uptake? How are these genes regulated in response to changes in environmental [Ca^{2+}]? How are these genes regulated in response to physiological factors known to stimulate or inhibit Ca^{2+} uptake?

Figure 1.1. Schematic diagram of the branchial Ca^{2+} uptake model in freshwater fish. This model suggests that Ca^{2+} enters mitochondria rich cells (MRCs) through apical non-voltage gated Ca^{2+} channels. At the basolateral membrane, Ca^{2+} is either actively transported by plasma membrane calcium ATPases (PMCA) or across the membrane via sodium-calcium exchangers (NCX). This putative model suggests the allocation of the calcium transport mechanism almost exclusively to MRCs. PVC = pavement cell.

Figure 1.1



In fish, the primary site for Ca^{2+} uptake is the gill (Fenwick 1989; Flik and Verbost 1993; Perry 1997; Perry and Flik 1988). This organ has a surface area 10 to 60 times that of the skin covering the body (Parry 1966). Although this large surface area is critical for gas exchange, inevitably it requires tight homeostatic control (Evans et al. 1999). As will be discussed shortly there are several types of cells that make up the gill epithelium, and it is now generally accepted that mitochondria rich cells (also termed chloride cells; see below) are the primary cells involved in Ca^{2+} uptake. In the environment, Ca^{2+} can also be found complexed to inorganic anions. This thesis will investigate the model for ionic calcium uptake at the gill and its regulation.

The intestine is also known to be a route for Ca^{2+} uptake in fish [e.g. Atlantic cod (Sundell et al. 1992)]. In seawater, where Ca^{2+} concentrations can reach approximately 10 mmol l^{-1}, drinking of water can be an important source of calcium. In freshwater, however, fish tend not to drink because they are in a hypoosmotic environment. Notwithstanding, Ca^{2+} can still be obtained from the diet, and studies of intestinal Ca^{2+} uptake in the proximal regions of the intestine in both freshwater and seawater species have confirmed this pathway (Flik et al. 1990; Flik et al. 1996; Schoenmakers et al. 1993). Although calcium uptake at the intestine is known to be affected by high demand or changes in stanniocalcin (Flik et al. 1990, Flik and Verbost 1993), branchial influx of Ca^{2+} appears to be sufficient for growth and homeostasis (Ichii and Mugiya 1983), therefore Ca^{2+} uptake at the intestine is most likely facultative.

Finally, the kidney is another site of Ca^{2+} regulation and its role can be vastly different depending on the environment. In fact, as a result of seawater adaptation, renal Ca^{2+} reabsorption tends to decrease (Flik et al. 1996). The highly segmented morphology

(and presumably specific regional physiological characteristics) of the kidney makes its biochemical analysis difficult. Nonetheless, evidence suggests that cytoplasmic Ca^{2+} extrusion is primarily driven by an ATP-dependent Ca^{2+} pump (Ca^{2+}-ATPase) with no apparent involvement of a Na^{+}-Ca^{2+} exchanger (NCX; Flik et al. 1996; van der Heijden 1999).

The rainbow trout (*Oncorhynchus mykiss*) was chosen as the experimental model for this thesis as it has been the subject of numerous studies on ion regulatory mechanisms with particular emphasis on the role of chloride cells (Perry 1997).

Calcium uptake at the gills

The fish gill is an important organ that is involved in numerous functions such as gas exchange and ion and acid-base regulation (Goss et al. 1998) as well as nitrogenous waste excretion (Pärt et al. 1999; Walsh et al. 2000; Walsh et al. 2001). The gill has a large surface area because it is subdivided into incrementally smaller structural elements (arches, filaments and lamellae). The various physiological functions of the gill are achieved via specialized cells that form a barrier between the external environment (water) and the internal environment (blood). The gill epithelium consists of at least five different cell types. The majority of cells (possibly covering more than 90% of the surface area) are pavement cells (PVCs) that are believed to be primarily involved in gas exchange (Goss et al. 1998). However, recent evidence suggests an additional role for PVCs in ion regulation and in particular Na^{+} uptake (Goss et al. 1994; Morgan et al. 1994; Perry 1998; Wilson et al. 2000). Mucous cells are not believed to be directly involved in ion or acid-base regulation (Goss et al. 1995). Another small fraction of gill

cells is made up of neuroepithelial cells (Dunel-Erb et al., 1982) and stem cells. A final group of cells that are characterized by being rich in mitochondria are the MRCs. These cells are larger than, and interspersed with, the PVCs and are often located on the trailing edge of the filament (Evans et al. 1999). Other distinctive ultrastructural features of these cells are a network of tubules continuous with the basolateral membrane and a vesiculotubular system in the apical portion of the cell where mitochondria are rare (Goss et al. 1995). Correlative evidence suggests that changes in total MRC apical surface area occur as a means to adjust gill ion transport capacity.

Ca^{2+} Entry

The initial barrier to Ca^{2+} uptake is thought to be the apical membrane of the MRC (figure 1.1). Identification of the route of entry for Ca^{2+} into the MRCs has not been conclusive. This is largely the result of the paucity of available techniques to isolate pure MRC apical membranes (Flik et al. 1995). Some evidence suggests that the route of entry is mediated through a lanthanum-sensitive, voltage-independent Ca^{2+} channel (Flik et al. 1995; Perry and Flik 1988). Stanniocalcin, the predominant hypocalcemic hormone in teleost fishes (Flik et al. 1995), is believed to induce its effects on the presumptive Ca^{2+} channel through a second messenger (possibly through cAMP). This may define the channel as a second messenger-operated Ca^{2+} channel (Flik et al. 1995). An interesting and potentially useful characteristic of these channels, other than their sensitivity to lanthanum, is the fact that they permit cadmium flux into the cells (Flik and Verbost 1993; Verbost et al. 1989). A similar mechanism has been identified in canine kidney epithelial cell (Flanagan and Friedman 1991) thus further emphasizing the similarities

between the mammalian kidney Ca^{2+} model and the fish gill Ca^{2+} uptake model. In the mammalian kidney, the apical influx of Ca^{2+} is the rate limiting step (Vennekens et al. 2000). Recently, an epithelial Ca^{2+} channel (ECaC) has been cloned (Hoenderop et al. 1999) from the rabbit kidney and is thought to be exclusively present in $1,25(OH)_2D_3$-responsive epithelia including intestine, kidney and placenta (Caterina et al. 1997; Kanzaki et al. 1999). ECaC possesses putative phosphorylation sites for protein kinase C, cAMP-dependent and cGMP-dependent protein kinase, calcium-calmodulin-dependent protein kinase, and structural domains, such as N-linked glycosylation sites and ankyrin repeats (Hoenderop et al. 1999).

The first fish (trout) ECaC was cloned and published in NCBI (Accession# AY256348) in 2003 by Shahsavarani et al. and will be, in part, the subject of Chapter 2. Partial sequences for 4 NCXs (with several possible splice variants) and 2 PMCAs were also identified; however, the complexity of simultaneously analyzing these genes and obtaining full length sequences proved overly time consuming and unproductive. Consequently, ECaC was chosen as the marker gene for the Ca^{2+} uptake model. Chapter 2 will also focus on the distribution of cells expressing the ECaC gene. Chapter 3 will demonstrate how ECaC expression and distribution change with environmental and physiological challenges.

Although other genes are also thought to play significant roles in Ca^{2+} uptake, ECaC is thought to be the rate limiting step and thus representative for the model. This thesis will not address the other two primary genes thought to be involved [ATP driven plasma membrane calcium ATPase (PMCA) and sodium calcium exchanger (NCX)].

However, the following section provides a general overview in order to present the entire model in context.

Trancellular transport

The transcellular transport of calcium has not been investigated in depth. The transport is believed to be facilitated through Ca-binding proteins (Ca-BP) such as calbindins (Feher et al. 1992). Vitamin D_3 metabolites stimulate expression of such proteins in both the mammalian kidney and intestine, and ^{45}Ca-binding studies suggest the presence of Ca-BP in fish gills, kidney and intestine. However the presence of calbindins in these tissues has yet to be demonstrated (van der Heijden 1999).

Ca^{2+} extrusion

At the basolateral surfaces, NCX and PMCA generate an outwardly directed Ca^{2+} flux which reduces the intracellular Ca^{2+} level to create an inwardly directed gradient for Ca^{2+} diffusion across the apical membrane (Perry 1997). Although physiological evidence strongly supports the presence of these mechanisms, their exact function, location and their interactions are yet to be elucidated. The NCX was first discovered in invertebrate nerves and since then it has been shown to be widespread (but not universally present) in the plasma membranes of excitable cells and epithelia (Baker and Allen 1986) and in numerous organisms such as mammals (Dyck et al. 1999; Fang et al. 1999; Kofuji et al. 1993; Kofuji et al. 1992; Van Eylen et al. 1997), lower vertebrates (van der Heijden 1999; Xue et al. 1999) and invertebrates (Bauer et al. 1999; Omelchenko et al. 1998).

Sodium calcium exchanger(NCX)

The NCX works in both forward and reverse modes and is dependent on the ionic conditions near the plasma membrane. NCX uses the energy stored in the transmembrane Na^+ gradient generated by the sodium pump to extrude Ca^{2+} from the cell (forward mode) against the electochemical gradient (intracellular $[Ca^{2+}] = 0.1$ μmol l^{-1} *versus* extracellular fluid $[Ca^{2+}] = 1.5$ mol l^{-1}). The exchange is electrogenic (1 Ca^{2+} : 3 Na^+) and can be reversed as a result of its sensitivity to plasma membrane potential (Reeves 1985). The affinity of the NCX for intracellular Ca^{2+} is much lower than the basal intracellular Ca^{2+} concentration (2 μmol.l^{-1} *versus* 0.1 μmol.l^{-1}) suggesting that this enzyme is not fully activated at basal Ca^{2+} levels (Schoenmakers et al. 1993). At the molecular level several NCXs have now been identified. The first vertebrate NCX to be cloned was the canine NCX (Nicoll et al. 1990) while Xue et al. (1999) were the first to clone and express a teleost NCX, derived from rainbow trout cardiac tissue (NCX-TR1). I have been able to identify several RNA fragments of 4 NCXs in trout with several potential splice variants for each (data not shown). Recently, Marshall et al. (2005) published a report that further supports the existence of 4 NCX families in fish species.

Ca^{2+}- ATPase (PMCA)

PMCA is probably a universal attribute of all cells (Schatzmann 1986). This pump is considered to have high affinity for Ca^{2+} (<1μmol l^{-1}) with a 1:1 Ca^{2+} to ATP stoichiometry (Carafoli et al. 1985). The Ca^{2+}-ATPase belongs to the family of P-type ATPases that include the Na^+ pump (Na^+/K^+-ATPase). The Ca^{2+} pump exhibits electrogenicity because 1 H^+ is exchanged for 1 Ca^{2+} (Guerini and Carafoli 1999).

Methodology

With the advent of "user-friendly" techniques such as polymerase chain reaction (PCR) and automated sequencing, molecular biology has become an important tool in physiological studies. The use of specific molecular probes can allow for the detection of various enzymes with such low expression or activity levels that would otherwise not be possible using conventional biochemical or physiological methods. Molecular biology will therefore be a major tool in the identification and characterization of the Ca^{2+} uptake model in this thesis.

The aim of this thesis was to contribute further to the existing body of evidence by providing:

1) Direct evidence for the existence of ECaC in the gill of rainbow trout
2) A phylogenetic analysis of rainbow trout ECaC (rtECaC) in relation to higher vertebrates
3) Provide evidence for the cellular distribution of ECaC particularly with relation to MRCs

Furthermore experiments were designed to answer the following question: Do environmental and physiological factors affect rtECaC gene expression as predicted through previous physiological experiments that suggest a regulatory role for ECaC in Ca^{2+} uptake at the gill?

Several attempts were made at functional characterization of ECaC (microinjections into zebrafish embryos, bacterial expression). In all cases the inclusion of the gene proved to be lethal to the organisms (results not shown).

Extensive data mining techniques were required for the identification of the trout ECaC. Chapter 2 presents the results of the procedures and experiments conducted to identify the target gene as well as the bioinformatics analysis used for the final sequence determination as well as the phylogenetic analysis of the gene with respect to other members of the gene family. The information obtained from these experiments was essential in the development of various molecular probes used for determining the distribution and cellular localization of ECaC in trout gills.

Following the identification of the ECaC gene and assessing its cellular distribution, experiments were conducted to assess gene expression responses to environmental (hypercapnia or exposure to altered levels of ambient Ca^{2+}) and physiological challenges (hypercalcemia or cortisol treatments). The results of these experiments are presented in Chapter 3.

The results of the studies presented here confirm that the ECaC is present in the gills as predicted by previous studies. However, the results also strongly suggest that unlike in the putative model for Ca^{2+} uptake, ECaC expression is not limited to MRCs and that perhaps a more diverse group of cells are involved in Ca^{2+} uptake.

CHAPTER 2

Characterization of a branchial epithelial calcium channel (ECaC) in freshwater rainbow trout (*Oncorhynchus mykiss*).

Shahsavarani A, McNeill B, Galvez F, Wood CM, Goss GG, Hwang PP, Perry SF., 2006. J Exp Biol. 209:1928-43

Statement of Contributions:

The work presented in this Chapter is the result of collaborative work between Arash Shahsavarani and the other authors. For clarification, original tissue preservation, *in situ* hybridization and immunocytochemistry were done by A. Shahsavarani. For the purpose of publication some additional imaging was done by B. McNeill and S.F. Perry. Cell isolations were originally performed by A. Shahsavarani and were repeated for verification by F. Galvez and C.M. Wood. Cell culture was done by F. Galvez and C.M. Wood while Western blots were completed by B. McNeill and S.F. Perry and separately by P.P. Hwang. Flow cytometry was conducted by B. McNeill and S.F. Perry. All remaining work was completed by A. Shahsavarani.

Abstract

The entry of calcium (Ca^{2+}) through an apical membrane epithelial calcium channel (ECaC) is thought to be a key step in piscine branchial Ca^{2+} uptake. In mammals, ECaC is a member of the transient receptor potential (TRP) gene family of which two members have been identified, TRPV5 and TPRV6. In the present study we have identified a single rainbow trout (*Oncorhynchus mykiss*) ECaC (rtECaC) that is similar to the mammalian TRPV5 and TRPV6 genes. Phylogenetic analysis of the protein sequence suggests that an ancestral form of the mammalian genes diverged from those in the lower vertebrates prior to the gene duplication event that gave rise to TRPV5 and TRPV6.

The putative model for Ca^{2+} uptake in fish proposes that the mitochondria rich cell (also termed ionocyte or chloride cell) is the predominant or exclusive site of trans-cellular Ca^{2+} movements owing to preferential localization of ECaC to the apical membrane of these cells. However, the results of real time PCR performed on enriched gill cell populations as well as immunocytochemistry and *in-situ* hybridization analysis of enriched cells, cell cultures and whole gill sections strongly suggest that ECaC is not exclusive to mitochondria rich cells but that it is also found in pavement cells. Not only was ECaC protein localized to areas of the gill normally expressing few mitochondria rich cells, but there was also no consistent co-localization of ECaC- and Na^+/K^+-ATPase-positive (a marker of mitochondria rich cells) cells. Taken together, the results of the present study suggest that although ECaC (mRNA and protein) does exist in trout gill, its cellular distribution is more extensive than previously thought thus suggesting that Ca^{2+}

uptake may not be restricted to mitochondria rich cells as was proposed in previous models.

Introduction

Unlike in mammals where Ca^{2+} is acquired principally through diet, fish obtain a significant component of their daily Ca^{2+} requirements directly from the aquatic environment. Ca^{2+} absorption from the water occurs predominantly at the gill (Flik et al. 1995; Flik and Verbost 1993; Perry 1997; Perry and Flik 1988), although the skin (Perry and Wood 1985) and intestine (Flik and Verbost 1993) may also be involved. Transepithelial Ca^{2+} uptake at the gill is a multi-step process that is believed to involve the passive entry of Ca^{2+} into epithelial cells through apical membrane non voltage-gated Ca^{2+} channels (Perry and Flik 1988) followed by its extrusion across the basolateral membrane by Na^{+}/Ca^{2+} exchange (Verbost et al. 1994) or Ca^{2+}-ATPase (PMCA) driven active transport (Flik et al. 1983; Flik et al. 1985). By analogy to the mammalian kidney (Hoenderop et al. 2002), it is possible that the rate-limiting step in transepithelial Ca^{2+} uptake is its entry through the apical membrane Ca^{2+} channel (Marshall 2002; Perry et al. 2003).

The discovery of a non-voltage gated epithelial Ca^{2+} channel (ECaC) in the rabbit kidney (Hoenderop et al. 1999) has led to the identification of a family of proteins (TRPV5 and TRPV6) that appears to be primarily involved in Ca^{2+} homeostasis (den Dekker et al. 2003; Hoenderop et al. 2003; Nijenhuis et al. 2005; Nilius et al. 2002). These proteins belong to the vanilloid subfamily of the transient receptor potential (TRP) superfamily (den Dekker et al. 2003; Vennekens et al. 2002). Previously, ECaC1 (epithelial calcium channel 1), CaT2 (calcium transport protein 2) and TRPV6 have all been used to refer to the same gene whereas ECaC2, CaT1 and TRPV5 have been used interchangeably for the second gene (den Dekker et al. 2003; Montell et al. 2002).

Because of the initial confusion surrounding the naming of these Ca^{2+} channel genes, there is now a growing momentum to adopt standard nomenclature (TRPV5 and TRPV6) (Montell et al. 2002).

In comparison to the vast body of literature on epithelial Ca^{2+} channels in mammals (Hoenderop et al. 2002), there is comparatively little known about the nature of the branchial Ca^{2+} channel in fish although it has been cloned from three teleost species; *Fugu rubripes* (Qiu and Hogstrand 2004), *Danio rerio* (Pan et al., 2005; NCBI GenBank Accession AY325807) and *Oncorhynchus mykiss* (A. Shahsavarani, B. McNeill, M. Bayaa and S.F. Perry, NCBI GenBank Accession AY256348). On the basis of extensive but largely correlative data (Ishihara and Mugiya 1987; MacKenzie and Perry 1997a; Marshall et al. 1992; McCormick et al. 1992; Perry et al. 1992; Perry and Flik 1988; Perry and Wood 1985), a model has been developed in which the branchial mitochondria rich cell (MRC; also termed ionocyte or chloride cell) is the principal site of transepithelial Ca^{2+} uptake in freshwater fish (Evans et al. 2005; Marshall 2002; Perry 1997).

With this background, the primary objectives of the present study were to provide direct evidence for the presence of ECaC in the gill of rainbow trout, to determine its phylogenetic relationship with ECaC genes of higher vertebrates and to test the hypothesis that the supposed preferential uptake of Ca^{2+} by branchial MRCs reflects the presence of ECaC that is mainly localized in these cells.

Materials and Methods

Animal care

Rainbow trout of both sexes were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). The fish were held at the University of Ottawa in large fibreglass tanks supplied with flowing, aerated, and dechloraminated city water and maintained at 13° C, on a 12 h:12 h light:dark photoperiod and were fed daily with a commercial trout diet. Adult rainbow trout used to obtain gill cell cultures were purchased from Humber Springs Trout Farm (Orangeville, ON, Canada). These fish were held at McMaster University at 12° C on a natural photoperiod and fed every second day with commercial trout feed.

Database searches and molecular cloning of ECaC

The identification of a potential Atlantic salmon (*Salmo salar*) ECaC sequence was achieved through a comparison of the predicted protein sequence of rabbit (*Oryctolagus cuniculus*) ECaC (GenBank Accession AJ133128) to the Atlantic salmon EST database library (http://snoopy.ceh.uvic.ca) using the tblastx program (Altschul et al. 1997).

Tissue samples were collected and stored at -80° C prior to RNA extraction. Frozen samples were homogenized under liquid nitrogen using a mortar and pestle. Total RNA was extracted using TRIZOL Reagent (Invitrogen Canada, Burlington, ON). All procedures were performed according to the manufacturer's instructions with the following modifications. No more than 30 mg of tissue was used per 1 ml of TRIZOL reagent. Following the re-suspension of the total RNA in 100 µl of nuclease-free water,

the RNA was re-extracted using 1 ml of TRIZOL by repeating the entire procedure. Finally, the RNA was re-suspended in 30 µl of nuclease-free water.

Reverse transcription was performed using Stratascript Reverse Transcriptase Kit (Stratagene, La Jolla, CA). Complementary DNA (cDNA) was synthesised as per the manufacturer's instructions with the following changes. Final reaction volume was reduced to 12.5 µl, 0.5 µg of total RNA was used with 0.25 µg of oligo-dT primers. For 3' and 5' rapid amplification of cDNA ends (RACE), oligo-dT primers were replaced by 3'AUAP (as provided by kit manufacturer; see below) or by ECaC –5RACE-RT (5'-CCACCAGGAACGCATAGGCAATAA-3') respectively (see Table 2.1).

PCR-generated amplicons were visualised using ethidium bromide staining. PCR conditions were adjusted according to the annealing temperature of each primer pair and as required in accordance with the estimated amplicon size. Various primers were designed (Table 2.1) for the initial cloning of a partial ECaC sequence as well as for 3' and 5' RACE. 3' and 5' RACE were performed using a commercial kit (GeneRacer; Invitrogen Canada, Burlington, ON) according to the manufacturer's instructions. Amplicons of interest (ECaC fragment candidates) were cloned using a TOPO-TA cloning kit using One Shot TOP 10 chemically competent cells (Invitrogen Canada, Burlington, Ontario). Clones were subsequently sequenced using a variety of commercial sequencing facilities.

ECaC sequence analysis

Full-length amino acid and nucleotide sequences for ECaC1, ECaC2, CaT1, CaT2, TRPV5 and TRPV6 were retrieved from the NCBI GenBank database (Table 2.2).

Table 2.1. Primers used for initial amplification of rainbow trout ECaC fragment, real time PCR, 3' and 5' RACE and cloning of the full-length coding region.

Name	Direction	Sequence 5'→ 3'
PCR of initial fragment		
260902-ECaC1F	FWD	GCCCTGGTTATTCTGCTACTG
260902-ECaC2R	REV	CAAACTGGGAGAACAGAGTGAT
Real time PCR		
ECaC-QPCR1	FWD	GGACCCTTCCATGTCATTCTTATT
ECaC-QPCR2	REV	ACAGCCATGACAACTGTTTCC
ß-actin	FWD	CCAACAGATGTGGATCAGCAA
ß-actin	REV	GGTGGCACAGAGCTGAAG GGTA
5'RACE		
ECaC-5RACE-RT	REV	CCACCAGGAACGCATAGGCAATAA
ECaC-5GSP1	REV	CAGGGCCGTTTGTCCAAAGTAATGCT
ECaC-5GSP2	REV	ATCTGGGACCTCCAGTAGCAGAATA
3'RACE		
ECaC-3GSP1	FWD	CCTCCCTGTGGGTGGTGTATATGA
ECaC-3GSP2	FWD	CCCCATCACTCTGTTCTCCCAGTT
Full length coding		
ECaC-full1	FWD	GCTTGACGTTGCATGTTCCCTTAGT
ECaC-full2	REV	CTACATTTCAACCCAGTCTTGCCCAT

Table 2.2. Genes used for sequence alignment and phylogenetic analysis of various epithelial calcium channels.

Gene names are based on those published for each accession number in GenBank.

Gene	Species	Accession Number
CaT1	*Xenopus laevis*	BAC24123.1
ECaC	*Danio rerio*	AAR30870.1
ECaC	*Oncorhynchus mykiss*	AAP12529.1
ECaC	*Oryctolagus caniculus*	CAB40138.1
ECaC	*Procambarus clarkii*	AAR19087.1
ECaC	*Takifugu rubripes*	AAP46137.1
TRPV4	*Gallus gallus*	NP_990023.1
TRPV5	*Canis familiaris*	XP_539860.1
TRPV5	*Homo sapiens*	EAL23777.1
TRPV5	*Mus musculus*	NP_001007573.1
TRPV5	*Rattus norvegicus*	Q9JIP0
TRPV6	*Danio rerio*	NP_001001849.1
TRPV6	*Gallus gallus*	XP_416530.1
TRPV6	*Homo sapiens*	CAC20416.2
TRPV6	*Mus musculus*	NP_071858.2
TRPV6	*Oryctolagus caniculus*	AAY34564.1
TRPV6	*Rattus norvegicus*	NP_446138.1

Representative amino acid sequences for TRPC, TRPM and other TRPV subfamilies were also retrieved for analysis (Table 2.3). *Gallus gallus* TRPV4 was used as an out-group for construction of phylogenetic trees. Alignment of sequences and neighbour joining (NJ) analysis were performed using ClustalW (v.1.8; Thompson et al. 1994). Maximum likelihood analysis was performed using PHYML (v.2.4.4; Guindon et al. 2005). Support for nodes in NJ analysis was performed on 100 pseudo data sets while PHYML analysis was performed on 1000 pseudo datasets using bootstrap analysis. Repeated analysis with and without gaps in the alignment did not affect the overall topology of the final phylogenetic trees. Ultimately, gaps were considered as missing characters (J. Felsenstein, PHYLIP documentation; http://evolution.genetics.washington.edu/phylip.html). Potential phosphorylation sites were identified using NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/; Blom et al. 1999).

Real time PCR analyses

Real time PCR was performed using a MX 4000 Multiplex Quantitative PCR System (Stratagene) with a Stratagene Brilliant SYBR Green QPCR Master Mix as per the manufacturers' instructions with the following modifications. The total reaction volume was reduced to 25 µl containing 0.5 µl cDNA and 150 nmol l^{-1} of each primer. All primers (Table 2.1) were designed and optimized for the following PCR reaction conditions; 15 min at 95° C, 45 cycles of 30 sec at 95° C, 30 sec at 60° C, 30 sec at 72° C. At the end of each run, a dissociation curve was established to determine the purity of the amplicon in each reaction. Those samples exhibiting more than 1 dissociation peak

Table 2.3. Genes used for sequence alignment and phylogenetic analysis of various members of the TRP gene family.

Gene names are based on those published for each accession number in GenBank.

Gene	Species	Accession Number	Gene	Species	Accession Number
TRPC1	*Gallus gallus*	NP_001004409.1	TRPM7	*Danio rerio*	NP_001025232.1
TRPC1	*Homo sapiens*	NP_003295.1	TRPM7	*Homo sapiens*	NP_060142.2
TRPC1	*Mus musculus*	AAB50622.1	TRPM7	*Mus musculus*	NP_067425.1
TRPC1	*Xenopus laevis*	AAD22978.1	TRPM8	*Gallus gallus*	NP_001007083.1
TRPC2	*Danio rerio*	NP_001025337.1	TRPM8	*Homo sapiens*	NP_076985.3
TRPC2	*Mus musculus*	NP_035774.1	TRPM8	*Mus musculus*	NP_599013.1
TRPC3	*Homo sapiens*	AAH93684.1	TRPV1	*Gallus gallus*	NP_989903.1
TRPC3	*Mus musculus*	NP_062383.1	TRPV1	*Homo sapiens*	NP_542437.1
TRPC4	*Homo sapiens*	CAI15562.1	TRPV1	*Mus musculus*	NP_001001445.1
TRPC4	*Mus musculus*	NP_058680.1	TRPV2	*Homo sapiens*	NP_057197.2
TRPC5	*Homo sapiens*	NP_036603.1	TRPV2	*Mus musculus*	NP_035836.1
TRPC5	*Mus musculus*	NP_033454.1	TRPV3	*Homo sapiens*	NP_659505.1
TRPC6	*Homo sapiens*	NP_004612.2	TRPV3	*Mus musculus*	NP_659567.1
TRPC6	*Mus musculus*	NP_038866.1	TRPV4	*Gallus gallus*	NP_990023.1
TRPC7	*Homo sapiens*	O94759	TRPV4	*Homo sapiens*	Q9HBA0
TRPC7	*Mus musculus*	NP_036165.1	TRPV4	*Mus musculus*	NP_071300.1
TRPM1	*Homo sapiens*	NP_002411.2	TRPV5	*Homo sapiens*	NP_062815.2
TRPM1	*Mus musculus*	AAH85168.1	TRPV5	*Mus musculus*	NP_001007573.1
TRPM2	*Homo sapiens*	AAY22174.1	TRPV6	*Danio rerio*	NP_001001849.1
TRPM2	*Mus musculus*	NP_612174.1	TRPV6	*Gallus gallus*	XP_416530.1
TRPM3	*Homo sapiens*	CAI96100.1	TRPV6	*Homo sapiens*	NP_061116.2
TRPM3	*Mus musculus*	NP_796315.2	TRPV6	*Mus musculus*	NP_071858.2
TRPM4	*Homo sapiens*	NP_060106.2	CaT1	*Xenopus laevis*	BAC24123.1
TRPM4	*Mus musculus*	NP_780339.1	ECaC	*Danio rerio*	AAR30870.1
TRPM5	*Homo sapiens*	NP_055370.1	ECaC	*Oncorhynchus mykiss*	AAP12529.1
TRPM5	*Mus musculus*	NP_064673.1	ECaC	*Procambarus clarkii*	AAR19087.1
TRPM6	*Homo sapiens*	NP_060132.3	ECaC	*Takifugu rubripes*	AAP46137.1
TRPM6	*Mus musculus*	NP_700466.1			

were eliminated. Control samples (diluted RNA samples) were assessed at random to test for the presence of genomic DNA contamination. ß-actin was used as an internal standard since analysis of each experiment demonstrated no significant difference between experimental samples and control samples on the average ß-actin Ct values (data not shown).

Tissue preservation, in situ hybridization and immunocytochemistry

Gill filaments were removed from freshly dissected gill arches and were placed in 4% paraformaldehyde (4% PFA, pH 7.4) at 4° C overnight. The filaments were transferred to phosphate buffered saline (PBS) containing 15% sucrose for 2 h at 4° C and finally transferred to PBS containing 30% sucrose for at least 2 h prior to sectioning. Tissue samples were embedded in ThermoShandon media (VWR International) and were sectioned (10 µm sections) at -18° C using a Leica CM 1850 cryostat. Sections were placed on SuperFrost^{++} (Fisher Scientific, Canada) electrostatic microscope slides, air dried for 10 min and stored at -20° C until use.

For *in-situ* studies, specific digoxigenin labelled oligonucleotide probes were designed (Table 2.4) and synthesised (Genedetect.com Limited, New Zealand). Sections on slides were hydrated (2 X 15 min) in 1X PBST (PBS with 0.1% Tween 20). Proteinase K (20 µg ml^{-1} in 1X PBST, Gibco BRL, Orand Island, NY) was used to de-proteinate samples for 20 min at room temperature. Following de-proteination, samples were fixed in 4% formaldehyde (in PBS) for 5 min. Fixed tissues were subsequently rinsed twice (10 min per wash) with 1X PBST and air-dried at 60° C for 15 min.

Probes (approximately 900 pg per reaction) were denatured for 3 min at 94° C in a solution containing 250 μg ml^{-1} of salmon sperm DNA, 250 μg of Poly A, topped up to 12.5 μl with DEPC (diethyl pyrocarbonate) H_2O. Probes were then quickly chilled on ice and centrifuged (7,500 g) for 1 min. Hybridization buffer (100 μl of 4X SSC, 20%

Table 2.4. Sequences of oligonucleotide probes used for *in situ* hybridization.

Gene	**Probe sequence**
ECaC	5′ - ACAGCCATGACAACTGTTTCCCCCTGCACCCCACTGACCCTGAACACA - 3′
Na^+/K^+-ATPase	5′ - TGGACCCCAGGGTCTCAACAGCTTCCGATTCTTCACCAGGCAGTTCT - 3′

dextran sulfate, 50% formamide, 250 μg ml^{-1} poly A, 250 μg ml^{-1} ssDNA, 0.1 mol l^{-1} DTT, 250 μg ml^{-1} tRNA, 0.5X Denhardts) was added to each probe. Each probe was then mixed well by vortex and placed onto the sections. Hybridization was performed overnight at 37° C in a humid chamber. Oligonucleotide probes were added to tissue sections at a concentration of 400 ng ml^{-1}.

Following overnight hybridization, sections were washed twice (15 min per wash, 58° C) with 2X SSC and twice (15 min per wash, 58° C) with 0.2X SSC, followed by one wash in 0.1X SSC for 10 min at room temperature and twice in 0.1X PBS (10 min per wash, room temperature). To detect hybridization, sections were incubated for 1 h at room temperature with 1% goat serum, 2g/mL BSA in 0.1M PBS with 0.3% triton-X, followed by overnight incubation at 4° C in anti-digoxigenin conjugated to alkaline phosphatase (1:1000 dilution; Roche Molecular Biochemicals). Slides were washed at room temperature in 0.1 mol l^{-1} PB for 15 min and then briefly rinsed in water. The slides were next washed twice (5 min per wash) 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 a single 5-bromocresyl-3-indolyl phosphate (BCIP) tablet (Sigma) were dissolved in 10 ml of H_20 and layered over the sections. Colour was allowed to develop in a humid chamber at room temperature for at least 4 h or until satisfactory coloration was observed. The slides were then washed twice with 0.1 mol l^{-1} PBS (15 min per wash). Cover-slips were mounted on the slides were using 60% glycerol as mounting medium.

Custom polyclonal antibodies (Abgent, San Diego) were raised in rabbits against an 18 amino acid region (SQFRFRLQNRKGWKEMLD) of the rainbow trout ECaC

protein. This region corresponded to amino acids 18 through 36 of rtECaC. Na^+/K^+-ATPase was detected using a mouse monoclonal antibody (α5; developed by Douglas M. Fambrough and obtained from the Developmental Studies Hybridoma Bank at The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). Tissue samples were prepared and sectioned as described above. Sections were hydrated three times (5 min per wash) with 0.1X PBS containing 0.1% Tween 20 (0.1X PBST). Sections were then incubated at room temperature with primary antibody for 2 h (ECaC 1:200 dilution, Na^+/K^+-ATPase 1:100 dilution with 0.1X PBST). Each section was then washed three times (5 min per wash) with 0.1 X PBST. Following the third wash, sections were incubated at room temperature (1 h) with appropriate secondary antibodies (either alexa 488 goat anti-rabbit or alexa 546 goat anti-mouse, both diluted 1:400 with 0.1 X PBST, Molecular Probes, Invitrogen Canada). Finally, sections were washed three times (5 min per wash) with 0.1X PBST and covered with cover-slips and mounting medium containing the nuclear marker DAPI (4'-6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA). Negative control sections were incubated with blocking buffer lacking primary antibodies or with antibodies pre-absorbed with excess peptide antigen (ECaC).

Once prepared, all specimens were observed and photographed using a Zeiss Axiophot (Zeiss, Jena, Germany) equipped with a Hamamatsu C5985 chilled CCD camera using Metamorph imaging software 4.01. In some instances, SSIs and DSIs were examined using a confocal microscope (Olympus Fluoview BX50W1).

Western blots

Gill filaments were homogenized in homogenization buffer (100 mmol l^{-1} imidazole, 5 mmol l^{-1} EDTA, 200 mmol l^{-1} sucrose, and 0.1% sodium deoxycholate; pH 7.6), and then centrifuged at 4° C and 10,000 rpm for 10 min. The supernatant (containing 50 μg total protein) was supplemented with 6X electrophoresis sample buffer (250 mmol l^{-1} Tris-base, 2 mmol l^{-1} Na_2EDTA, 2% SDS, and 5% dithiothreitol), and then incubated at 95° C for 10 min. The denatured samples were subjected to 6-8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 2 h. After being transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), the blots were incubated in 5% nonfat milk for 2 h and then washed 2X with PBST buffer (0.01 mol l^{-1}phosphate, 0.09% NaCl (pH 7.5), and 0.05% Tween 20). Blots were incubated over night at 4° C with the primary ECaC antibody at a 1: 6000 dilution. After washing 2 times with PBST buffer, the blots were incubated for another 2 h with alkaline-phosphatase (AP)-conjugated anti-rabbit IgG (Pierce, Rockford, IL, USA, diluted 1:1000) at room temperature. After 2 washings with PBST buffer, immunoreactive proteins were visualized with NBT and BCIP in staining buffer.

Gill cell isolation

To obtain mixed gill cell populations, fish were euthanised by a sharp blow to the head and the entire gill basket was quickly removed and placed in cold PBS. Filaments were removed from gill arches and placed in a 50-ml Falcon tube containing 10 ml of cold trypsin-EDTA dissolved in PBS (0.25% trypsin, 1 mM EDTA; Canadian Life Technologies). Filaments were cut into small pieces and the tube was placed on an orbital shaker (300 rpm) for 8 min. The dissociated cells were mixed and filtered through

a 100 μm cell culture filter directly into a cold stop solution (10% foetal bovine serum in PBS). The cells were centrifuged (300 g for 8 min) and re-suspended in distilled water for 30 sec to lyse red blood cells. PBS was added to each tube in a 3:1 ratio to the volume of distilled water. The cells were once again re-suspended and washed 3 times with PBS. Cells utilised for immuno-staining were fixed and treated as described for tissue sections except that isolated cells were maintained in suspension and were washed through centrifugation (300g for 8 min) and re-suspension. Enriched populations of cells [pavement cells (PVCs), peanut lectin agglutinin positive mitochondria rich cells (PNA^+ MRCs) and PNA^- MRCs) were isolated at University of Alberta according to previously described protocols (Galvez et al. 2002; Goss et al. 2001).

RNA was extracted using 1 ml of Trizol Reagent as previously described.

Flow cytometry

Cells were prepared as described above and after final centrifugation, the cell pellet was re-suspended to yield a final concentration of at least 1.5 million cells ml^{-1} and fixed in 4% PFA at 4° C for 4 h. After fixation, cells were filtered through 70 μm mesh and washed (2 X 5 min) with 0.1X PBS. Fixed gill cells were labelled using primary (alone or in combination) and secondary antibodies as described above with the following exception. Alexa 633 goat anti-mouse (1:400 dilution) was used instead of Alexa 546 to detect Na^+/K^+-ATase. Cells were re-suspended in 0.5 ml of 0.1X PBS and analyzed by flow cytometry (Beckman Coulter FC 500 flow cytometry series with CXP software). Forward scatter and side scatter were used to measure relative cell size and granularity respectively. To measure Alexa 488 fluorescence the FL1 channel was used which

detects a wavelength of 525 ± 25 nm. To measure Alexa 633, the FL4 channel was used which detects a wavelength of 675 ± 25 nm. Samples were ran at a medium flow rate (30 $\mu l\ min^{-1}$) until the desired 10000 events had been captured within the selected gate. Analysis of calibration beads prior to each run allowed the estimation of cell size. To reduce the possibility of recording data from cellular debris or clumps of cells, the capture gate was set to exclude events < 5 μm and > 25 μm.

Cell culture

Gill cultures were derived from adult rainbow trout held in Hamilton at McMaster University. Gill cell isolations were performed using sterile techniques as outlined by Part et al. (1993). Gill epithelia were subsequently grown on semi-permeable membrane inserts using methods for single-seeded inserts (SSI) comprised of PVCs only (Wood and Part 1997) or that for double-seeded insert (DSI) preparations composed both of PVCs and MRCs (Fletcher et al. 2000). In brief, gill cells for the SSI cultures were first grown in flasks for approximately 6 days allowing for an enrichment of PVCs alone. This was followed by a passaging and reseeding onto filter inserts. DSI cultures were developed by the sequential seeding of gill cells from two separate fish over two days onto the same type of filter insert. SSI and DSI cultures were grown into functional epithelia at 18 °C for at least 6 days before preparation for fixation. Single and double seeded filter inserts were washed 3-times in PBS and fixed in 4% PFA for 2 h (see above). Immuno-staining of inserts was performed using the same protocol as that described for tissue sections.

Statistical analysis

Statistical analysis was completed using Sigma Stat (v2.03, SPSS Inc, Chicago). One way Analysis of Variance (One Way ANOVA) was used when comparing relative ECaC mRNA expression in the enriched cell. In flow cytometry experiments, significant differences between the PVC and PNA^- cell fractions were determined by rank sum test. In all cases, significance was set at $P < 0.05$.

Results

The final trout ECaC sequence was obtained through multiple cloning and sequencing steps. Based on sequences derived from 3' and 5' RACE, primers were designed to amplify the entire coding region (Figure 2.1, GenBank Accession #: AY256348) and to obtain full-length clones. The complete coding region is composed of 2184 nucleotides corresponding to a protein of 727 amino acids with a predicted molecular mass of 82.3 kDa. Analysis of the sequence identified a large domain representative of ankyrin sites as well as the identification of an ion transport domain and pore-forming region (Figure 2.2). Twenty nine potential phosphorylation sites were identified; 16 serine (S) sites, 8 threonine (T) sites and 5 tyrosine (Y) sites.

The results of phylogenetic analysis of rtECaC are depicted in Figure 2.3. These findings suggest that the mammalian TRPV5 and TRPV6 form distinct groups. Furthermore, it appears that the non-mammalian genes do not belong to either of these groups (Figure 2.3). There is evidence that invertebrate, fish and amphibian and avian genes each form their own individual and distinct branches both at the amino acid level (Figure 2.3) as well as at the nucleotide level (data not shown). Based on these analyses, TRPV5 and TRPV6 appear to have diverged more recently from one another than from the calcium transport channels found in invertebrates, fish, amphibians and birds. A comparison of 53 different genes belonging to the TRP family (Figure 2.4) through bootstrapped (100 pseudo data sets) NJ analysis showed that rtECaC is most closely related to the TRPV subfamily but does not group with either TPV5 or TRPV6.

Figure 2.1. Nucleotide and predicted amino acid sequence of the rainbow trout (*Oncorhynchus mykiss*) gill epithelial calcium channel (ECaC).

The sequence shown represents the predicted coding region including the start (underlined) and stop (asterisk) codons. The sequence was obtained by RACE and verified by full length PCR and cloning.

Figure 2.1

```
O.mykiss ECaC      ATGGCCCCGGCCTTGGCAAGATCTGCTCCAGGTGAGCTCAACCATTGGTGGAGCCAGTTTAGGTTCCGCCTCCAGAACAGG
                     M  A  P  A  L  A  R  S  A  P  G  E  L  N  H  W  W  S  Q  F  R  F  R  L  Q  N  R

AAGGGGTGGAAAGAAATGCTGGATGAAACTTTTTTGCTGCAGAACAAAAGGACGAATGGCGTCCTCTCTTTTTTGCCGCCAAAGAGAGCAGTGCAGGTT
 K  G  W  K  E  M  L  D  E  T  F  L  L  Q  N  K  R  T  N  G  V  P  L  F  F  A  A  K  E  S  S  A  G

GCATTAAGAAACTTCTGGACTGTGCATCCACTAACATCTTTGAAAGAAGGGCTCTGGGGGAGACCGCGCTGCATGTGGCAGTTATGAATGATAACATGGA
C  I  K  K  L  L  D  C  A  S  T  N  I  F  E  R  R  A  L  G  E  T  A  L  H  V  A  V  M  N  D  N  M  E

AGCTGCTTTAGCTCTGATGGACGGAGCACCTGAACTCATCAATGAGCCCATGACCTCTGAACTCTTCCTTGGCATGAAACCTCTCCACATTGCCGTGGTG
  A  A  L  A  L  M  D  G  A  P  E  L  I  N  E  P  M  T  S  E  L  F  L  G  M  K  P  L  H  I  A  V  V

AATCAGAACTTTAACCTAGTCCGCAGTCTGATTGGTAAAGGGGCGGATGTAGCCACGCCCAGACTCACAGGCCTGTACTTCAGGAAGAGAAGAGGAGGGC
 N  Q  N  F  N  L  V  R  S  L  I  G  K  G  A  D  V  A  T  P  R  V  T  G  L  Y  F  R  K  R  R  G  G

TGCTCTACTATGGTGAGCACATCCTGGCATTTGCGGCCTGTGTGGGGAATCAGGACATCATCTCATGGTGATCAACGTAGGAGCCAGCACCAGGGCCCA
L  L  Y  Y  G  E  H  I  L  A  F  A  A  C  V  G  N  Q  D  I  I  S  M  V  I  N  V  G  A  S  T  R  A  Q

GGACTCCATTGGTAACACAGTGCTCCACATCCTGGTCCTGCAGCCCAATAAGACTATAGCATGCTGGTGTTGGATCTGCTGTTGGCACGTGACATTGAG
  D  S  I  G  N  T  V  L  H  I  L  V  L  Q  P  N  K  T  I  A  C  L  V  L  D  L  L  L  A  R  D  I  E

TGGACCAGGCTGTGCCACTGGACATGGTGCCCAACTACCATGGCTTAACACCCTTCAAACTGGCTGCCAAGGAGGGCAACCTTGTGGCCTTCCAGCACC
 L  D  Q  A  V  P  L  D  M  V  P  N  Y  H  G  L  T  P  F  K  L  A  A  K  E  G  N  L  V  A  F  Q  H

TGGTCAATCGGAGGCGAATCAACCAGTGGAACCTGGGACCCTTGACCTCTAACCTCTATGACCTACAGGGATCGACTCCTTGGTTGCCGACGACGACTG
L  V  N  R  R  R  I  N  Q  W  N  L  G  P  L  T  S  N  L  Y  D  L  T  G  I  D  S  L  V  A  D  D  D  C

CTCTGTGCTTGAGCACATCGTGGGCAGCAAGAGGAGAGAGGCAAAGAGGATACTGGAAGTGACTCCTGTTAGGCAATTGGTCAGTTTCAAGTGGAACCTC
  S  V  L  E  H  I  V  G  S  K  R  R  E  A  K  R  I  L  E  V  T  P  V  R  Q  L  V  S  F  K  W  N  L

TATGGAAAACACTACTTTAGGTTGTTGCTGCTGCTGTACCTCCTGTACATTGGGACCTTCACACTGTGTTGTGTGTATCGCCCCCTAAAGGACGCTCCAG
 Y  G  K  H  Y  F  R  L  L  L  L  L  Y  L  L  Y  I  G  T  F  T  L  C  C  V  Y  R  P  L  K  D  A  P

AGAATTACACTGTATCTGACATGGACAAAACCATCCGCGTGCAGAAAACTCTGAAGGAGAGTTATGTGACCTATGGGGACAACTTGCGTCTGGCAGGAGA
E  N  Y  T  V  S  D  M  D  K  T  I  R  V  Q  K  T  L  K  E  S  Y  V  T  Y  G  D  N  L  R  L  A  G  E

GATGATCAGCGTCCTGGGTGCCCTGGTTATTCTGCTACTGGAGGTCCCAGATATGCTGAGAGTAGGGGCCAAGCATTACTTTGGACAAACGGCCCTGGGG
  M  I  S  V  L  G  A  L  V  I  L  L  L  E  V  P  D  M  L  R  V  G  A  K  H  Y  F  G  Q  T  A  L  G

GGGCCCTTCCATGTCATTCTTATTGCCTATGCGTTCCTGGTGGTGCTGCTGTGTGTGTTCAGGGTCAGTGGGGTGCAGGGGGAAACAGTTGTCATGGCTG
 G  P  F  H  V  I  L  I  A  Y  A  F  L  V  V  L  L  C  V  F  R  V  S  G  V  Q  G  E  T  V  V  M  A

TGTGTCTGGTGCTGGGCTGGAGCAATGTTATGTTCTTCGCCCGAGGCTTTCAGATGCTGGGGGCTTACGTCATCATGATACAGAAGATTATATTTGGAGA
V  C  L  V  L  G  W  S  N  V  M  F  F  A  R  G  F  Q  M  L  G  P  Y  V  I  M  I  Q  K  I  I  F  G  D

CCTGACCAAGTTCATGTGGCTGAGCTTCATCGTGCTCATAGGGTTTTCCACCTCCCTGTGGGTGGTGTATATGACTCAGGACCCAGACTCTCTACCTGCG
  L  T  K  F  M  W  L  S  F  I  V  L  I  G  F  S  T  S  L  W  V  V  Y  M  T  Q  D  P  D  S  L  P  A

TACCGCTCCTTCCCCATCACGCTGTTCTCCCAGTTTGAGCTGAGTGTGGGTCTGATAGACCTGCCAGTGGACCACACCATCACAACGCCCCCTATTGTCC
 Y  R  S  F  P  I  T  L  F  S  Q  F  E  L  S  V  G  L  I  D  L  P  V  D  H  T  I  T  T  P  P  I  V

ATGTGCTGCACTGCACCTTCTCTGTGGTCTCCTACATACTGCTGCTCAACCTGCTCACAGCCATGATGAGTGATACACAATGGAGAGTTGCCCAGGAGAG
H  V  L  H  C  T  F  S  V  V  S  Y  I  L  L  L  N  L  L  T  A  M  M  S  D  T  Q  W  R  V  A  Q  E  R

GGACGAGCTCTGGAGGACACAGGTGGTGGCCACTACCCTGATGTTGGAGAGAAGGTTGCCCCGCTGCCTGTGGCCCCGGCTGGGGGTGTGTGGACTGCTC
  D  E  L  W  R  T  Q  V  V  A  T  T  L  M  L  E  R  R  L  P  R  C  L  W  P  R  L  G  V  C  G  L  L

TACGGCCTGGGGGAGCGTTGGTACCTCCGGGTTGAGGATCGCAACGACCCACTGGTGCAAAAGATGCGTCGCTACGTGCAAGCCTTCTCTAAGGATGAGG
 Y  G  L  G  E  R  W  Y  L  R  V  E  D  R  N  D  P  L  V  Q  K  M  R  R  Y  V  Q  A  F  S  K  D  E

ACCAGAGCAAGGAGCGGGAGGAGATGGAGAACACTGACATGTCAAAAGGACCTGGAAGCCCTCTGATCAGAACCAAACACAGGGGTGGGATAGATGGAAA
D  Q  S  K  E  R  E  E  M  E  N  T  D  M  S  K  G  P  G  S  P  L  I  R  T  K  H  R  G  G  I  D  G  N

CAGGAAGTCCCTGGCATGCTGGCAGATGATTCGCCACAGCGCTCTGGGTTTAGATGTGGAACAGGAAGAGCCTGAGGATGACCAGGAAGTAAGATACGTC
  R  K  S  L  A  C  W  Q  M  I  R  H  S  A  L  G  L  D  V  E  Q  E  E  P  E  D  D  Q  E  V  R  Y  V

TGA
 *
```

Figure 2.2. Predicted rainbow trout (*Oncorhynchus mykiss*) ECaC protein functional domains.

Bold-underlined sequence represents possible ankyrin repeats. Outlined sequence areas represent ion transport regions. Outlined-bold sequence represents pore-forming region. Asterisks (*) represent predicted phosphorylation sites: Serine (S) = 16; threonine (T) = 8; tyrosine (Y) = 5.

Figure 2.2


1 MAPALARSAPGELNHWWSQFRFRLQNRKGWKEHLDETFLLQNKRTNGVPLFFAAKESSAG
61 IKKLLDCASTNIFERRALGETALHVAVHNDNMEAALALMDGAPELINEPMTSELFLGMK
121 PLHIAVVNQNFNLVRSLICKGADVATPRVTGLYFRKRRGCLLYYGEHILAFAACVGNQDI
181 ISMVINVGASTRAQDSIGNTVLHILVLQPNKTIACLVLDLLLARDIELDQAVPLDMVFNY
241 BGLTPFKLAAKEGNLVAFQHLVNRRRINQWNLGPLTSNLYDLTGIDSLVADDDCSVLEHI
301 VGSKRREAKRILEVTPVRQLVSFKWNLYGKHYFRLLLLLYLLYIGTFTLCCVYRPLKDAP
361 ENYTVSDMDKTIRVQKTLKESYVTYGDNLRLAGEMISVLGALVILLLEVPDMLRVGAKHY
421 FGQTALGGPFHVILIAYAFLVVLLCVFRVSGVQGETVVMAVCLVLGWSNVMFFARGFQML
481 GPYVIMIQKIIFGDLTKFMWLSFIVLIGFSTSLWVVYMTQDPDSIPAYRSFPITLFSQFE
541 ISVGLIDLPVDETITTPPIVHVLHCTFSVVSYILLLNLLTAMMSDTQWRVAQERDELWRT
601 QVVATTIMLERRLPRCLWPRLGVCGLLYGLGERWYLRVEDRNDPLVQKMRPYVQAFSKDE
661 DQSKEREEMENTDMSKGPGSPLIRTKHRGGIDGNRKSLACWQMIRHSALGLDVEQEEPED
721 DQEVRYV

Figure 2.3. Phylogenetic analysis of rainbow the trout (*Oncorhynchus mykiss*) ECaC. The phylogenetic tree was constructed using PHYML (maximum likelihood) with support for nodes determined through bootstrapping of 1000 pseudo-datasets. Branches are drawn to scale as the scale represents replacement of 5% of the amino acids in the protein alignment. See Table 2.2 for the description of genes used in this tree, c = chicken, cf = crayfish, d = dog, f = *Fugu*, h = human, m = mouse, r = rat, rb = rabbit, rt = rainbow trout, x = *Xenopus*, z = zebrafish.

Figure 2.3

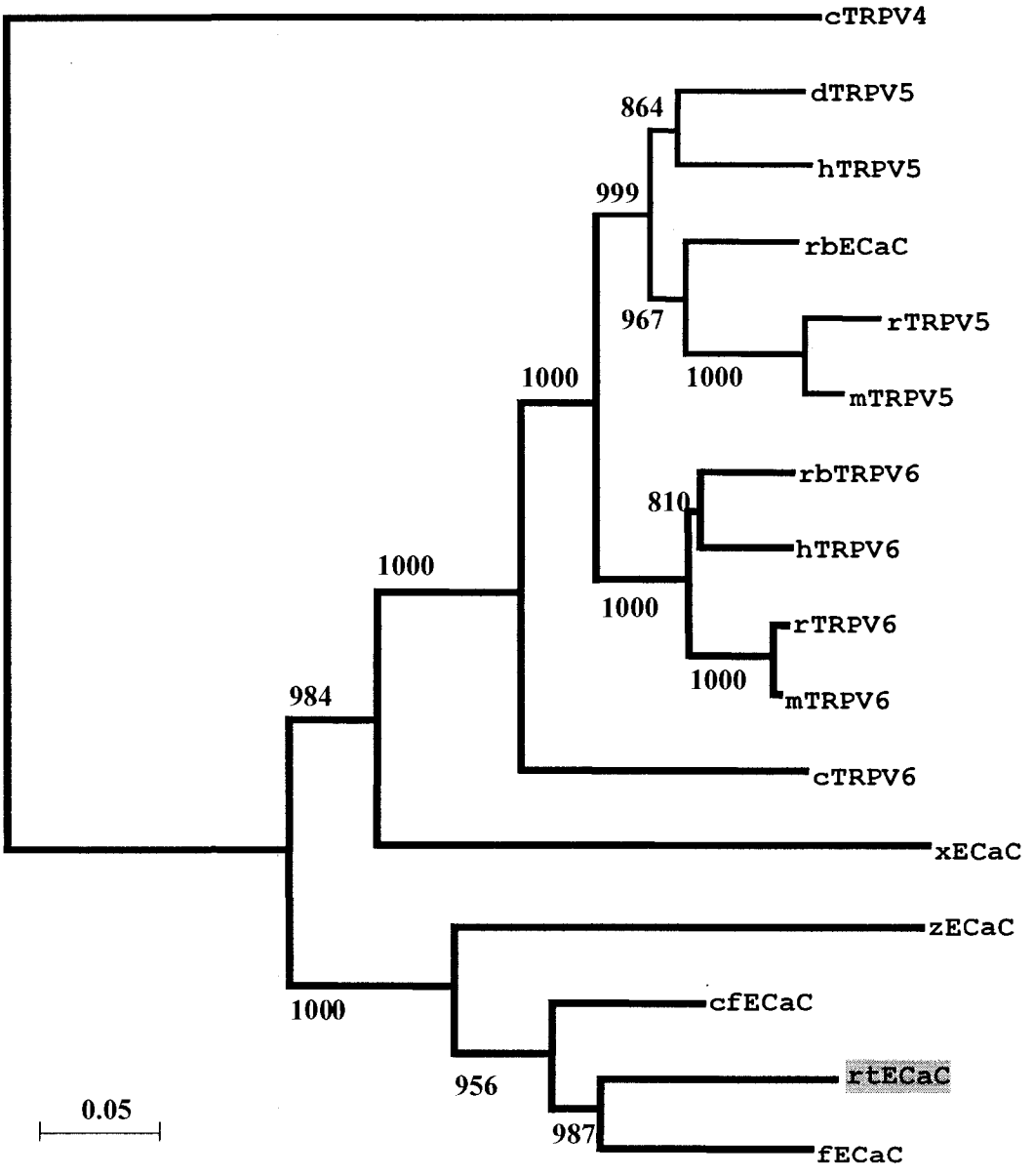
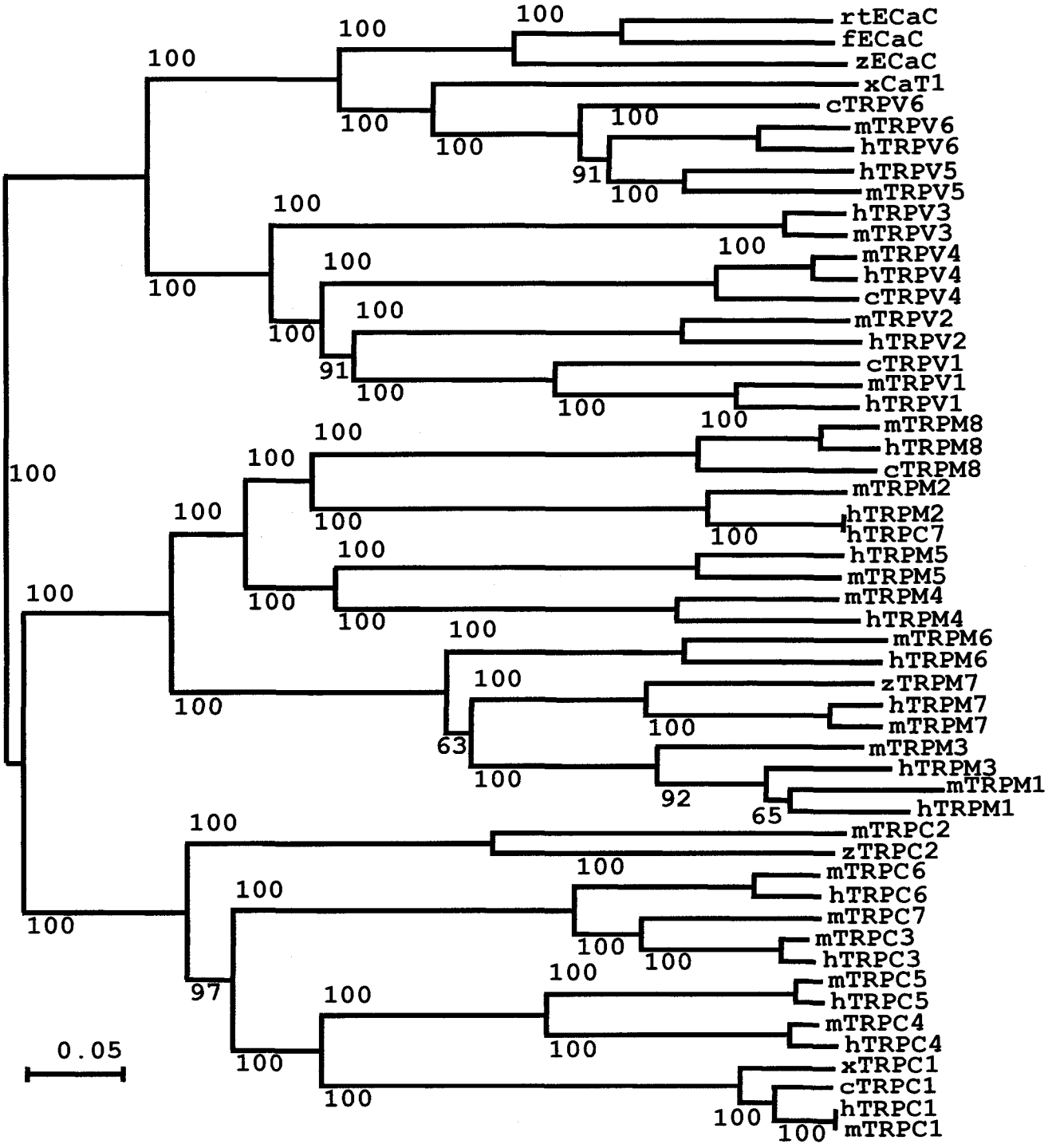

cTRPV4
dTRPV5
hTRPV5
rbECaC
rTRPV5
mTRPV5
rbTRPV6
hTRPV6
rTRPV6
mTRPV6
cTRPV6
xECaC
zECaC
cfECaC
rtECaC
fECaC
864
999
967
1000
1000
810
1000
1000
1000
984
1000
956
987
0.05

Figure 2.4. Phylogenetic analysis of the TRP gene family.

The phylogenetic tree was constructed using neighbour joining algorithms with bootstrap analysis of 100 pseudo-datasets for node support. Scale represents replacement of 5% of the amino acid in the protein alignment. See Table 2.3 for description of genes used in this tree; c = chicken, cf = crayfish, f = *Fugu*, h = human, m = mouse, rt = rainbow trout, x = *Xenopus*, z = zebrafish.

Figure 2.4


rtECaC
fECaC
zECaC
xCaT1
cTRPV6
mTRPV6
hTRPV6
hTRPV5
mTRPV5
hTRPV3
mTRPV3
mTRPV4
hTRPV4
cTRPV4
mTRPV2
hTRPV2
cTRPV1
mTRPV1
hTRPV1
mTRPM8
hTRPM8
cTRPM8
mTRPM2
hTRPM2
hTRPC7
hTRPM5
mTRPM5
mTRPM4
hTRPM4
mTRPM6
hTRPM6
zTRPM7
hTRPM7
mTRPM7
mTRPM3
hTRPM3
mTRPM1
hTRPM1
mTRPC2
zTRPC2
mTRPC6
hTRPC6
mTRPC7
mTRPC3
hTRPC3
mTRPC5
hTRPC5
mTRPC4
hTRPC4
xTRPC1
cTRPC1
hTRPC1
mTRPC1
0.05

Based on standard RT-PCR (35 cycles) and visualization using ethidium bromide staining, the gill displayed the highest expression of ECaC (Figure 2.5A). Of all other tissues examined using this technique, only the heart exhibited detectable ECaC. The more sensitive technique of real time PCR demonstrated that that ECaC expression was approximately 10-fold greater in the gills compared to other tissues (Figure 2.5B). Except for spleen, all extra-branchial tissues which were examined (kidney, intestine, heart, white muscle, liver, brain and blood) displayed detectable, but low levels of ECaC mRNA (Figure 2.5B).

Immunocytochemical analysis of gill sections clearly demonstrated the presence of ECaC associated with the apical surface of lamellar and to a lesser extent, filamental epithelial cells (Figure 2.6A). Indeed, the majority of lamellar epithelial cells appeared to express ECaC. A variety of staining patterns were observed; first, there were cells exhibiting obvious co-localization of ECaC and Na^+/K^+-ATPase, second, there were cells expressing only ECaC and third, there were cells expressing only Na^+/K^+-ATPase (Figure 2.6A). Figure 2.6D illustrates two Na^+/K^+-ATPase-positive cells in close proximity on the same lamella with only one showing the presence of ECaC on the apical membrane. Pre-absorption of the ECaC primary antibody with the peptide antigen (Figure 2.6B) or omission of the ECaC primary antibody (Figure 2.6C) clearly prevented the detection of ECaC along the apical surfaces. A representative western blot showing a single immunoreactive band at 90 kDa in trout gill is shown in Figure 2.6E; the immunoreactive band was eliminated after preabsorbing the primary antibody with immunizing peptide (Shahsavarani et al. 2006)

Figure 2.5. Tissue distribution of ECaC in rainbow trout (*Oncorhynchus mykiss*). Representative and mean tissue distributions as determined through (A) RT-PCR and (B) real time RT-PCR. ECaC-QPCR1 and ECaC-QPCR2 primers were used for PCR detection of ECaC while β-actin-FWD and β-actin-REV primers were used for the detection of β-actin (see Table 2.1 for primer sequences). For each tissue, a no template control (NTC) sample was tested (data not shown for real time PCR results). For real time PCR, the results are presented as the expression of ECaC relative to ß-actin and standardised to ECaC expression in the gill (N = 4).

Figure 2.5

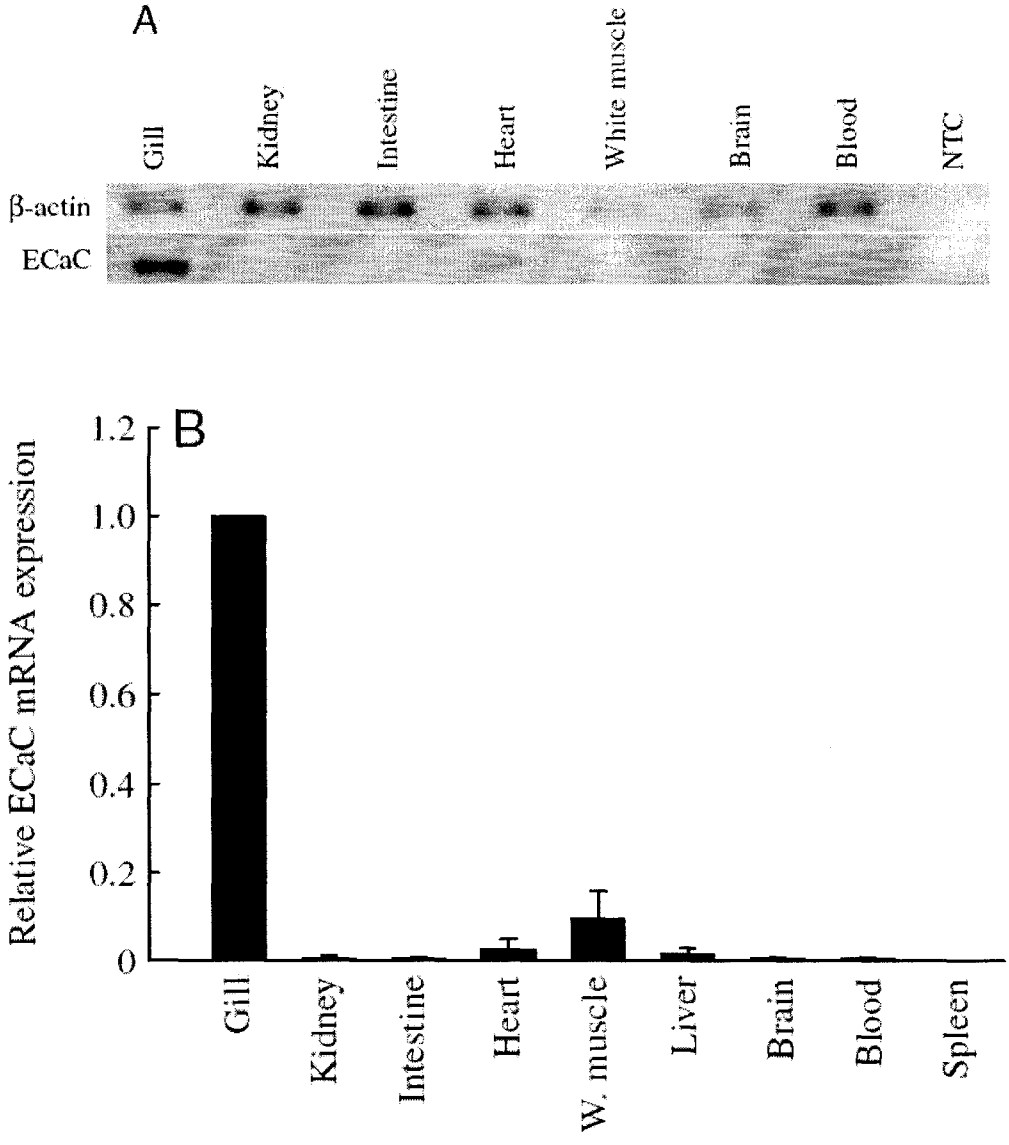
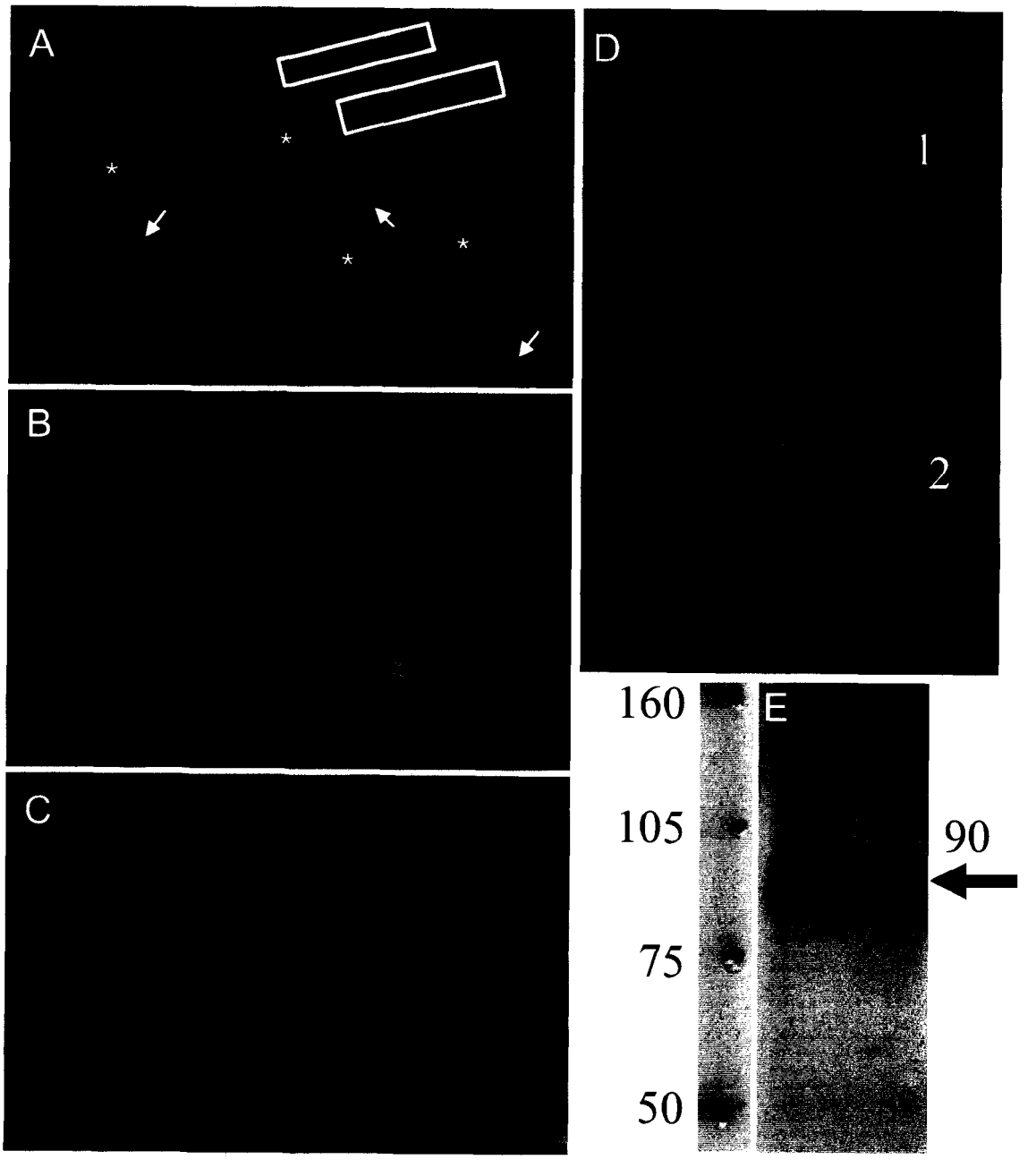

A
Gill
Kidney
Intestine
Heart
White muscle
Brain
Blood
NTC
β-actin
ECaC
B
Relative ECaC mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
Gill
Kidney
Intestine
Heart
W. muscle
Liver
Brain
Blood
Spleen

Figure 2.6. Localization of epithelial calcium channel (ECaC), Na^+K^+-ATPase and nuclei in rainbow trout (*Oncorhynchus mykiss*) gill epithelia.

The cell nuclei appear as blue, ECaC appears as green and Na^+/K^+-ATPase appears as red while yellow represents co-localization of ECaC with Na^+/K^+-ATPase. Panel A is a representative image of a gill section treated with primary antibodies against ECaC and Na^+/K^+-ATPase. Areas within the boxes illustrate cells exhibiting only ECaC immunoreactivity; arrows indicate cells exhibiting only Na^+/K^+-ATPase immunoreactivity; asterisks indicate cells exhibiting co-localization of ECaC and Na^+/K^+-ATPase. Panel B is a representative image of a gill section pre-absorbed with the ECaC peptide antigen. Panel C is a representative image of a gill section on which the ECaC primary antibody was omitted. Panel D is a higher magnification image showing two Na^+/K^+-ATPase-positive cells with one displaying no ECaC immunoreactivity (1) and the other exhibiting co-localization (2). Panel E is a representative western blot showing the presence of a single immunoreactive band at 90 kDa in trout gill; the sizes of the protein markers (kDa) are indicated on the left.

Figure 2.6



Results of *in-situ* hybridization experiments confirmed the presence of ECaC mRNA along lamellar and filament surfaces (Figure 2.7A). Furthermore, there appeared to be a higher concentration of ECaC mRNA-positive cells towards the tip of each lamella (Figure 2.7A and B). Although co-localizing ECaC and Na^+/K^+-ATPase mRNA was not possible using *in situ* hybridization, a comparison of Figure 2.7A and C clearly demonstrates that the staining pattern for ECaC mRNA was much more diverse than for Na^+/K^+-ATPase mRNA that appeared to be restricted to a single cell type (presumably the MRC).

Further characterization of ECaC distribution was achieved using cell isolation techniques. Figure 2.8 illustrates representative micrographs showing ECaC and Na^+/K^+-ATPase distribution in (A, B) crude cell suspensions, (C) enriched populations of PVCs and (D, E) enriched populations of PNA^- MRCs. Despite the varying levels of purification, each fraction displayed a mosaic of 4 cell types based on immuno-staining: ECaC-positive cells, Na^+/K^+-ATPase-positive cells, cells expressing both ECaC and Na^+/K^+-ATPases as well as cells displaying no immunoreactivity. Of particular interest was the large number of cells in the enriched PVC fraction expressing ECaC and the relatively large numbers of cells in the PNA^- fraction exhibiting co-localization of ECaC and Na^+/K^+-ATPase. These qualitative observations were confirmed by quantitative flow cytometry (Figure 2.8F). Approximately 30% of all cells displayed no immunoreactivity and 60-70% displayed detectable levels of ECaC regardless of whether the cells were derived from enriched PVC fractions of enriched PNA^- fractions (Figure 2.8F). However, the cells derived from PNA^- fractions exhibited significantly greater co-localization of ECaC and Na^+/K^+-ATPase compared to cells obtained from PVC fractions

Figure 2.7. Localization of ECaC and Na^+/K^+-ATPase mRNA and protein in rainbow trout (*Oncorhynchus mykiss*) gill epithelia.

Panel A is a representative image of a gill section probed with a homologous ECaC oligonucleotide probe; (a) indicates cells expressing detectable levels of ECaC mRNA, (b) indicates MRCs expressing detectable levels of ECaC mRNA and (c) indicates MRCs that do not appear to express high levels of ECaC mRNA. Panel B is a representative image of a gill section treated with primary antibodies against ECaC and Na^+/K^+-ATPase; the cell nuclei appear as blue, ECaC appears as green and Na^+/K^+-ATPase appears as red while yellow represents co-localization of ECaC with Na^+/K^+-ATPase; asterisks indicate areas of intense ECaC mRNA or protein at the tips of lamellae where MRCs are rarely found. Panel C is a representative image of a gill section probed with a homologous Na^+/K^+-ATPase oligonucleotide probe.

Figure 2.7

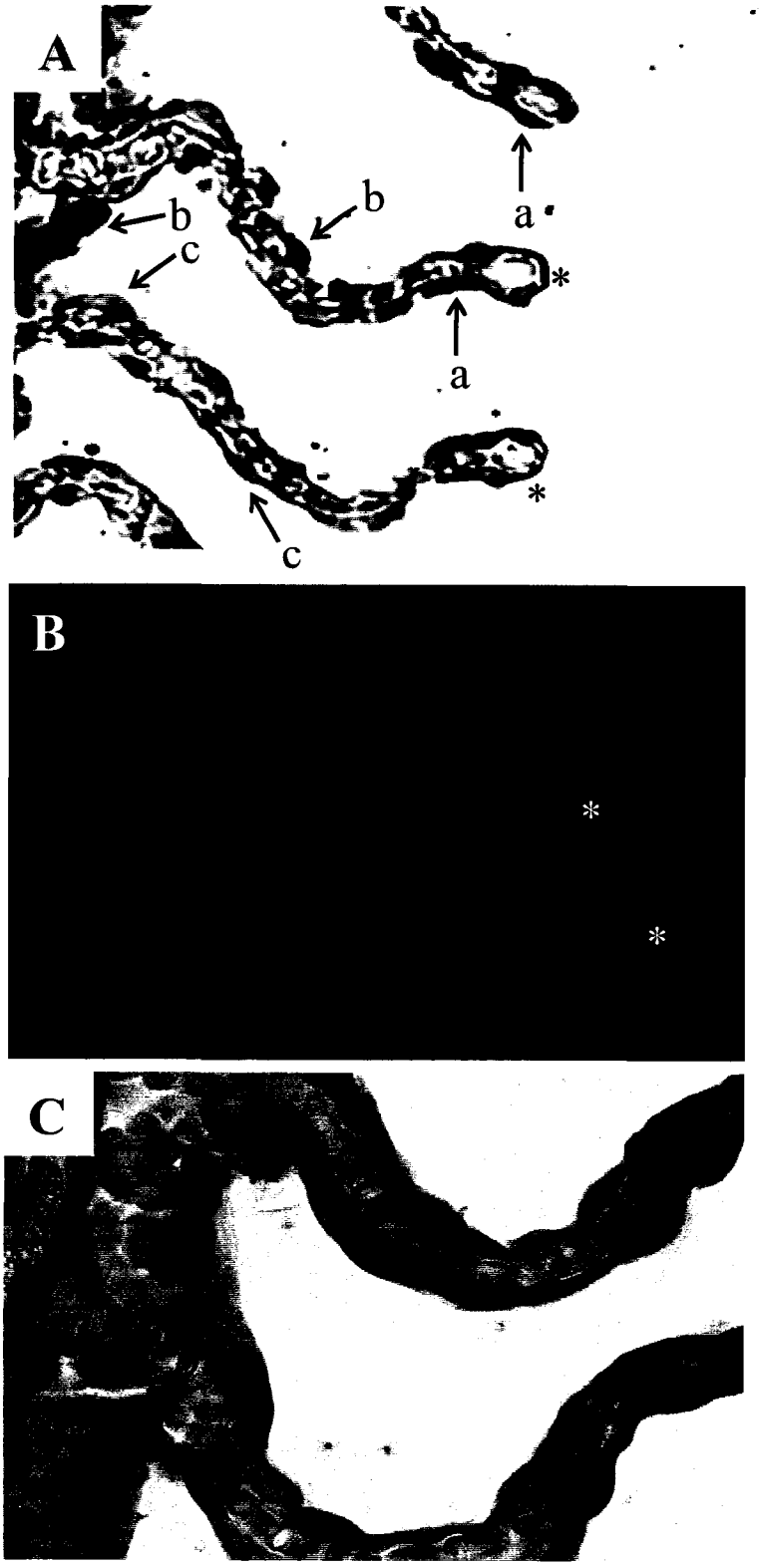
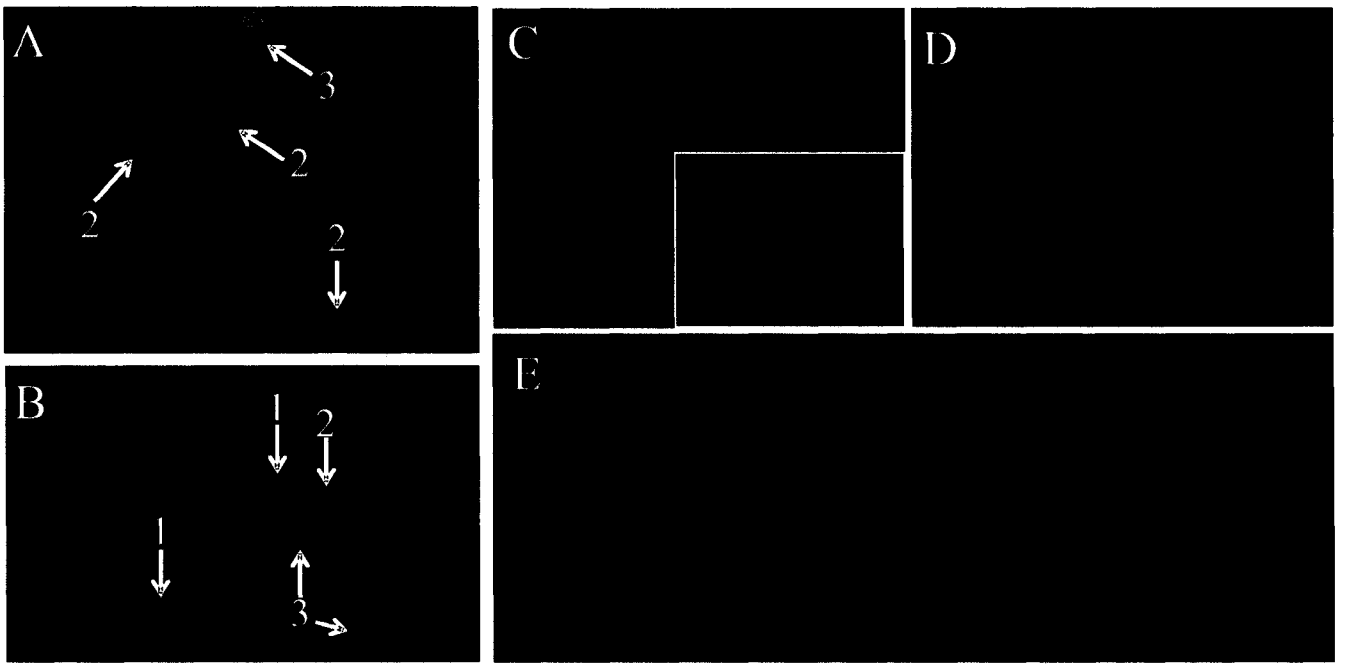
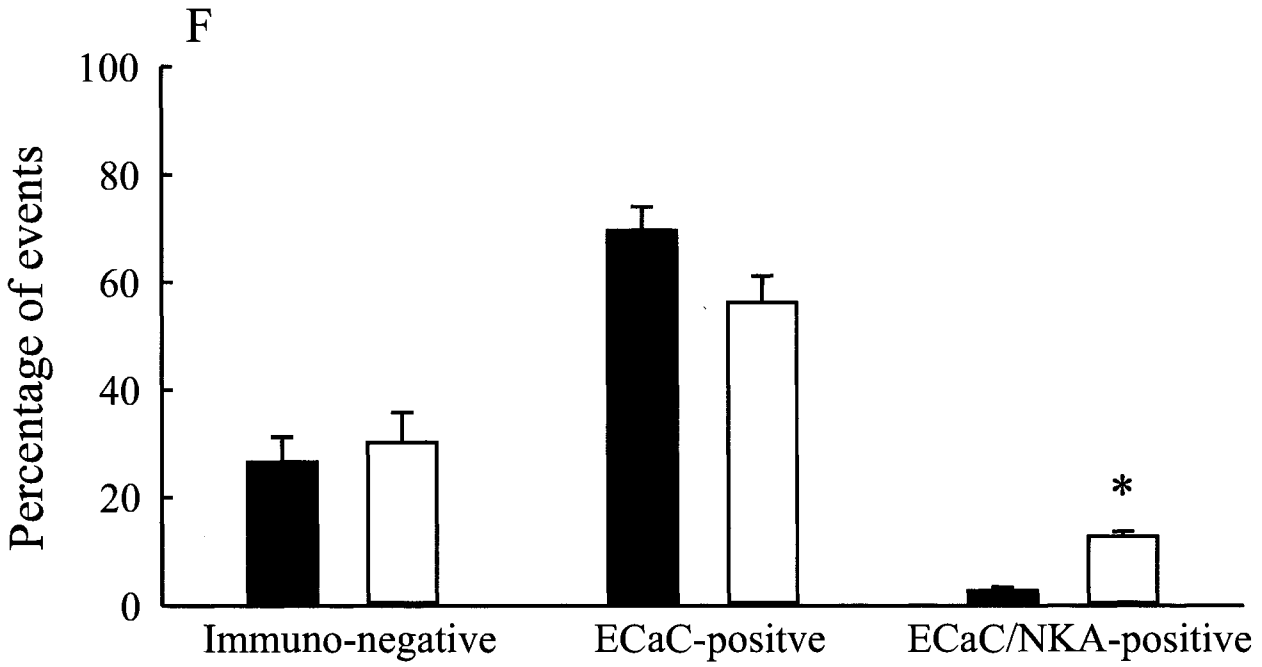


Figure 2.8. Localization and quantitative distribution of epithelial calcium channel (ECaC) and Na^+K^+-ATPase protein in suspensions of rainbow trout (*Oncorhynchus mykiss*) gill epithelial cells.

(A-E) immunocytochemistry and (F) flow cytometry. In panels A-E, the cell nuclei appear as blue, ECaC appears as green and Na^+/K^+-ATPase appears as red while yellow represents co-localization of ECaC with Na^+/K^+-ATPase. Panels A-B are representative images of crude cell suspension prior to separation of different cell types; (1) indicates ECaC-positive cells, (2) indicates Na^+/K^+-ATPase-positive cells and (3) indicates cells co-expressing ECaC and Na^+/K^+-ATPase. Panel C is a representative image from a cell suspension enriched with PVCs. Panels D and E are representative images from purified cell suspensions enriched with PNA^- mitochondria rich (MR) cells. Panel F illustrates the distribution of single- and double-labelled events (cells) as determined by flow cytometry in cell suspensions enriched with PVCs (filled bars; N = 4) or PNA^- MRCs (unfilled bars; N = 4). Data are shown as means ± 1 SEM; an asterisk indicates a significant difference between the two cell populations (rank-sum test, $P < 0.05$).

Figure 2.8


A
3
2
2
2
B
1
2
1
3
C
D
E


F
100
80
60
40
20
0
Percentage of events
*
Immuno-negative
ECaC-positve
ECaC/NKA-positive

(13.1 ± 0.7% *versus* 3.1 ± 0.3%; P = 0.029, rank-sum test). It was not possible to obtain great enough numbers of PNA^+ cells to analyze this population by flow cytometry. An analysis of the relative levels of ECaC mRNA in the three cell populations (PVCs, PNA^- and PNA^+) demonstrated that ECaC expression was similar among these fractions (Figure 2.9).

Single seeded cell cultures containing only PVCs displayed both ECaC-positive and ECaC-negative cells (Figure 2.10). Based on qualitative assessment, the majority of cells were ECaC-positive. Double-seeded cell cultures, containing a mixture of PVCs and MRCs also displayed a mosaic of 4 cell types based on immuno-staining; ECaC-positive cells, Na^+/K^+-ATPase-positive cells, cells expressing both ECaC and Na^+/K^+-ATPase as well as cells displaying no immunoreactivity (Figure 2.11). As was observed on whole gill sections (Figure 2.5) and crude cell suspension (Figure 2.8), the double-seeded cell cultures clearly contained two distinct types of Na^+/K^+-ATPase-positive cells, those expressing ECaC and those not expressing ECaC. Similarly, using MitoTracker™, two distinct populations of MRC's were identified; those exhibiting ECaC and those not exhibiting ECaC (Figure 2.11D).

Figure 2.9. Relative epithelial calcium channel ECaC mRNA expression in enriched populations of PVCs, PNA^- mitochondria rich (MR) cells and PNA^+ MR cells. Data are shown as means ± 1 SEM (N = 6).

Figure 2.9

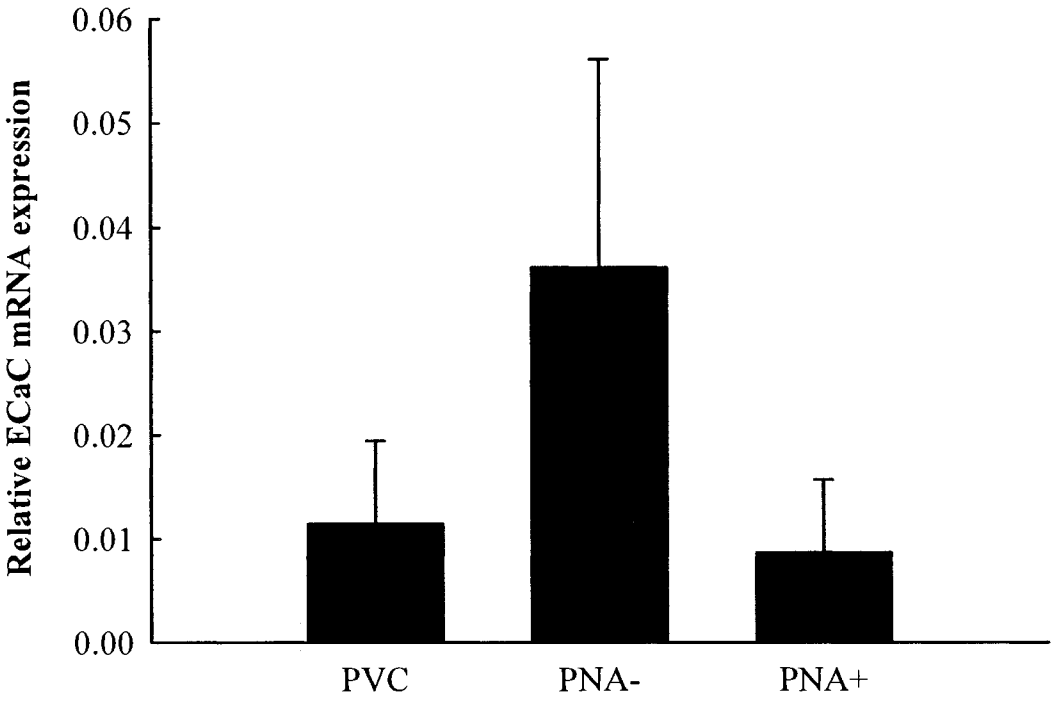

Relative ECaC mRNA expression
0.06
0.05
0.04
0.03
0.02
0.01
0.00
PVC
PNA-
PNA+

Figure 2.10. Immunocytochemical localization of epithelial calcium channel (ECaC) in single seeded rainbow trout gill epithelial cell culture.

The cell nuclei appear as blue and ECaC appears as green. Note the presence of both ECaC-positive and ECaC-negative (denoted by asterisk) cells. Omission of primary antibody eliminated all fluorescence (C).

Figure 2.10

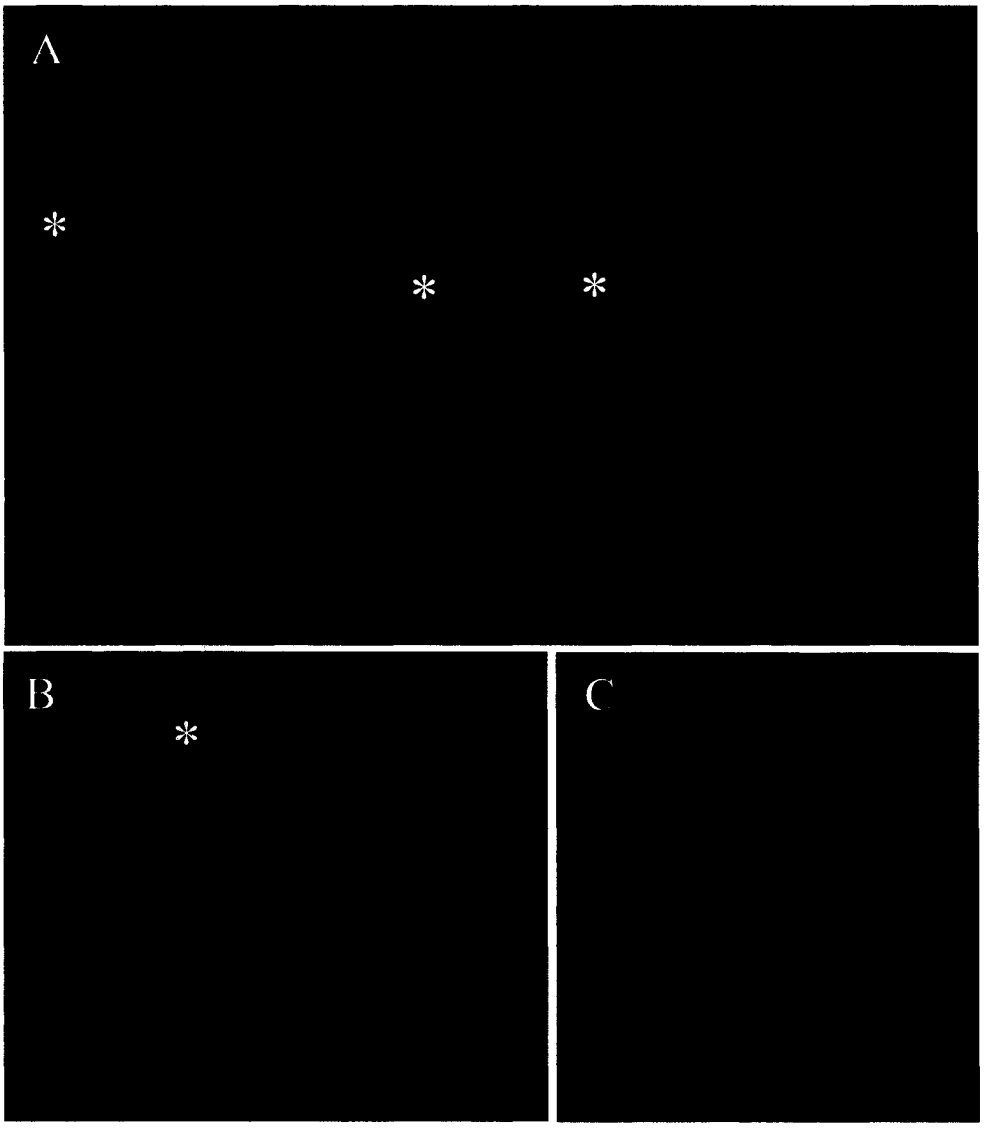


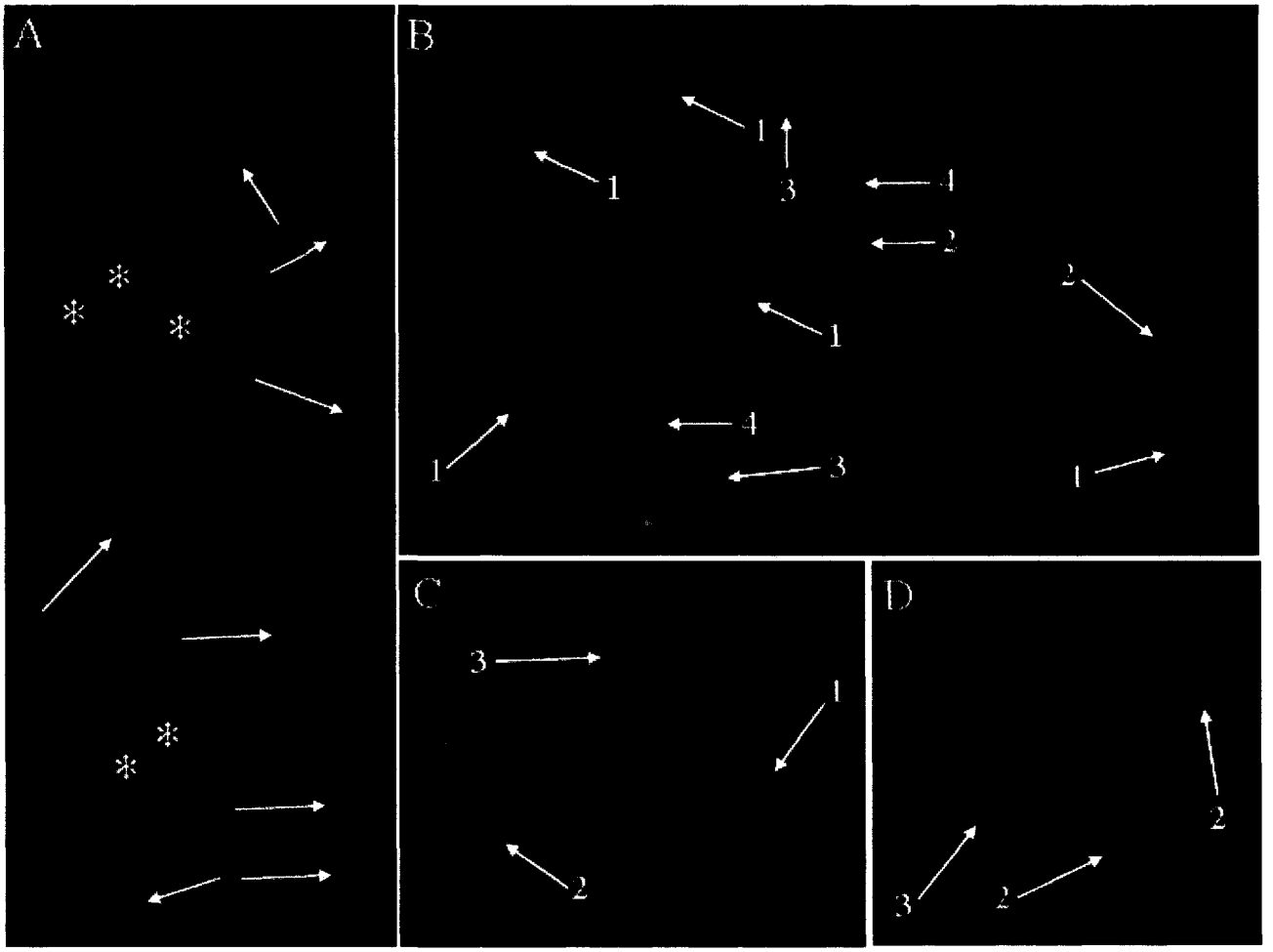
Figure 2.11. Immunocytochemical localization of epithelial calcium channel (ECaC), Na^+/K^+-ATPase in double seeded rainbow trout gill epithelial cell cultures using (A) confocal or (B – D) or epifluorescence microscopy.

In panels A – C, the cell nuclei appear as blue, ECaC appears as green and Na^+/K^+-ATPase appears as red while yellow represents co-localization of ECaC with Na^+/K^+-ATPase.

In panel D, MitoTracker™ was used to localize mitochondria rich cells (stained green) and ECaC appears as red.

Panel A is a reconstruction of 22 optical sections showing the presence of ECaC-positive mitochondria rich cells (MRCs) lying on top of a confluent layer of ECaC-positive and ECaC-negative (asterisks) pavement cells. In panels B and C, (1) indicates ECaC-positive cells, (2) indicates Na^+/K^+-ATPase-positive cells, (3) indicates cells co-expressing ECaC and Na^+/K^+-ATPase and (4) indicates cells neither positive for ECaC or Na^+/K^+-ATPase. In panel D, (2) indicates MRCs and (3) indicates MRCs co-expressing ECaC.

Figure 2.11



Discussion

The results of the present study demonstrate that the epithelial calcium channel found in fish, although similar in sequence and topology, does not belong to either the TRPV5 or TRPV6 family of genes. Furthermore, it would appear that the mammalian TRPV5 and TRPV6 have only recently diverged from one another.

The rainbow trout ECaC (rtECaC) has a coding region of 2184 base pairs with a predicted protein sequence of 727 amino acids. At the amino acid level, rtECaC displayed 77 and 66% identity with pufferfish (*Takifugu rubripes*) and zebrafish ECaC, respectively. The rtECaC protein was 51 and 50% identical to mouse TRPV6 and TRPV5, respectively.

Genomic analysis has shown that human TRPV5 and TRPV6 are both located on chromosome 7q35, juxtaposed to one another (indicative of gene duplication; (Muller et al. 2000b, 2000a). Despite extensive examination of the zebrafish (*Danio rerio*) genome, it has not been possible to identify more than a single ECaC gene located on chromosome 16. A similar conclusion was reached following analysis of the pufferfish genome (Qiu and Hogstrand 2004). These results are consistent with the phylogenetic analysis presented here (Figure 2.3) and suggest that unlike in mammals, there is but a single gene for ECaC in rainbow trout, zebrafish, pufferfish and presumably other fish species. The results of a more complete phylogenetic analysis of 53 genes from TRPC, TRPM and TRPV families suggest that fish ECaC and TRPV sub-families diverged prior to a possible gene duplication giving rise to TRPV5 and TRPV6 (Figure 2.4). This conclusion is slightly different than the scheme presented by Qiu and Hogstrand (2004)

who proposed that the mammalian TRPV5 and TRPV6 sub-families may have originated from a single ancestral TRPV6 gene. Interestingly, neither the amphibian (*Xenopus*) nor the avian (*Gallus*) calcium channels appear to group with either the fish or the mammalian channels. Thus, the gene duplication giving rise to TRPV5 and TRPV6 likely occurred recently in mammalian evolution.

In mammals, evidence suggests that there may be a significant tissue dependent distribution for TRPV5 and TRPV6 (Hoenderop et al. 2001; Muller et al. 2000a; Nijenhuis et al. 2005; Nijenhuis et al. 2003; Song et al. 2003; van Abel et al. 2003). In contrast to the mammalian system where Ca^{2+} is acquired exclusively from the diet, fish may obtain Ca^{2+} directly from the aqueous environment through the gills as well as from the diet (Bindels 1993; Flik and Verbost 1993; Perry 1997; Perry and Flik 1988). Mammals, therefore, are consistently faced with the challenge of finding a dietary source of calcium whereas fish can always exploit the alternate waterborne supply if dietary supply becomes limiting (Rodgers, 1984). Therefore, mammals are not only faced with having to possess an optimal Ca^{2+} absorption mechanism at the intestinal tissue but must also minimize Ca^{2+} loss; this may explain the difference in TRPV5 and TRPV6 distribution in various tissues. In fish, it is possible that Ca^{2+} loss is not a major factor under normal circumstances and that as long as the uptake mechanism meets physiological needs, Ca^{2+} homeostasis can be maintained. This may have led to gene deletion after any genome duplication event.

Consistent with its presumed critical role in Ca^{2+} uptake, the gill displayed the highest levels of ECaC mRNA as determined either by standard RT-PCR or real time RT-PCR. The predominance of ECaC mRNA expression in gill was also reported for

pufferfish (Qiu and Hogstrand 2004). In contrast to mammals where ECaC expression is high in vitamin D_3 sensitive tissues (den Dekker et al. 2003; Hoenderop et al. 2000b; Hoenderop et al. 1999; Van Ball et al. 1996; Wood et al. 2001), ECaC expression was low in rainbow trout intestine and kidney (Figure 2.5). Previous studies on the effect of vitamin D_3 in fish have suggested a possible regulatory role for this hormone in Ca^{2+} uptake at the intestine (Sundell et al. 1993). Therefore it is surprising that ECaC expression is extremely low (Figure 2.5) in a tissue that appears to be physiologically sensitive to vitamin D_3. Further studies are required to determine how vitamin D_3 affects ECaC expression in the various Ca^{2+} transporting tissues including the intestine and kidney.

According to current models, the MRC of the gill is the principal cell type involved in Ca^{2+} uptake (Copp 1982; Fenwick 1989; Flik et al. 1995; Flik et al. 1996; Ichii and Mugiya 1983; Ishihara and Mugiya 1987; Jie 1997; Marshall et al. 1992; McCormick et al. 1992; Moron et al. 2003; Perry et al. 1992). In apparent disagreement with the putative model, the results presented in this study suggest that MRCs are neither the sole, nor the primary cell types expressing ECaC, thought to represent the initial step in Ca^{2+} absorption across the gill.

Homologous polyclonal antibodies raised against rtECaC were used in conjunction with a heterologous mouse monoclonal Na^+/K^+-ATPase antibody (α5) to examine the cellular distribution of rtECaC in the gill epithelium. Previous studies have established that α5 can be used to detect Na^+/K^+-ATPase in numerous species ranging from invertebrates to mammals and it has been used extensively in previous studies examining fish (e.g. Wilson et al. 2000). Because the MRC is vastly enriched with

Na^+/K^+-ATPase, the presence or absence of Na^+/K^+-ATPase immunoreactivity is routinely used to discriminate the MRC from other cell types of the gill epithelium. Thus, to interpret the results of the present study, we have assumed that all cells displaying Na^+/K^+-ATPase immunoreactivity are MRCs. Several control experiments were performed to evaluate the specificity of the ECaC antibody including western blots and preabsorption of the primary antibody with peptide antigen and omission of primary antibody in immunocytochemistry and flow cytometry experiments. The results of the western blot revealed a single immunoreactive band at 90 kDa, only slightly higher than the predicted mass for trout ECaC of 83 kDa. The larger than predicted size can be explained by post-translational modification(s) such N-glycosidic linkage. This type of modification has been previously identified in the mammalian TPRV6 with the presence of an Asn-glycosylation site at position 358 of the human protein between transmembrane domains 1 and 2 (Hirnet et al. 2003). In the trout protein, a possible site has been identified using NetNGlyc 1.0 at position 362 located between transmembrane domains 1 and 2. Although the results of the preabsorption and primary antibody omission experiments indicated that the antibody was specific, they do not conclusively demonstrate that the antibody is detecting ECaC. It is also acknowledged that minor residual fluorescence can still be observed following preabsorption (figure 2.6B) which may be the result of non-specific binding or perhaps more likely the result of incomplete preabsorption. However, in addition to the single immunoreactive band of approximately the predicted molecular weight on western blots, several indirect findings support our contention that the antibody used in this study was indeed detecting ECaC. First, there was the similarity between the results obtained using immunocytochemistry and *in situ*

hybridization. Second, using the same antibody, it was demonstrated (by western blots and immunocytochemistry) that conditions known to increase Ca^{2+} uptake (elevated cortisol, softwater exposure and hypercapnia) caused a marked increase in the intensity of the immunoreactive protein (Chapter 3).

The results of the immunocytochemistry experiments clearly demonstrated an apical distribution of ECaC on gill epithelial cells. However, the overall distribution of ECaC was more extensive than anticipated. Although ECaC was co-localized with a subset of MRCs, there were numerous lamellar PVCs exhibiting ECaC immunoreactivity. The results of *in-situ* hybridization support a broad cellular distribution of ECaC throughout the gill lamellae. The finding that ECaC is not restricted to MRCs would appear to be inconsistent (at least at first glance) with the prevailing view that the MRCs are the predominant site of branchial Ca^{2+} uptake. Although these findings do not rule out a role for the MRC in Ca^{2+} uptake, they do suggest that PVCs may also be involved (Zia and McDonald 1994).

An interesting result of the present study was the observation that only a sub-population of gill MRCs exhibited ECaC immunoreactivity. Recently, Goss and co-workers described two sub-types of MRC in rainbow trout on the basis of presence (PNA^+) or absence (PNA^-) of peanut lectin agglutinin (PNA) binding sites (Galvez et al. 2002; Goss et al. 2001). The PNA^+ cells appeared to resemble the typical MRC (chloride cell) of the freshwater fish gill (Perry 1997) whereas the PNA^- cells exhibited characteristic similar to PVCs and were likely identical to the MR PVCs described in earlier literature (Goss et al. 1992b; Goss et al. 1992a; Goss et al. 1994). Clearly, the PNA^- cells express ECaC on the apical membrane (see below) but because it was not possible to examine ECaC

protein expression in enriched PNA^+ cell populations, we cannot exclude that these cells also express ECaC. On the basis of detectable ECaC mRNA levels in the PNA^+ cells, it would be surprising if they did not express ECaC protein. Thus, further experimentation will be required to explain the heterogeneous distribution of ECaC among the MR cells.

To further describe ECaC distribution in the various cell types, gill cells were isolated and re-suspended prior to fixation and staining. The immunocytochemistry results were in accord with those previously obtained using gill cross sections. The total cell population could be visually classified into four groups; a) ECaC-positive, b) Na^+/K^+-ATPase-positive, c) ECaC and Na^+/K^+-ATPase-positive or d) ECaC and Na^+/K^+-ATPase-negative. Further examination of enriched population of PVC's, PNA^- cells and PNA^+ (mRNA analysis only) cells confirmed the broad distribution of ECaC amongst the various cell types. Interestingly 56% of PNA^- cells expressed only ECaC with only 13% exhibiting ECaC and Na^+/K^+-ATPase co-localization. Thus, within the PNA^- cell fraction, there would appear to be several cell sub-types of which only a small percentage are enriched with Na^+/K^+-ATPase. The heterogeneity of this cell fraction is consistent with the findings of Galvez et al. (2002) which demonstrated several unidentified cell types in addition to the MR PVC's in the PNA^- fraction.

Fletcher et al (2000) developed a technique to co-culture rainbow trout PVCs and MRCs on permeable inserts (referred to as double-seeded inserts or DSIs). These preparations differed from the single seeded insert (SSIs) that contained only PVCs. Although both preparations exhibited equivalent rates of Ca^{2+} uptake, only the DSI's displayed active Ca^{2+} transport (Fletcher et al. 2000). It was concluded that the presence of MRCs in the DSIs was responsible for conferring the capacity for active Ca^{2+} transport

(Fletcher et al. 2000). Immunocytochemical analysis of DSIs in the present study revealed a similar mosaic of four cell types as observed in the cell suspensions (see above). Thus, the transport differences between the two preparations could be explained by the presence of several cell types not present in the SSI including PNA^+ MRC's and PNA^- MRC's (with or without enrichment of Na^+/K^+-ATPase). However, because PVC's contain apical membrane ECaC, the capacity for epithelial active transport of Ca^{2+} is unlikely to reflect the presence or absence of ECaC, alone. Thus, it is likely another component of the overall Ca^{2+} transport system (perhaps NCX or PMCA) is being uniquely expressed in the DSIs.

Conclusions and perspectives

The results of this study clearly demonstrate that some members of both the PVC and MRC populations possess apical membrane Ca^{2+} channels and thus could potentially contribute to Ca^{2+} uptake at the gill. However, although entry of Ca^{2+} through apical membrane channels is clearly a crucial step in Ca^{2+} uptake in the fish gill and other Ca^{2+} transporting epithelia, other steps are required including extrusion of Ca^{2+} across the basolateral membrane by Ca^{2+}-ATPase (PMCA) or Na^+/Ca^{2+} exchange (NCX). Thus, the presence or absence of these proteins (in sufficient quantities) in addition to apical membrane ECaC, may be a prerequisite for transcellular Ca^{2+} movements. Because of the extensive indirect evidence that the MRC is the site of Ca^{2+} uptake and the recent finding that rates of Ca^{2+} uptake *in vitro* are highest in suspensions of PNA^+ cells (Galvez et al., 2006), it is conceivable that the PVC has a lower intrinsic rate of Ca^{2+} uptake

compared to the MRCs. However, because the PVC is, by far, the most abundant epithelial cell type, it is possible that the bulk of Ca^{2+} uptake *in vivo* may be occurring via the PVCs. Clearly, further molecular, physiological as well as morphological investigations are required at the cellular level to define the relative roles of the various gill epithelial cell types in branchial Ca^{2+} uptake.

Regardless of its location, the regulation of ECaC by hormones or other signals is likely to be a key process maintaining Ca^{2+} balance in fish. The hormonal regulation of ECaC is likely to involve both post-translational and transcriptional control mechanisms. For example, rapid adjustments of Ca^{2+} uptake across the apical membrane largely reflect post-translational modifications of ECaC leading to rapid changes in Ca^{2+} conductance. The anti-hypercalcemic hormone, stanniocalcin, is arguably the most important hormone regulating the Ca^{2+} conductance of ECaC and plays a crucial role in reducing the rate of Ca^{2+} entry across the gill during acute hypercalcemia (Wendelaar Bonga and Pang, 1991). Chronic regulation of Ca^{2+} uptake is probably predominantly achieved via transcriptional mechanisms and may involve adjustments in the number of ECaC proteins expressed on the apical membrane and/or Ca^{2+} transporting proteins (NXC, PMCA) on the basolateral membrane.

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CHAPTER 3

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

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Statement of Contributions:

The work presented in this Chapter is the result of collaborative work between Arash Shahsavarani, S.F. Perry and B. McNeill. For clarification, original tissue preservation and immunocytochemistry were done by A. Shahsavarani. For the purpose of publication some additional imaging was done by B. McNeill and S.F. Perry. Western blots were completed by B. McNeill and S.F. Perry. All remaining work was completed by A. Shahsavarani.

Abstract

We indirectly tested the idea that the epithelial Ca^{2+} channel (ECaC) of the trout gill is regulated in an appropriate manner to adjust rates of Ca^{2+} uptake. This was accomplished by assessing the levels of gill ECaC mRNA and protein in fish exposed to treatments known to increase or decrease Ca^{2+} uptake capacity. Exposure of trout to soft water ($[Ca^{2+}] = 20 - 30$ nmol l^{-1}) for 5 days (a treatment known to increase Ca^{2+} uptake capacity) caused a significant increase in ECaC mRNA levels and an increase in ECaC protein expression. The inducement of hypercalcemia by infusing fish with $CaCl_2$ (a treatment know to reduce Ca^{2+} uptake) was associated with a significant decrease in ECaC mRNA levels yet protein levels were unaltered. ECaC mRNA and protein expression were increased in fish treated with the hypercalcemic hormone cortisol. Finally, exposure of trout to 48 h of hypercapnia (~7.5 mm Hg; a treatment known to increase Ca^{2+} uptake capacity) elicited an approximate 100-fold increase in the levels of ECaC mRNA and a significant increase in protein expression. Immunocytochemical analysis of the gills from hypercapnic fish suggested a marked increase in the apical expression of ECaC on pavement cells and a sub-population of mitochondria rich cells. The results of this study provide evidence that Ca^{2+} uptake rates are, in part, regulated by the numbers of apical membrane Ca^{2+} channels that in turn modulate the inward flux of Ca^{2+} into gill epithelial cells.

Introduction

Unlike other vertebrates that rely exclusively on dietary calcium to fulfill nutritional requirements, fish absorb a substantial component of their calcium needs from the surrounding water (Fenwick 1989). The gill is considered to be the predominant site of calcium uptake although the skin may play a supplementary role (Perry and Wood 1985). The current model for calcium uptake across the fish gill (Flik et al. 1995; Flik and Verbost 1993; Marshall 2002; Perry and Flik 1988) proposes that the initial step in transepithelial uptake is the entry of Ca^{2+} into gill epithelial cells through apical membrane calcium channels (Perry et al. 2003). The final step involves the movement of Ca^{2+} across the basolateral membrane either by Ca^{2+}-ATPase mediated active transport (Flik et al. 1983) or via Na^{+}/Ca^{2+} exchangeer (Verbost et al. 1994). It is possible that by analogy to renal or intestinal Ca^{2+} uptake in mammals (Brown et al. 2002; Clapham et al. 2001; Hoenderop et al. 2000a; Hoenderop et al. 2002; Hoenderop et al. 1999; Song et al. 2003; Vennekens et al. 2000), the entry of Ca^{2+} through the apical calcium channel is the rate-limiting step in branchial Ca^{2+} uptake. Therefore, modification in the kinetic properties or changes in the number of calcium channels may underlie acute and chronic modifications of Ca^{2+} uptake rates across the gill.

The branchial epithelial calcium channel (ECaC) has been cloned from several fish species including pufferfish [*Fugu rubripes* (Qiu and Hogstrand 2004); NCBI GenBank Accession AY232821], zebrafish [*Danio rerio* (Pan et al. 2005); NCBI GenBank Accession AY325807] and rainbow trout [*Oncorhynchus mykiss* (Shahsavarani et al. 2006); GenBank Accession AY256348]. The epithelial calcium channel belongs to the vanilloid subfamily of the transient receptor potential (TRP) superfamily (den Dekker

et al. 2003; Vennekens et al. 2002). Unlike in mammals where two distinct forms of ECaC are encoded by different genes (TRPV5 and TRPV6), there would appear to be but a single gene in fish (Qiu and Hogstrand 2004; Shahsavarani et al. 2006; Chapter 2). It has been suggested that fish ECaC and TRPV sub-families diverged prior to a possible gene duplication giving rise to TRPV5 and TRPV6 (Shahsavarani et al. 2006; Chapter2). Although the site of Ca^{2+} uptake in freshwater fish is believed to be the chloride cell (also termed mitochondria rich cell) (Ishihara and Mugiya 1987; Marshall et al. 1992; McCormick et al. 1992; Perry et al. 1992; Perry and Flik 1988; Perry and Wood 1985), ECaC appears to be ubiquitously expressed in all of the gill epithelial cell types including pavement cells and chloride cells (Shahsavarani et al. 2006).

If Ca^{2+} entry through ECaC were indeed an important regulatory step in overall transepithelial Ca^{2+} flux, one would expect predictable changes in the levels of ECaC mRNA and protein in response to physiological cues known to modify branchial Ca^{2+} uptake rates. Thus, in the present study it was predicted that conditions known to increase Ca^{2+} transport capacity such as low environmental Ca^{2+} levels (Perry and Wood 1985), cortisol treatment (Flik and Perry 1989) or hypercapnia (MacKenzie and Perry 1997b) would be associated with increased ECaC expression. Hypercalcemia, a condition known to reduce branchial Ca^{2+} uptake (Lafeber and Perry 1988; Perry et al. 1989), on the other hand, would be expected to reduce ECaC expression. ECaC mRNA expression was monitored using real time PCR whereas protein levels were assessed by western blots and immunocytochemistry using a homologous polyclonal antibody.

Materials and Methods

Animal care

Rainbow trout (*Oncorhynchus mykiss*) of both sexes were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). The fish were held at the University of Ottawa in large fiberglass tanks supplied with flowing, aerated, and dechloraminated city water and maintained at 13°C, on a 12 h:12 h light:dark photoperiod and were fed daily with a commercial trout diet.

All procedures involving animals were carried out according to institutional guidelines, which are in accordance with those of the Canadian Council on Animal Care (CCAC).

Effect of environmental calcium

Three aquaria (30 cm H x 30 cm W x 61 cm L, 54 l volume) were used with water temperature maintained at 13° C by using a re-circulating water bath and a stainless steel cooling coil. All sides of the aquaria were blackened to minimize visual stress. Three environmental Ca^{2+} levels were established (low = 20 - 30 nmol l^{-1}; normal = 200 – 300 nmol l^{-1} or high = 2 - 2.5 mmol l^{-1}). Calcium concentrations were adjusted using calcium nitrate as needed. Six fish [mean mass for all 18 fish 97.4 ± 10.9 (SEM) g] were placed into each environment for a period of 5 days. Water chemistry was monitored on a daily basis to ensure that Ca^{2+} levels were maintained within the target range. Following the five-day exposure, the animals were euthanized by a sharp blow to the head. The gill basket was quickly removed and gill filaments were isolated. Samples were immediately placed into liquid N_2 and stored at -86° C until use. Tissue samples were also preserved

for microscopy using standard procedures (see below). Water [Ca^{2+}] was determined by flame emission spectrophotometry (Varian, model Spectra AA 250 Plus).

Effect of calcium infusion

Benzocaine (ethyl-*p*-aminobenzoate; 2.4 x 10^{-4} mol l^{-1}) was used to anesthetize each fish (weighing between 180 and 260 g). The fish were then placed on a surgical table where the gills were irrigated continuously with anesthetic solution. The dorsal aorta was cannulated (Soivio et al. 1975) using a polyethylene cannula (Clay-Adams, PE 50). For recovery, fish were placed into individual opaque acrylic boxes provided with continuous flow of aerated fresh water (13° C) for 24 h prior to the commencement of the experiments.

Following the recovery period, each fish was infused via the dorsal aorta cannula for a period of 24 h using a syringe pump (Model 355, Sage Instruments). Fish were infused with either saline (140 mmol l^{-1} NaCl, pH 7.8) or with calcium enriched saline (0.01 mol l^{-1} $CaCl_2$) at a rate of 1.0 ml h^{-1} for 24 h. At the conclusion of the experimental period, blood samples were collected for plasma Ca^{2+} measurements. Blood samples were immediately centrifuged (12 000 g for 1 min) and the plasma was collected and stored at -20° C until analysis. Plasma [Ca^{2+}] was determined by flame emission spectrophotometry (Varian, model Spectra AA 250 Plus).

Each animal was euthanized by a sharp blow to the head and tissue samples were collected as previously described.

Effect of increased plasma cortisol levels

Fish (60 – 207 g) were anesthetized and implanted with 5 ml kg^{-1} of either cocoa butter (sham) or with cocoa butter containing cortisol (22 mg hydrocortisone 21-hemisuccinate ml^{-1} cocoa butter). The fish were returned to holding tanks where they were kept for 5 days without disturbance. Following the experimental period, fish were quickly collected, euthanized and sampled as previously described. Blood samples were also collected for cortisol measurements. These samples were centrifuged (12 000 g for 1 min) and the plasma was collected rapidly, frozen in liquid N_2 and stored at -80° C until analysis using a commercial RIA kit (ICN pharmaceuticals).

Effect of 48 h hypercapnia

Fish (205 - 327 g) were placed into individual opaque acrylic boxes with continuous flow of aerated fresh water (13° C) for 24 h prior to the start of the experiment. Following this acclimation period, one group of fish was exposed to flowing water containing 1% CO_2 in air (hypercapnic water PCO_2 = 7.5 mm Hg) while the other group was exposed to normally aerated water for 48 h. The desired level of hypercapnia was achieved by gassing a water equilibration column with appropriate mixtures of CO_2 in air (Cameron gas mixer). The PCO_2 of the water exiting the column was continuously monitored using a PCO_2 electrode (Cameron Instruments) connected to a blood gas meter (Cameron Instruments). The PCO_2 electrode was calibrated using solutions of water (13° C) equilibrated with mixtures of 0.5 or 1.0% CO_2 achieved using the Cameron gas mixer. The final PCO_2 of the water exiting the column was controlled by adjusting the flows of gas and water and the percentage of CO_2 gassing the column. At the conclusion of the

exposure period, fish were euthanized and samples were collected as previously described.

Real time PCR analysis

Tissue samples were powdered under liquid N_2 using a mortar and pestle. Tissue total RNA was extracted from 30 mg of tissue using Invitrogen TRIZOL Reagent. All procedures were followed as per the manufacturer's instructions with the following modifications. No more than 30 mg of powdered tissue was used per 1 ml of TRIZOL and following the re-suspension of the total RNA in 100 µl of nuclease-free water, the RNA was re-extracted using 1 ml of TRIZOL by repeating the entire procedure. The RNA was finally re-suspended in 30 µl of nuclease-free water.

Reverse transcription was performed using Stratascript Reverse Transcriptase Kit (Stratagene). Complementary DNA was synthesized as per kit manufacture's instructions with the following changes. Final reaction volume was adjusted to 12.5 µl, while 0.5 µg of total RNA was used with 0.15 µg of random hexamer primers.

Real time polymerase chain reaction was performed using a MX 4000 Multiplex Quantitative PCR System (Stratagene) using a Brilliant SYBR Green QPCR Master Mix (Stratagene) as per the instructions of the manufacturer with the following modifications. The total reaction volume was reduced to 25 µl; 0.5µg of cDNA template was used and primer concentrations were 0.150 nmol l^{-1} for each primer. All primers (see below) were designed and optimized for the following PCR reaction conditions: 15 min at 95° C, 45 cycles of 30 sec at 95° C, 30 sec at 60° C and 30 sec at 72° C. At the end of each run a dissociation curve was established to determine the purity of the amplicons in each

reaction. Those samples exhibiting more than 1 dissociation peak (indicative of multiple products) were eliminated. Control samples (diluted RNA samples) were examined at random to test for the presence of genomic DNA contamination.

Primers for real time PCR:

ECaC-QPCR1 FWD	5'-GGACCCTTCCATGTCATTCTTATT-3'
ECaC-QPCR2 REV	5'-ACAGCCATGACAACTGTTTCC-3'
ß-actin FWD	5'-CCAACAGATGTGGATCAGCAA-3'
ß-actin REV	5'-GGTGGCACAGAGCTGAAG GGTA-3'

Tissue preservation and immunocytochemistry

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

Sections were incubated *in situ* (3 X 5 min) with a blocking buffer containing 2% normal goat serum, 0.1 mol 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 two 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) or trout ECaC (1:200). For ECaC, custom polyclonal antibodies were raised in rabbit (Abgent, San Diego) against an 18 amino acid region (SQFRFRLQNRKGWKEMLD) of rainbow trout ECaC protein. This region corresponded to amino acids 18 through 36 (see Shahsavarani et al., 2006; Chapter 2). For negative controls, sections were incubated with blocking buffer lacking primary antibodies, with pre-immune serum (ECaC) or with antibodies pre-absorbed with excess peptide antigen (ECaC). 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 slides were then washed (3 X 5 min) in 0.1 mol l^{-1} PB. For double immunofluorescence staining, the trout anti-rabbit ECaC was detected with a 1:400 dilution of Alexa 488-coupled goat anti-rabbit IgG (Fisher) and α5 was detected with a 1:400 dilution of Alexa 546-coupled to goat anti-mouse IgG (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 and mounted with a mounting medium (Vector Laboratories) containing 4',6'-diamidino-2-phenylindole (DAPI) to stain nuclei.

Specimens were observed and photographed using a Zeiss Axiophot light microscope and a Hamamatsu C5985 chilled CCD camera. Images were captured using Metamorph v4.01 imaging system.

Western blotting

Proteins were prepared from frozen tissues by homogenization on ice in 1 ml of extraction buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium

deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS and protease inhibitor cocktail (Roche). The samples were incubated on ice for 10 min and briefly sonicated to break up any DNA that might have been extracted. The samples were centrifuged at 14 000g for 10 min at 4° C and the supernatants were stored at -80° C before use. Protein concentrations were determined using a micro bicinchoninic acid protein assay (Pierce) using BSA as standard. Samples (50 µg protein) were size fractionated by reducing SDS-PAGE using 7% separating and 5% stacking polyacrylamide gels and transferred to nitrocellulose membranes (BioRad). After transfer, each membrane was blocked for 1 h in TBS-T (1X PBS, 0.1% Tween 20), 5% milk and probed with a dilution of 1:3000 rabbit anti-trout ECaC overnight at 4° C. The membranes were then probed for 1 h at room temperature with 1:4000 goat anti-rabbit antibody (Pierce). After each exposure to antibody, the membranes were washed 3 X 5 min in TBS-T. The specific bands were detected by enhanced chemiluminescence (ECL; Pierce SuperSignal West Pico Chemiluminescent Substrate), and blots were exposed to Kodak X-Omat, Blue XB-1 film (Fisher). The protein size marker used was obtained from Fermentas Life Sciences. To demonstrate specificity of the trout ECaC antibody, primary antiserum was combined with excess (20 µg) of the peptide against which the antibody was raised. Additional negative controls included incubating blots with blocking buffer lacking antibodies or with pre-immune serum.

To assess for equal loading, blots were stripped using Re-Blot Plus mild stripping solution (CHEMICON). The blot was incubated in 1X stripping solution for 20 min at room temperature and then rinsed for 10 minutes in PBST. Following rinsing, the blot was then blocked twice in 5% PBST-milk for 10 min each. The blot was then probed

with an anti-β-tubulin antibody (1:1000, Sigma-Aldrich Canada Ltd) for one hour at 37°C. The blot was then incubated in anti-mouse Ig, horseradish peroxidase (1:5000) for one hour at room temperature. Following additional washings, the proteins were visualized using enhanced chemiluminescence (ECL; Pierce SuperSignal West Pico Chemiluminescent Substrate) as above.

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

Statistical analysis

Statistical analysis was performed using Sigma Stat (Version 2.03, SPSS Inc. Chicago). One way Analysis of Variance (One Way ANOVA) was used to determine the effect of environmental calcium and cortisol implant on ECaC expression. In all other experiments the Student's t-test was used; significance was set at $P < 0.05$.

Results

Localization of ECaC in the gill

Figure 3.1 depicts the typical pattern of ECaC expression in the trout gill epithelium that was observed throughout the course of this study. ECaC was localized to the apical membranes of lamellar pavement cells as well as to cells expressing Na^+/K^+-ATPase (presumed to be MRCs). Interestingly, not all of the cells expressing Na^+/K^+-ATPase co-expressed ECaC (Figure 3.1A). Pre-absorption of the primary antibody with excess peptide antigen eliminated nearly all fluorescence signal (Figure 3.1B). On a western blot (Figure 3.1C), the ECaC antibody recognized a single immunoreactive band at 90 kDa; the band was not observed after pre-absorption with peptide antigen.

Effect of environmental calcium

Real time PCR analysis of rainbow trout gill tissue from fish exposed to low (20 - 40 nmol l^{-1}), normal (200 - 300 nmol l^{-1}) or high (2 - 2.5 mmol l^{-1}) environmental Ca^{2+} levels revealed a significant 10-fold increase in ECaC mRNA expression in fish exposed to low Ca^{2+} when compared to the control fish (Figure 3.2A). ECaC protein levels also were significantly increased in the fish exposed to low levels of Ca^{2+} but unaltered in the animals kept in high Ca^{2+} water (Figure 3.2A). Immunocytochemical analysis of gill sections indicated increased amounts of ECaC protein at the tips of the lamellae with an apparent increase in Na^+/K^+-ATPase rich cell population at the base of the lamellae (Figure 3.2B). While not quantified, there also appeared to be a reduction in the intensity and extent of ECaC fluorescence and in the numbers of Na^+/K^+-ATPase enriched cells in the fish kept in high Ca^{2+} water (Figure 3.2D).

Figure 3.1. Co-localization of epithelial calcium channel (ECaC), sodium potassium ATPase (Na^+/K^+-ATPase) and nuclei in rainbow trout (*Oncorhynchus mykiss*) gill. The cell nuclei appear as blue, ECaC appears as green and Na^+/K^+-ATPase appears as red. Yellow represents co-localization of ECaC and Na^+/K^+-ATPase. Panel A is a representative image of gill lamellae; note that most Na^+/K^+-ATPase -positive cells exhibit apical ECaC immunoreactivity (asterisks). Additionally, some cells appeared to exclusively express ECaC (arrows) or Na^+/K^+-ATPase (arrowheads) while other cells appeared to lack either protein. Panel B is a representative image of a gill section pre-absorbed with ECaC peptide antigen (blocking peptide). The scale bars in panels A and B represent 20 μm. Panel C is a representative western blot showing the presence of a single immunoreactive band at 90 kDa in trout gill (lane 1) that was not observed when the antibody was preabsorbed with excess blocking peptide (lane 2).

Figure 3.1

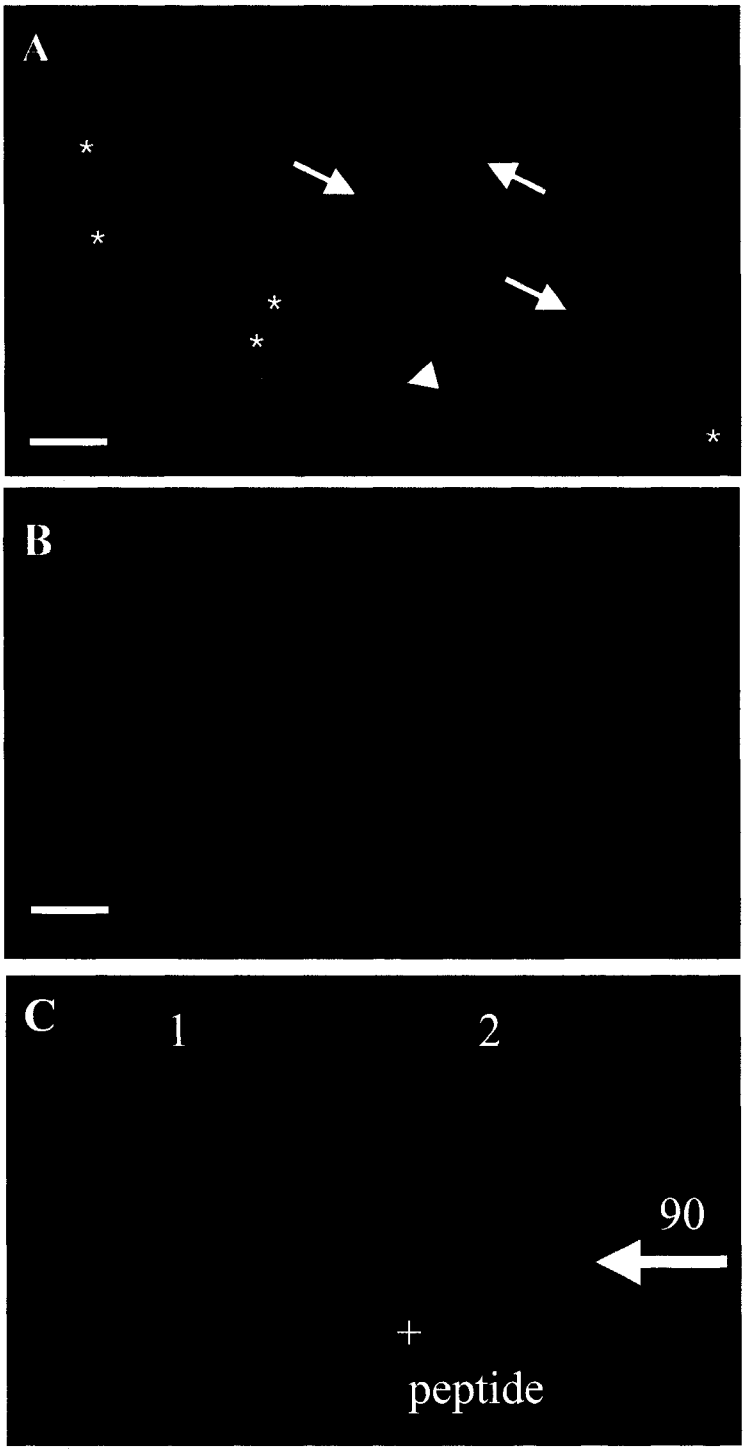


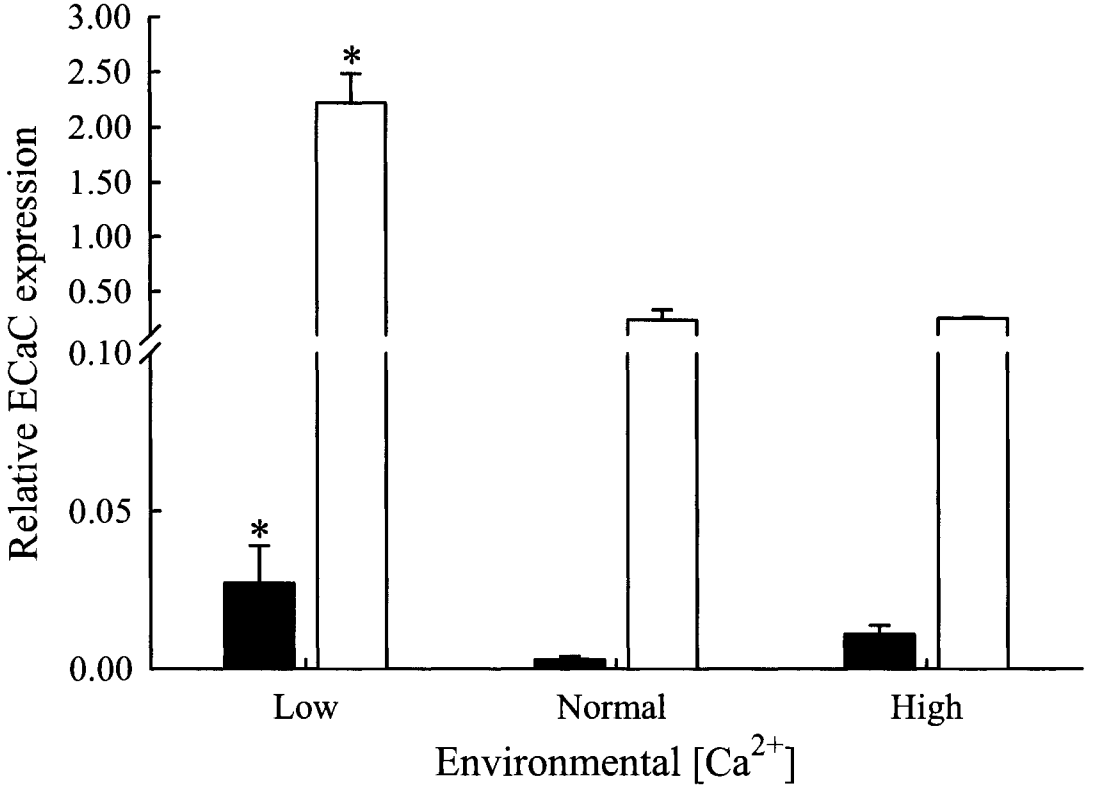
Figure 3.2. Effects of 5-day exposure to low (20 - 40 nmol l^{-1}), normal (200 – 300 nmol l^{-1}) or high (2 - 2.5 mmol l^{-1}) environmental calcium levels on (A) epithelial calcium channel (ECaC) mRNA (filled bars) and protein (unfilled bars) expression or (B – F) distribution of ECaC and Na^+/K^+-ATPase immunoreactivity in rainbow trout (*Oncorhynchus mykiss*) gills.

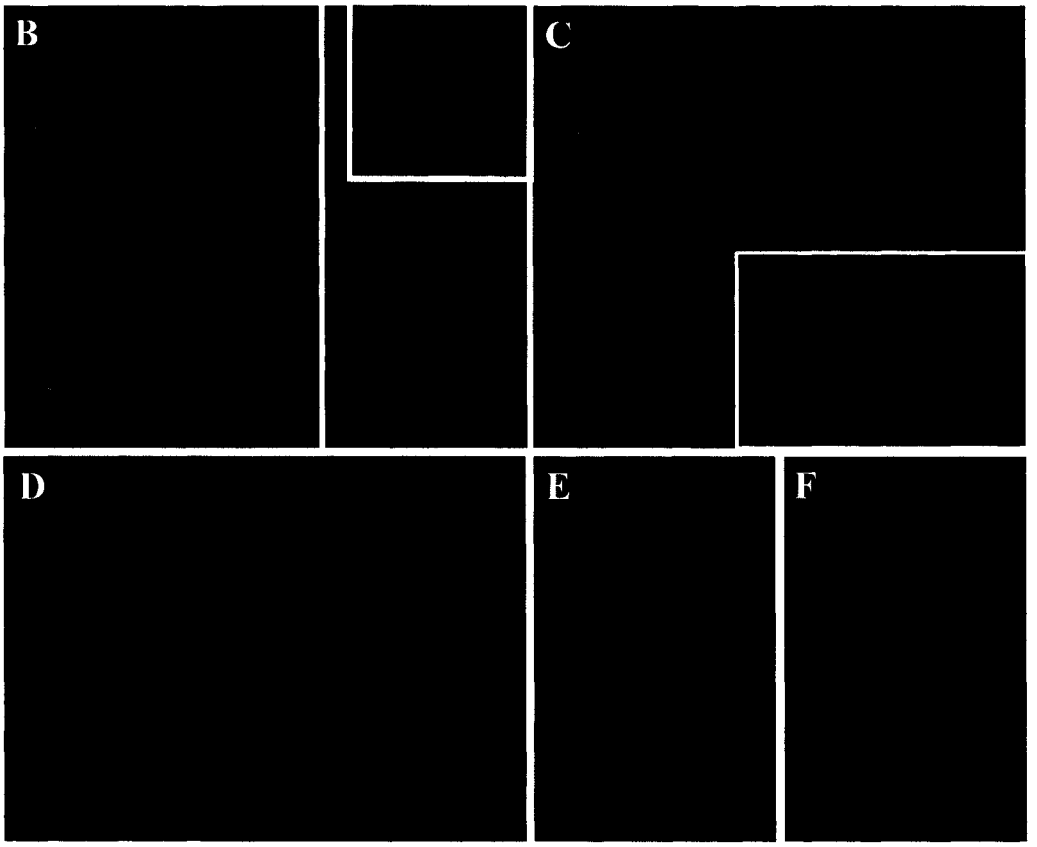
(A) Results for mRNA (N = 6 for each treatment) are presented relative to β-actin expression within the given sample. Densitometry results for protein (N = 5 for controls, N = 6 for low Ca^{2+}) were normalized to the reference protein, β-tubulin. All data are expressed as means ± 1 SEM. Significant differences from the normal Ca^{2+} (control) group are indicated by asterisks ($P < 0.05$; one way ANOVA for mRNA; Student's t-test for protein).

(B – F) Co-localization of ECaC, Na^+/K^+-ATPase and nuclei following (B) 5-day exposure to low (20 - 40 nmol l^{-1}), (C) normal (200 - 300 nmol l^{-1}) and (D) high (2 - 2.5 mmol l^{-1}) environmental calcium levels. The cell nuclei appear as blue, ECaC appears as green and Na^+/K^+-ATPase appears as red. Yellow represents co-localization of ECaC and Na^+/K^+-ATPase. All photos were taken with the same exposure times and adjusted identically for contrast and brightness. However, the image in panel B is the superposition of two pictures in which the intensity of green signal was significantly reduced in the right panel (distal filaments) to produce a more uniform pattern of fluorescence intensity. The top right square is enlarged in panels E and F. Panel E shows an enlarged and enhanced image of the tip of a lamella in which the cells expressing high levels of ECaC appear to be enlarged. Panel F is the same image digitally adjusted to

more clearly show the stained nuclei. The inset in panel C depicts a section in which ECaC primary antibody was omitted.

Figure 3.2


3.00
2.50
2.00
1.50
1.00
0.50
0.10
0.05
0.00
Relative ECaC expression
*
*
Low
Normal
High
Environmental [Ca2+]


B
C
D
E
F

Effect of intravascular calcium infusion

Fish infused with $CaCl_2$ for 24 h exhibited a 2.5 fold increase in plasma Ca^{2+} levels (from 3.31 ± 0.24 to 8.11 ± 0.74 mmol l^{-1}). Gene expression analysis revealed a significant decrease in ECaC mRNA expression after 24 h of infusion yet protein levels were unchanged (Figure 3.3).

Effect of cortisol treatment

Fish treated with cortisol implants exhibited an approximate 50-fold increase in plasma cortisol levels when compared to the control (untreated) fish (116.2 ± 32.2 *versus* 2.3 ± 0.9 ng ml^{-1}). The sham treated fish (cocoa butter only) also demonstrated high plasma cortisol levels (94.4 ± 19.0 ng ml^{-1}) and thus the data from these fish were not included in the analysis. The fish given cortisol implants displayed significant 3-fold increases in relative ECaC mRNA expression and protein levels in relation to the control fish (Figure 3.4A). Qualitatively, the gills of cortisol-treated fish possessed greater numbers of Na^+/K^+-ATPase rich cells especially on lamellae; the majority of these cells appeared to express apical ECaC (Figure 3.4B).

Figure 3.3. The effects of 24 h of intra-arterial infusion of $CaCl_2$ on epithelial calcium channel (ECaC) mRNA (filled bars) and protein (unfilled bars) expression in rainbow trout (*Oncorhynchus mykiss*) gills.

Results for mRNA (N = 4 for each treatment) are presented relative to β-actin expression within the same sample. Densitometry results for protein (N = 4 for each treatment) were normalized to the reference protein, β-tubulin. All data are expressed as means ± 1 SEM. An asterisk denotes a statistical difference from the control (infused with 140 mmol l^{-1} NaCl) group ($P < 0.05$; Student's t-test). NS = not significant.

Figure 3.3

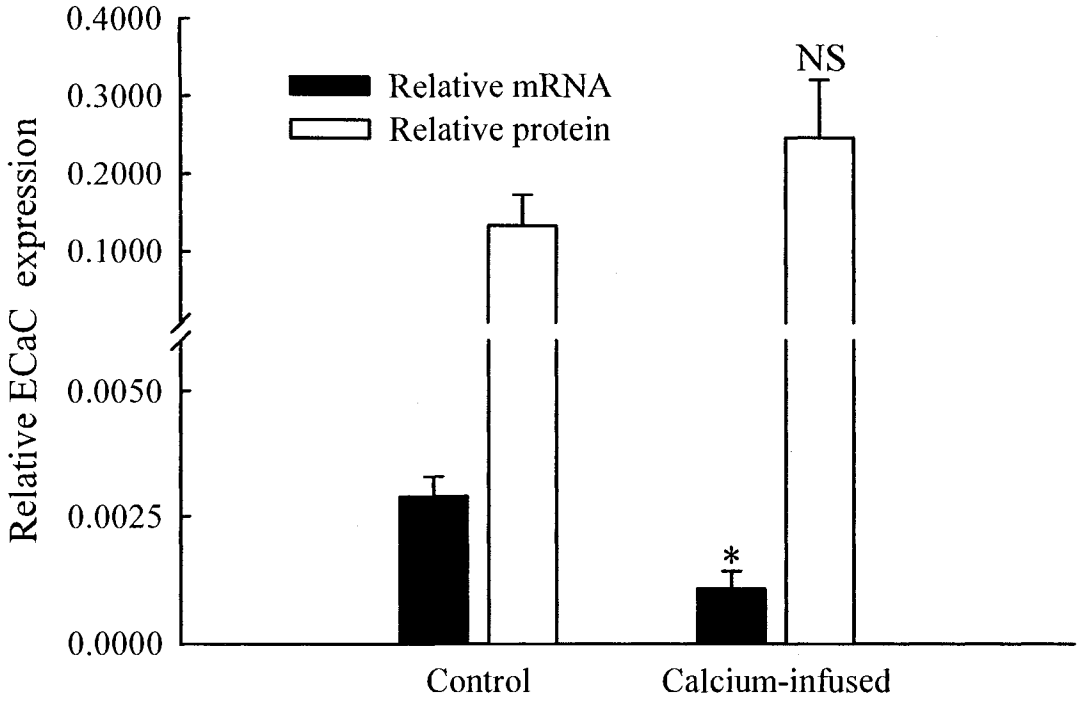

0.4000
0.3000
0.2000
0.1000
0.0050
0.0025
0.0000
Relative ECaC expression
Relative mRNA
Relative protein
NS
*
Control
Calcium-infused

Figure 3.4. The effect of increased plasma cortisol concentrations on (A) epithelial calcium channel (ECaC) mRNA (filled bars) and protein (unfilled bars) expression or (B and C) distribution of ECaC and Na^+/K^+-ATPase immunoreactivity in rainbow trout (*Oncorhynchus mykiss*) gills.

Animals were implanted for 5 days with cocoa butter containing cortisol and compared with fish containing no implant.

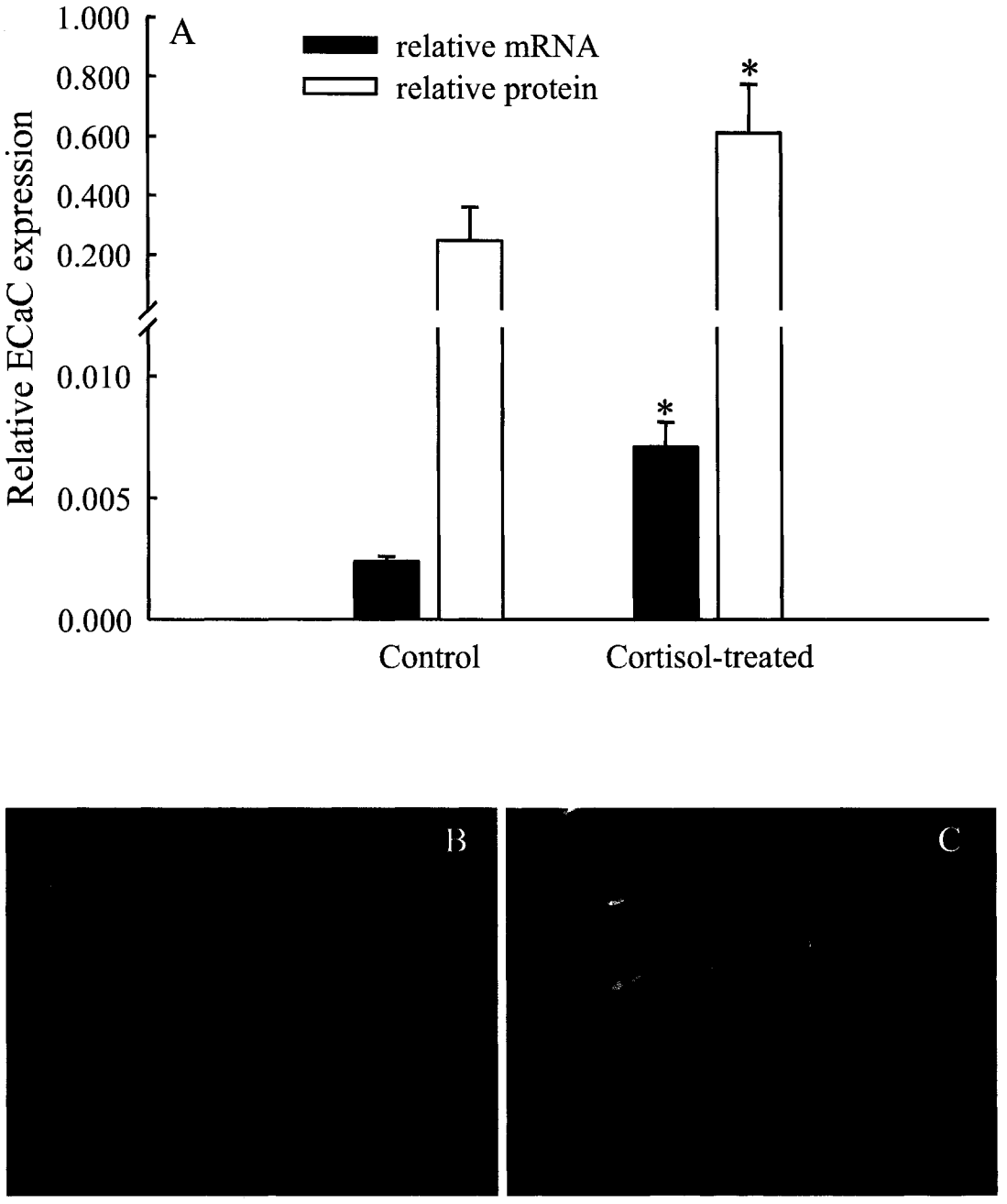
(A) Results for mRNA (N = 7 for each treatment) are presented relative to β-actin expression within the same sample. Densitometry results for protein (N = 7 for each treatment) were normalized to the reference protein, β-tubulin. All data are expressed as means ± 1 SEM. Significant differences from the control group are indicated by asterisks ($P < 0.05$; Student's t-test).

(B) Co-localization of ECaC, Na^+/K^+-ATPase and nuclei in fish with no cortisol implant.

(C) Co-localization of ECaC, Na^+/K^+-ATPase and nuclei in fish following 5 days of treatment with cortisol implant.

The cell nuclei appear as blue, ECaC appears as green and Na^+/K^+-ATPase appears as red. Yellow represents co-localization of ECaC and Na^+/K^+-ATPase. All photos were taken with the same exposure times and adjusted identically for contrast and brightness.

Figure 3.4


A
relative mRNA
relative protein
Relative ECaC expression
1.000
0.800
0.600
0.400
0.200
0.010
0.005
0.000
*
*
Control
Cortisol-treated
B
C

Effect of hypercapnia

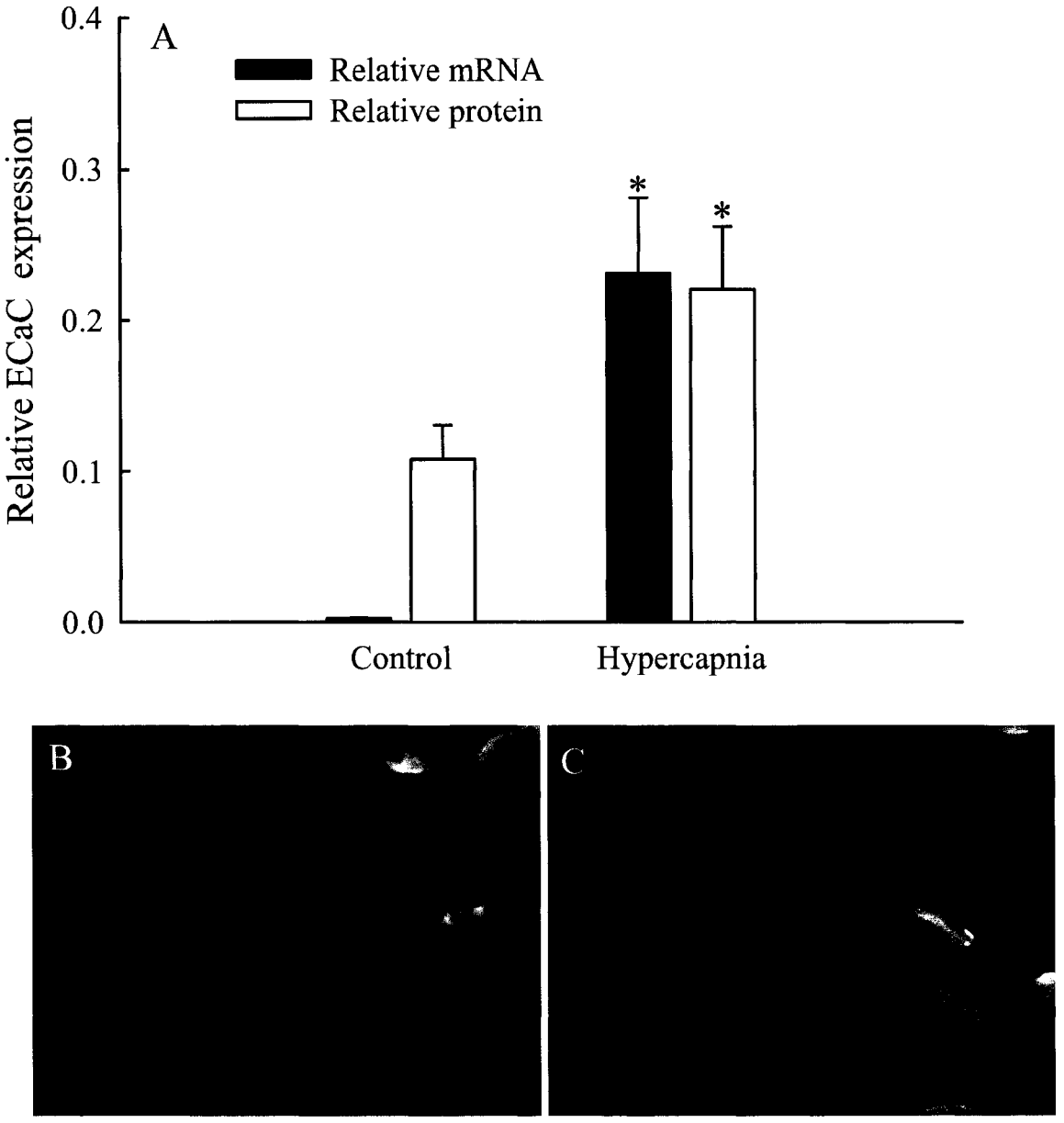
Exposure of fish to hypercapnia (approximately 7.5 mm Hg) for a period of 48 h significantly increased ECaC mRNA expression by approximately 100-fold (Figure 3.5A) while ECaC protein levels were increased only by 2.1 fold. Immunocytochemical analysis of gill sections revealed a striking increase in the intensity of apical ECaC expression after hypercapnia on both Na^+/K^+-enriched MRCs and lamellar pavement cells (Figure 3.5 B, C).

Figure 3.5. The effects of 48 h hypercapnia (N = 6) on (A) epithelial calcium channel (ECaC) mRNA (filled bars) or protein (unfilled bars) expression or (B – C) distribution of ECaC and Na^+/K^+-ATPase immunoreactivity in rainbow trout (*Oncorhynchus mykiss*) gills.

(A) Results for mRNA (N = 6 for each treatment) are presented relative to β-actin expression within the same sample. Densitometry results for protein (N = 4 for each treatment) were normalized to the reference protein, β-tubulin. All data are expressed as means ± 1 SEM. Significant differences from the control group are indicated by asterisks ($P < 0.05$; Student's t-test).

(B) Co-localization of ECaC, sodium potassium ATPase (Na^+/K^+-ATPase) and nuclei. The cell nuclei appear as blue, ECaC appears as green and Na^+/K^+-ATPase appears as red. Yellow represents co-localization of ECaC and Na^+/K^+-ATPase.

Figure 3.5


A
Relative mRNA
Relative protein
Relative ECaC expression
0.4
0.3
0.2
0.1
0.0
*
*
Control
Hypercapnia
B
C

Discussion

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

The results of real time PCR analyses clearly demonstrated that the levels of ECaC mRNA varied in direct relation to the Ca^{2+} transporting capacity of the gill, increasing in fish exposed to low environmental [Ca^{2+}], elevated plasma [cortisol] or hypercapnia while decreasing in fish experiencing experimentally induced hypercalcemia. Except for the fish experiencing hypercalcemia there was a matching change in ECaC protein levels associated with the mRNA changes. The changes in protein levels were not always of the same magnitude as the mRNA changes.

The results of the immunocytochemistry, while not quantitative, suggest that the altered levels of ECaC detected in this study reflected modifications of ECaC protein within both chloride cells and pavement cells.

Low environmental Ca^{2+} levels

After exposure of trout to low environmental Ca^{2+} concentrations for 5 days, ECaC gene expression increased by approximately 10 fold while protein levels were increased by 2.5 fold. Adult zebrafish, exposed to similar experimental conditions, exhibited a similar response in ECaC gene expression (A. Shahsavarani and S.F. Perry; unpublished data). Additionally, Pan et al. (2005) recently demonstrated that exposure of zebrafish embryos to water containing low levels of Ca^{2+} (0.02 mmol l^{-1}) caused an increase in ECaC

mRNA expression in gills and skin. Thus, ECaC clearly is being affected (directly or indirectly) by environmental Ca^{2+} levels and thus suggests that the increased capacity of the trout gill (Perry and Wood 1985) or zebrafish embryo (Pan et al. 2005) to absorb Ca^{2+} after exposure to softwater may indeed reflect increased numbers of apical membrane Ca^{2+} channels.

The results of previous studies (Marshall et al. 1992; McCormick et al. 1992) provided evidence that gill mitochondria rich cells are involved in Ca^{2+} uptake. Based on these and other indirect or correlative studies (Ishihara and Mugiya 1987; Payan et al. 1981; Perry et al. 1992; Perry and Wood 1985), a model was constructed (Perry and Flik 1988) in which the MRC was implicated as the principal (potentially exclusive) cell type responsible for Ca^{2+} uptake. In the present study, however, immunocytochemical analysis of gill cross sections in fish exposed to softwater failed to demonstrate strong co-localization of Na^+/K^+ ATPase (representative of MRCs) and ECaC-positive cells (Figure 3.2). Under control conditions, ECaC appeared to be widely distributed to both PVCs and a subset of MRCs. A similar conclusion was reached by Shahsavarani et al. (2006; Chapter 2) upon examination of both gill sections and cultured cells. During exposure to softwater, there appeared to be a shift in the cell types expressing ECaC. Specifically, ECaC appeared to be highly expressed in a sub-population of enlarged cells located at the tips of lamellae (Figure 3.2B). This regional increase in ECaC expression in fish exposed to a low Ca^{2+} environment suggests the presence of a previously unidentified cell type that may be playing a significant role in Ca^{2+} uptake.

Exposure of trout to the varied levels of environmental Ca^{2+} used in the present study could potentially lead to transient or longer-term alterations in plasma Ca^{2+} levels.

Therefore, the changes in ECaC mRNA expression observed in this study could reflect sensing of ambient and/or internal Ca^{2+}. The presence of a Ca^{2+} sensing receptor (CaR) has been demonstrated in the trout gill (Radman et al. 2002) and thus it is conceivable that CaR is involved the sensing of low ambient Ca^{2+} levels and the initiation of events leading to increased transcription of ECaC. Further, it is well established that increased levels of internal Ca^{2+} can be sensed and lead to downstream effects to reduce branchial Ca^{2+} uptake (Lafeber and Perry 1988). Clearly, further work will be required to elucidate the relative importance of external *versus* internal changes in Ca^{2+} in promoting transcriptional changes to ECaC expression.

Various studies have clearly demonstrated an increase in MRC surface area (through cellular enlargement as well as cellular proliferation) with exposure to softwater (Greco et al. 1996; Perry and Wood 1985). Similar results were noted in the present study (compare Figure 3.2B and C). The more important observation, however, was that the MRCs in the softwater fish did not display any obvious increase in ECaC expression. Although the levels of ECaC mRNA and protein were increased in softwater fish, there was no reduction in ECaC mRNA or protein associated with exposing fish to Ca^{2+}-enriched water. It has been established previously (Perry and Wood 1985) that maximal Ca^{2+} transport capacity (J_{MAX}) is reduced in trout exposed to high ambient Ca^{2+}. Thus, the absence of any regulation of ECaC mRNA (and presumably protein) in these fish suggest that the reduced Ca^{2+} uptake reflects non-transcriptional control of ECaC or modulation of another component of the overall transepithelial Ca^{2+} absorption process (basolateral PMCA or NCX).

Hypercalcemia

Infusing fish with Ca^{2+}-enriched saline for 24 h resulted in a marked elevation of plasma Ca^{2+} levels and a concomitant reduction in ECaC mRNA levels. Because ambient Ca^{2+} concentration was unchanged, the results provide strong evidence for an internal Ca^{2+} sensing mechanism linked to transcriptional control of ECaC. This mechanism may be similar to the one proposed to initiate the release of the hypocalcemic hormone, stanniocalcin (STC), in which the CaR is thought to be involved (Radman et al. 2002). The release of STC from the corpuscles of Stannius during acute hypercalcemia is a critical mechanism leading to rapid reductions in the rate of branchial Ca^{2+} uptake (Fenwick and Brasseur 1991; Lafeber and Perry 1988; Perry et al. 1989; Wagner et al. 1997) thought to involve modification of Ca^{2+} conductance though existing Ca^{2+} channels. The results of the present study demonstrate that chronic hypercalcemia may lower Ca^{2+} uptake by an additional mechanism, a reduction in the number of epithelial Ca^{2+} channels. However, because the reduced levels of ECaC mRNA were not accompanied by a reduction in ECaC protein levels, it is possible that a longer period of hypercalcemia is required for transcriptional changes to significantly impact protein levels.

Elevated plasma cortisol levels

Plasma cortisol levels have been shown to increase in fish exposed to softwater (Flik and Perry 1989; Perry and Laurent 1989; Perry and Wood 1985; Sloman et al. 2001). Owing to the hypercalcemic action of cortisol (Flik and Perry 1989; Perry and Wood 1985), this response presumably helps to maintain Ca^{2+} homeostasis in softwater environments. The hypercalcemic actions of cortisol have been attributed to increased branchial Ca^{2+} uptake associated with MRC proliferation (Laurent and Perry 1990) and increased activity of the basolateral plasma membrane Ca^{2+}-ATPase (PMCA) (Flik and Perry 1989). In this study, we present evidence for an additional mechanism underlying the hypercalcemic effects of cortisol, which involves a transcriptional increase in ECaC. The increased levels of ECaC appeared to be a direct result of MRC proliferation. Thus, although the apparent expression of ECaC per MRC did not change, the numbers of MRCs expressing ECaC was increased by cortisol treatment. It is possible, therefore, that the increased rates of Ca^{2+} uptake observed (Flik and Perry 1989) after cortisol treatment *in vivo* reflects the combined effects of increased PMCA activity and a greater number of apical membrane Ca^{2+} channels.

Hypercapnia

Exposure of trout or bullhead (*Ictalurus nebulosus*) to hypercapnia has been shown to cause a re-modeling of the gill epithelium whereby the surface area of MRCs exposed to the water is markedly reduced owing to their apparent covering by neighboring PVCs (Goss et al. 1992a; 1992b; 1994; Goss and Perry 1993;). Assuming that the MRCs are the predominant sites of Ca^{2+} uptake one would expect hypercapnia

exposure to cause a reduction in the rate of Ca^{2+} uptake because MRC apical surface area is being reduced. Indeed, this prediction was tested by MacKenzie and Perry (1997b) who exposed rainbow trout to hypercapnia while monitoring branchial and renal Ca^{2+} fluxes. Despite a 68% reduction in the surface area of exposed MRCs, the hypercapnic trout in that study (MacKenzie and Perry 1997b) actually exhibited a significant increase in the rate of Ca^{2+} uptake owing to an increase in maximal transport capacity (J_{MAX}). The increase in J_{MAX} for Ca^{2+} uptake was not associated with any changes PMCA because the ATP-dependent Ca^{2+}-transporting capacity of basolateral membrane vesicles was unaffected by hypercapnia. Instead, it was suggested that the increased rates of Ca^{2+} uptake in hypercapnic fish might reflect modification of apical membrane Ca^{2+} channels. The results of the present study provide compelling evidence that the mechanism underlying the increased rates of Ca^{2+} uptake observed by MacKenzie and Perry (1997b) in hypercapnic trout is transcriptional up-regulation of branchial ECaC. It is possible that the number of Ca^{2+} channels on MRC apical membranes is being increased to enhance the transporting capacities of cells still exposed to the water. Alternatively, the numbers of channels may be increasing on PVCs to compensate for the loss of exposed MRC surface area. Given the widespread cellular localization of ECaC (e.g Figure 3.1) and the likelihood that both MRCs and PVCs are involved in Ca^{2+} uptake (Shahsavarani et al., 2006; Chapter 2), it is probable that ECaC expression is being increased in both cell types during hypercapnia. Although it is not possible to distinguish between exposed and covered MRCs from light micrographs, the results of the immunocytochemistry suggest that ECaC expression is increasing in a subset of both MRCs and PVCs.

Summary and Perspectives

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

Acknowledgements

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CHAPTER 4

GENERAL DISCUSSION

The research presented in this thesis focused on the identification, distribution and regulation of the rainbow trout (*Oncorhynchus mykiss*) epithelial calcium channel (ECaC). Indeed, without functional expression data, it cannot be unequivocally concluded that the trout ECaC that was cloned and characterized in this thesis functions as a calcium channel. However, the presence of conserved domains, its cellular distribution and its physiological response to calcium challenges (see below) strongly suggest that rtECaC is in fact an epithelial calcium channel.

Although trout ECaC is similar to other epithelial calcium channels it does not appear to group with the mammalian TRPV5 and TRPV6 subfamilies. Distribution and cellular localization of the channel does confirm that the channel is primarily present at the gills. The findings reveal a broad cellular distribution within the gill epithelium thus suggesting that cells other than the MRCs may also be playing a role in Ca^{2+} uptake. ECaC gene expression was clearly influenced by environmental as well as physiological challenges yet in accordance with its broad distribution, the changes were not restricted to MRCs.

The trout ECaC coding region is composed of 2184 base pairs (coding for 724 amino acids). The presence of conserved domains such as multiple ankyrin sites, ion transport region, 6 transmembrane domains as well as multiple phosphorylation sites primarily located in the C-terminus intracellular region of the protein, strongly suggests similarities between trout ECaC and mammalian TRPV5 (transient receptor protein, vanilloid receptor subtype 5) and TRPV6 subfamilies of calcium channels.

Phylogenetic analysis using Neighbor Joining analysis with bootstrap support (as well as Maximum Likelihood analysis) indicates that trout ECaC, much like other

lower vertebrate epithelial calcium channels, is distinctly different from the mammalian TRPV5 and TRPV6 subfamilies. However, within the TRP family of genes, trout ECaC (much like other lower vertebrate ECaC) are more closely related to the TRPV5 and TRPV6 subfamilies than to any of the other subfamilies. In *homo sapiens*, TRPV5 and TRPV6 are both located in close proximity to one another on chromosome 7q35 (Muller et al. 2000b; Muller et al. 2000a). This suggests a recent gene duplication that has given rise to the two subfamilies (Muller et al. 2000a; 2000b). Extensive examination of the zebrafish (*Danio rerio*) genome has proven to be unsuccessful at identifying more than one ECaC gene (located on chromosome 16). These findings in conjunction with the phylogenetic analysis presented here suggests that unlike in mammals, the epithelial calcium channel is perhaps found in only one form in the rainbow trout, the zebrafish and perhaps other fish species. Qiu and Hogstrand (2004) were also able to only identify 1 form of ECaC in their analysis of the pufferfish (*Fugu rubripes*) genome. One can speculate that changes that appear to have occurred in the epithelial calcium channel may have effectively been the result of a move from the aquatic environment to the terrestrial environment. Furthermore, given that neither the avian nor the amphibian epithelial calcium channels appear to belong to TRPV5 or TRPV6 subfamilies one may venture to suggest the gene duplication event giving rise to TRPV5 and TRPV6 occurred after the separation of the mammalian ancestral form from that of the lower vertebrates.

In mammals, TRPV5 and TPV6 have been shown to have significant tissue distribution profiles (Hoenderop et al. 2001; Muller et al. 2000a; Nijenhuis et al. 2005; 2003; Song et al. 2003; van Abel et al. 2003). In contrast to the situation in mammals where Ca^{2+} is acquired from diet (Bindels 1993; Flik and Verbost 1993; Perry 1997;

Perry and Flik 1988), fish obtain their Ca^{2+} directly from the environment through the gills (Bindels 1993; Flik and Verbost 1993; Perry 1997; Perry and Flik 1988). In aquatic environments where calcium levels are extremely low, fish may rely more significantly on dietary sources of calcium. Regardless, the environment can still provide an almost limitless supply of calcium. The relative abundance of calcium may significantly lessen the possibility of fish facing sporadic availability of calcium, a condition faced by mammals or other organisms relying solely on dietary calcium.

Mammals are faced with the requirements of having to possess an optimal Ca^{2+} absorption mechanism at the intestinal tissue as well as a closely regulated Ca^{2+} reabsorption mechanism at kidney. Although these two mechanisms may appear to be similar in function, the physical and chemical environments in which they function are different. In fish, it may very well be that calcium loss is not a major factor under normal circumstances and that as long as the uptake mechanism meets physiological needs and a functional (and not necessarily fine tuned) reabsorption mechanism exists at the kidney, calcium homeostasis can be maintained. The need for a tighter control of calcium uptake and regulation (more specifically the loss of calcium) may have been the effective evolutionary pressure that kept the duplicated gene in mammals with its resulting tissue distribution.

In trout, ECaC was primarily detected in the gills using both RT-PCR and RT-QPCR techniques (10 fold higher expression in the gills as compared to any other tissue examined). The putative Ca^{2+} uptake model contends that the MRCs (that constitute roughly 10% of the gill epithelium) are largely responsible for whole body Ca^{2+} uptake. Using immunohistochemistry and *in-situ* hybridization techniques, it was shown that

ECaC distribution is more extensive than predicted by this model. Analysis of tissue cross-sections, dispersed cells as well as primary cell cultures revealed that not all ECaC expressing cells were MRCs and that not all MRCs expressed ECaC. Using a Na^+/K^+ ATPase antibody and oligonucleotide probe as markers for MRCs, it was demonstrated that, under normal conditions, the majority of cells expressing ECaC were either expressing Na^+/K^+ ATPase at very low levels or that they were not expressing them at all. Furthermore, MRCs are normally distributed within the inter-lamellar regions or at the base of lamellae whereas ECaC expressing cells tended to be more widely dispersed. These findings suggest that the Ca^{2+} uptake model should be revised with respect to the cellular distribution of the Ca^{2+} uptake mechanism. Given that MRCs comprise less than 10% of the entire gill cell population and that the evidence presented in this thesis suggests a wider distribution of ECaC, it should not be surprising that under normal conditions a larger number of ECaC positive cells did not co-express Na^+/K^+-ATPase and these were likely PVCs. These findings do not necessarily mean that the PVCs are the primary sites of calcium uptake. They simply demonstrate that under normal circumstances PVCs may make up a large proportion of ECaC positive cells.

To further examine the putative Ca^{2+} uptake model, ECaC expression and cellular distribution were monitored following environmental and physiological challenges. Under low environmental Ca^{2+} concentrations, trout ECaC gene expression increased by almost 10 fold. A similar effect was also seen in adult zebrafish. These findings suggest that ECaC may play a role in the increased Ca^{2+} uptake that had previously been observed following softwater exposure. Although gene expression analysis supports predictions of the role that ECaC plays in Ca^{2+} uptake, there does not appear to be a

strong co-localization between Na^+/K^+ ATPase rich cells (representative of MRCs) and ECaC rich cells. In softwater acclimated fish, ECaC appeared to be predominantly present in enlarged cells located at the tip of the lamellae rather than at the inter-lamellar region where MRCs are found. This pattern may therefore be indicative of specialised cells that are needed for increased Ca^{2+} uptake.

During acute hypercalcemia, calcium uptake is reduced at the gill owing to the release of Stanniocalcin, the principal hypocalcemic hormone of fish. Stanniocalcin is believed to influence calcium uptake by reducing the conductance of Ca^{2+} through apical membrane calcium channels. The results of this thesis have revealed a complementary system to that of Stanniocalcin that operates over a longer time scale. Namely, long-term hypercalcemia caused a significant reduction in ECaC mRNA, a response that would presumably reduce the number of apical membrane calcium channels and thus serve to lower the conductance of Ca^{2+} across this membrane. In contrast to Stanniocalcin, vitamin D_3 is thought to have hypercalcemic effects in fish. In mammals, ECaC distribution has been correlated to tissues sensitive to vitamin D_3. However unlike in mammalian studies, daily injection of vitamin D_3 did not cause a significant increase in ECaC expression (results not shown). It is important to note that vitamin D_3 affects TRPV5 and TRPV6 differently with TRPV5 demonstrating greater sensitivity than TRPV6 (Song et al. 2003). In fact, although TRPV5 is also present in renal tissue, it appears to be less sensitive to vitamin D_3 in this tissue (recall that TRPV5 is more prevalent in the intestine) as compared to when it is located in the intestine. Notwithstanding the mammalian results, the lack of significant response to vitamin D_3 treatments is likely due to low dosage. A recent study by C. Hogstrand (personal

communication through Dr. Perry) has clearly demonstrated a significant effect of a higher dose of vitamin D_3 (10 μg kg^{-1}) on levels of ECaC mRNA in juvenile rainbow trout. That result is consistent with the presence of Vitamin D response elements on fish ECaC (Qiu and Hogstrand, 2003). Given that the effect of Vitamin D_3 on ECaC expression in this thesis was just below the level of statistical significance ($P = 0.059$), a re-examination of the effects of vitamin D_3 on ECaC expression would seem warranted. This re-examination should include analysis of gastrointestinal tissue to determine if rtECaC is also differentially sensitive to vitamin D_3 as has been observed in mammal TRPV5.

Other hormones are also known to have varying effects on Ca^{2+} regulation through physiological and/or gill morphological changes. For example, plasma cortisol levels has been shown to increase with exposure to softwater (Flik and Perry 1989; Perry and Laurent 1989; Perry and Wood 1985) in conjunction with an increase in MRC surface area (Laurent and Perry 1991; Sloman et al. 2001). The results of experiments presented here suggest that both MRC surface area as well as ECaC expression increased as a result of increased plasma cortisol levels. However, although these results support the correlative relationship between MRCs and Ca^{2+} uptake, the findings suggest that ECaC expression appears to persist in PVCs following high cortisol treatment. These findings further emphasize the possibility that although MRCs may be playing a significant role in calcium uptake under certain condition, the role of PVCs or other specialised cells must also be considered and that their contribution to the overall calcium uptake mechanism should be quantified.

Proliferation of MRCs, as a result of increased plasma cortisol levels ultimately changes gill morphology and increases the blood-to-water diffusion barrier (Greco et al. 1996). In contrast, hypercapnia reduces MRC surface. Under hypercapnic conditions and with the resulting decrease in MRC apical surface area one would anticipate a reduction in calcium influx. However, MacKenzie and Perry (1997b) demonstrated that calcium influx was actually increased during hypercapnia despite an associated loss in MRC apical membrane surface area exposed to the water. The results of this thesis provide additional support to the findings by MacKenzie and Perry (1997b) by demonstrating a significant increase in ECaC expression after 48 h of hypercapnia. It is possible that the increased expression of ECaC is in response to the reduction of MRC apical surface area and is an attempt at increasing the number of ECaCs per MRC. However, the possibility of an increase in ECaC expression in PVCs is equally plausible. Given the wide distribution of ECaC in both MRCs and PVCs it is conceivable that expression in both cell types was increasing during hypercapnia; indeed, this would appear to be supported qualitatively by the immunocytochemistry results.

As has been previously mentioned, the evidence presented in this thesis strongly suggests that the distribution of ECaC is more widespread in gill epithelial cells than initially anticipated. It has been demonstrated for the first time that ECaC is expressed in a subset of MRCs and PVCs. Although this thesis only sheds new light on the cellular distribution of ECaC, much remains to be done in clearly redefining the Ca^{2+} uptake model in fish. Given the vast body of information implicating MRCs in Ca^{2+} uptake, including the evidences presented in this thesis, MRCs are likely to be playing an

important role. However, given that PVCs can make up over 90% of the gill epithelial cells, their role in overall Ca^{2+} uptake may be significant.

Although the results presented in this thesis strongly suggest a key role for ECaC, particularly as a regulated step in calcium uptake, they do not explain the entire correlative body of evidence suggesting almost exclusivity of calcium uptake to MRCs. The oversimplification of the gill epithelium into 5 cell types (see general introduction) is misleading as it has been shown that MRCs can be subdivided into at least two groups either morphologically (Chang et al. 2001; Perry 1997; Pisam et al. 1987; Sasai et al. 1998; van der Heijden et al. 2001) or by their apical membrane protein composition (Galvez et al. 2002; Goss et al. 2001). The results of this thesis present a new perspective to the characterization or classification of MRCs. It is now apparent that only a subset of MRCs is ECaC positive at their apical membrane. Furthermore, separation of MRCs through cell fractionation has demonstrated that both PNA^- and PNA^+ fractions contain cells expressing ECaC. This finding has been supported by flow cytometry analysis showing that just over 13% of PNA^- cells were co-expressing ECaC and Na^+/K^+ ATPase. Although sufficient cells were not obtained for the analysis of the PNA^+ fraction, it is safe to assume that some co-localization must exist within this fraction. Therefore, based on functionality and particularly calcium transport, MRCs may exist in more than the proposed 2 groups, as the results presented here clearly suggest the possibility of 4 types of MRCs (PNA-/ECaC positive, PNA-/ECaC negative, PNA+/ECaC positive, PNA+/ECaC negative, the last two not having been tested). Furthermore, it is clear that only a subset of PVCs is ECaC positive. The significance of the cell type distribution of ECaC remains to be elucidated. Does this cellular distribution represent a physiological

need for a variable calcium uptake capacity under differing conditions (physiological or environmental) that would be more adequately addresses by PVCs, MRCs or their specific subtypes? Perry and Flik (1988) clearly showed that the inorganic calcium channel blocker lanthanum trichloride ($LaCl_3$) was preferentially (but not exclusively) deposited on MRC apical membrane. This finding clearly demonstrated the difference in electrochemical composition between PVCs and MRCs. The results presented in this thesis do not contradict these findings, however they do suggest that the lower levels of deposition of $LaCl_3$ on PVCs may be related to the presence of ECaC on PVCs but at a lower level relative to MRCs. Furthermore, it would be interesting to determine the extent of $LaCl_3$ deposits on MRCs not exhibiting ECaC expression so as to further clarify the correlation between $LaCl_3$ deposition and cells thought to be actively involved in calcium uptake.

It also remains to be determined which calcium extrusion mechanisms are prevalent at the basolateral membrane of each cell type. It was demonstrated by MacKenzie and Perry (1997b) that the increase in calcium flux following hypercapnia was not the result of a change in the ATP-dependent Ca^{2+}-transporting capacity of basolateral membrane vesicles. This may suggest that the mechanisms involved in calcium extrusion from gill epithelial cells have a greater capacity than that used during basal activity or that a shift from PMCA activity to NCX may take place under certain conditions. Yamauchi et al. (2005) have shown that 1,25-dihydroxyvitamin D3 induces increased gene expression in rat ECaC1, calbindin-D9K and calbindin-D28K but not in ECaC2 or any of the NCXs or PMCAs investigated. However, given the difference in the affinity for Ca^{2+} and the transport capacity of Ca^{2+} between PMCA and NCX, further

analysis is required in determining under which circumstances either of these transport mechanisms may be more significant. It also remains to be seen whether, much like in the mammalian system, ECaC, PMCA and NCX are always co-localized or whether a fraction of ECaC positive cells are lacking or are enriched by one or both of the transporters. Furthermore given the evidence in the mammalian system demonstrating fluctuations of calcium binding proteins such as calbindins, the role of intracellular shuttling of Ca^{2+} during uptake must be more aggressively investigated. Given that NCX has a lower affinity but higher capacity for Ca^{2+} transport, a localized increase in $[Ca^{2+}]_i$ may be the actual driving force under circumstances such as low environmental calcium. By increasing the shuttling capacity (through an increase in calcium binding proteins), $[Ca^{2+}]_i$ can be lowered locally, near the apical membrane (thus near ECaC), which would be required to allow entry of calcium into the cell, and to increase $[Ca^{2+}]_i$ near the basolateral membrane.

Taken together, these results suggest that although the putative Ca^{2+} uptake model accurately presents the gill as the primary site of calcium uptake, it requires further investigation as well as re-formulation with respect to the characterization of cell populations involved. Moreover, there is also strong evidence for the presence of only a single form of ECaC in the trout (and perhaps in other fish such as pufferfish and zebrafish). Further investigations are required to better understand the evolution of ECaC as well as for the overall understanding of the Ca^{2+} uptake mechanism and its various components.

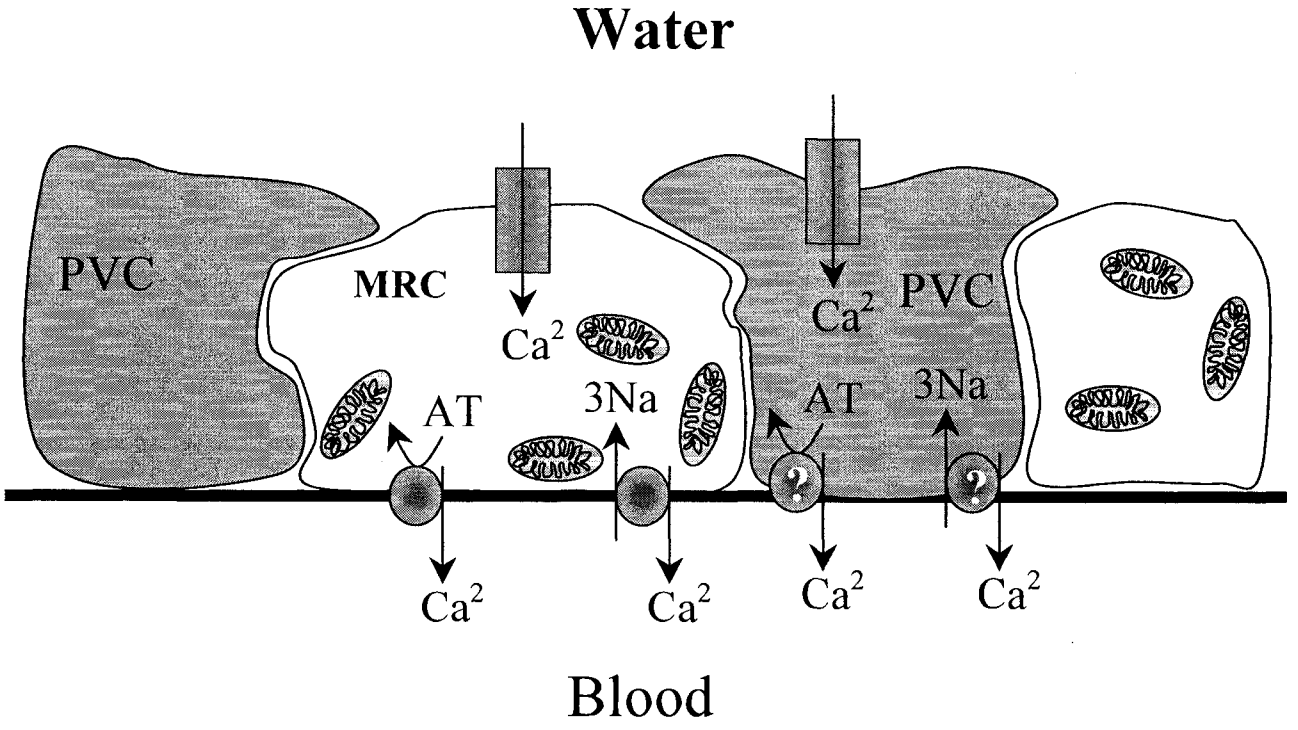
Future Perspectives

The results presented in this thesis shed light on a small component of the overall calcium transport mechanism at the gill epithelium. The original model presented in Chapter 1 (Fig. 1.1) clearly shows that the transfer of calcium from the external to the internal environment requires not only the epithelial calcium channels but also the participation of mechanisms at the basolateral membrane such as the plasma membrane calcium ATPase (PMCA) and the sodium calcium exchanger (NCX). The key contribution of this thesis is that the putative model needs considerable revision at a minimum with respect to the overall distribution of ECaC (Fig. 4.1). Furthermore, although I have been able to detect the presence of mRNA related to the basolateral components of the model in trout branchial tissue, their distribution and their overall response to physiological challenges remain to be examined. Greater effort is needed to obtain additional sequence information of the over a dozen different fragments of NCX that have been detected as well as the possible 4 isogenes of PMCA. Marshall et al. (2005) have confirmed the presence of 4 NCX isogenes in zebrafish and the Japanese pufferfish. Their findings support my unreported identification of fragments of 4 different NCXs with numerous potential splice variants in rainbow trout. Co-localization of all three components of the model need to be completed to further demonstrate the overall distribution of the Ca^{2+} uptake mechanism itself. With the development of the appropriate molecular tools, experiments such as those presented in this thesis will better demonstrate the interaction of these components in the overall transport system.

Figure 4.1. Proposed schematic diagram of the branchial Ca^{2+} uptake model in freshwater fish.

This model suggests that ECaC is present in a fraction of both pavement cells and mitochondria rich cells. This implies therefore that Ca^{2+} entry may be occurring through both these cell types. At the basolateral membrane, Ca^{2+} is either actively transported by plasma membrane calcium ATPases (PMCA) or across the membrane via sodium-calcium exchangers (NCX). Although the putative model suggests the allocation of the calcium transport mechanism at the basolateral membrane almost exclusively to MRCs their presence in pavement cells must now be examined. PVC = pavement cell.

Figure 4.1



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