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

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Summary

The entry of calcium (Ca^{2+}) through an apical membrane epithelial calcium channel (ECaC) is thought to 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 sub-families 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. 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 transcellular Ca^{2+} movements owing to preferential localisation of ECaC to the apical membrane of these cells. However, the results of real-time PCR performed on enriched gill

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 (Perry and Flik, 1988; Flik and Verbost, 1993; Flik et al., 1995; Perry, 1997), 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⁺/Ca²⁺ exchange (Verbost et al., 1994) or Ca²⁺-ATPase (PMCA) driven active transport (Flik et al., 1983; Flik et al., 1985). By analogy to the cell populations as well as immunocytochemistry and *in* situ hybridisation 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 having 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 Ca²⁺ uptake may not be restricted to mitochondria-rich cells as was proposed in previous models.

Key words: TRPV, mitochondria-rich cell, pavement cell, calcium uptake, chloride cell, rainbow trout, *Oncorhynchus mykiss*.

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 Ca²⁺ 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 Ca²⁺ homeostasis (Nilius et al., 2002; Hoenderop et al., 2003; den Dekker et al., 2003; Nijenhuis et al., 2005). These proteins belong to the vanilloid subfamily of the transient receptor potential (TRP) superfamily (Vennekens et al., 2002; den Dekker et al., 2003). 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 (Montell et al., 2002; den Dekker et al., 2003). 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 Ca²⁺ channels in mammals (Hoenderop et al., 2002), there is comparatively little known about the nature of the branchial Ca²⁺ 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 no. AY325807) and Oncorhynchus mykiss (A. Shahsavarani, B. McNeill, M. Bayaa and S. F. Perry; NCBI GenBank accession no. AY256348). On the basis of extensive but largely correlative data (Perry and Wood, 1985; Ishihara and Mugiya, 1987; Perry and Flik, 1988; Perry et al., 1992; Marshall et al., 1992; McCormick et al., 1992; MacKenzie and Perry, 1997), a model has been developed in which the branchial mitochondriarich cell (MRC; also termed ionocyte or chloride cell) is the principal site of transepithelial Ca²⁺ uptake in freshwater fish (Perry, 1997; Marshall, 2002; Evans et al., 2005).

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 (*Oncorhynchus mykiss* Walbaum) 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 dechlorinated city water. They were 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* L.) ECaC sequence was achieved through a comparison of the predicted protein sequence of rabbit (*Oryctolagus cuniculus*) ECaC (GenBank accession no. AJ133128) to the Atlantic salmon EST database library (http://snoopy.ceh.uvic.ca) using the tblasx 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, Canada). 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 μ l 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 μ l of nuclease-free water.

Reverse transcription was performed using a Stratascript Reverse Transcriptase Kit (Stratagene, La Jolla, CA, USA). Complementary DNA (cDNA) was synthesised as per kit manufacturer's instructions with the following changes. Final reaction volume was reduced to 12.5 μ l, whereas 0.5 μ g of total RNA was used with 0.25 μ g of oligo(dT) primers. For 3' and 5' rapid amplification of cDNA ends (RACE), oligo(dT) primers were replaced with 3'AUAP (as provided by the kit manufacturer; see below) or with ECaC–5RACE–RT (5'-CCACCAGGAACGCATAGGCAATAA-3') respectively (see Table 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 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, Canada) 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, ON, Canada). 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). Representative amino acid sequences for TRPC, TRPM and other TRPV subfamilies were also retrieved for analysis (Table 3). Gallus gallus TRPV4 was used as an out-group for construction of phylogenetic trees. Alignment of sequences and neighbourjoining (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 and 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).

			8
Nai	me	Direction	Sequence $5' \rightarrow 3'$
PC	R of initial fragment		
2	260902-ECaC1F	FWD	GCCCTGGTTATTCTGCTACTG
2	260902-ECaC2R	REV	CAAACTGGGAGAACAGAGTGAT
Rea	al-time PCR		
H	ECaC-QPCR1	FWD	GGACCCTTCCATGTCATTCTTATT
H	ECaC-QPCR2	REV	ACAGCCATGACAACTGTTTCC
j.	3-actin	FWD	CCAACAGATGTGGATCAGCAA
j.	3-actin	REV	GGTGGCACAGAGCTGAAG GGTA
5'R	RACE		
H	ECaC-5RACE-RT	REV	CCACCAGGAACGCATAGGCAATAA
I	ECaC-5GSP1	REV	CAGGGCCGTTTGTCCAAAGTAATGCT
H	ECaC-5GSP2	REV	ATCTGGGACCTCCAGTAGCAGAATA
3'R	RACE		
H	ECaC-3GSP1	FWD	CCTCCCTGTGGGTGGTGTATATGA
I	ECaC-3GSP2	FWD	CCCCATCACTCTGTTCTCCCAGTT
Ful	l-length coding		
H	ECaC-full1	FWD	GCTTGACGTTGCATGTTCCCTTAGT
I	ECaC-full2	REV	CTACATTTCAACCCAGTCTTGCCCAT
FWD forward. P	FV reverse		
1 11 D, 101 walu, N	L v, 10 v0130.		

 Table 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

 Table 2. Genes used for sequence alignment and phylogenetic analysis of various epithelial calcium channels

Gene	Species	Accession number
CaTl	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

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

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 $25 \,\mu$ l containing 0.5 μ l of cDNA and 150 nmol l⁻¹ of each primer. All primers (Table 1) were designed and optimized for the following PCR reaction conditions; 15 min at 95°C, 45 cycles of 30 s at 95°C, 30 s at 60°C, 30 s 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 one dissociation peak were eliminated. Control samples (diluted RNA samples) were assessed at random to test for the presence of genomic DNA contamination.

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 medium (VWR International, Ville Mont-Royal, QC, Canada) and were sectioned (10 μ m sections) at -18°C using a Leica CM 1850 cryostat. Sections were placed on SuperFrost⁺⁺ (Fisher Scientific, Ottawa, ON, 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 4) and synthesised (Genedetect.com Limited, New Zealand). Sections on slides were hydrated (2×15 min) in 1× PBST (PBS with 0.1% Tween 20). Proteinase K (20 μ g ml⁻¹ in 1× PBST; Gibco-BRL, Orand Island, NY, USA) was used to de-

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 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	CaTl	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			

Table 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.

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 $1 \times$ 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 μ g ml⁻¹ of salmon sperm DNA, 250 μ g of poly(A)⁺, topped up to 12.5 μ l with DEPC (diethyl pyrocarbonate) H₂O. Probes were then quickly chilled on ice and centrifuged (7500 g) for 1 min. Hybridization buffer (100 μ l of 4× SSC, 20% dextran sulfate, 50% formamide, 250 μ g ml⁻¹ poly(A)⁺, 250 μ g ml⁻¹ ssDNA, 0.1 mol l⁻¹ DTT, 250 μ g ml⁻¹ tRNA, 0.5× Denhardt's solution) was added to each probe. Each probe was then mixed well by vortexing and placed onto 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 hybridisation, sections were washed twice (15 min per wash, 58°C) with $2 \times$ SSC and twice (15 min per wash, 58°C) with $0.2 \times$ SSC, followed by one wash in $0.1 \times$ SSC for 10 min at room temperature and twice in $0.1 \times PBS$ (10 min per wash, room temperature). To detect hybridisation, sections were incubated for 1 h at room temperature with 1% goat serum, 2 mg ml⁻¹ BSA in 0.1 mol l⁻¹ PBS with 0.3% Triton X-100, followed by overnight incubation at 4°C in antidigoxigenin-conjugated to alkaline phosphatase (1:1000 dilution; Roche Molecular Biochemicals, Temecula, CA, USA). 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 MgCl₂, 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 H₂O and layered over the sections. Colour was allowed to develop in a humid chamber

Table 4. Sequences of oligonucleotide probes used for in situ hybridization

Gene	Probe sequence
ECaC	5'-ACAGCCATGACAACTGTTTCCCCCTGCACCCCACTGACCCTGAACACA-3'
Na ^{+/-} K ⁺ -ATPase	5'-TGGACCCCAGGGTCTCAACAGCTTCCGATTCTTCACCAGGCAGTTCT-3'

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). Coverslips were then placed on the slides using 60% glycerol as mounting medium.

Custom polyclonal antibodies (Abgent, San Diego, CA, USA) were raised in rabbits against an 18 amino acid region (SQFRFRLQNRKGWKEMLD) of 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 ($\alpha 5$; 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, USA; 52242). Tissue samples were prepared and sectioned as described above. Sections were hydrated three times (5 min per wash) with $0.1 \times$ PBS containing 0.1% Tween 20 ($0.1 \times$ 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 $0.1 \times PBST$). Each section was then washed three times (5 min per wash) with $0.1 \times PBST$. Following the third wash, sections were incubated at room temperature (1 h) with appropriate secondary antibodies (either Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 546 goat anti-mouse, both diluted 1:400 with $0.1 \times$ PBST; Molecular Probes, Invitrogen, Burlington, ON, Canada). Finally, sections were washed three times (5 min per wash) with $0.1 \times PBST$ and coverslips were then placed on the slides with mounting medium containing the nuclear marker DAPI (4'-6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA). 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 microscope (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 $(100 \text{ mmol } l^{-1} \text{ imidazole}, 5 \text{ mmol } l^{-1} \text{ EDTA}, 200 \text{ mmol } l^{-1}$ sucrose, and 0.1% sodium deoxycholate, pH 7.6), and then centrifuged at 4°C and 12 000 g for 10 min. The supernatant (containing 50 μ g total protein) was supplemented with 6× electrophoresis sample buffer (250 mmol l⁻¹ Tris-base, 2 mmol l⁻¹ Na₂EDTA, 2% SDS and 5% dithiothreitol), and then incubated at 95°C for 10 min. The denatured samples subjected were 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 twice with PBST buffer [0.01 mol l⁻¹ phosphate buffer, 0.09% NaCl (pH 7.5) and 0.05% Tween 20]. Blots were incubated for overnight at 4°C with the primary ECaC antibody at a 1:6000 dilution. After washing twice with PBST buffer, the blots were incubated for another 2 h with alkaline phosphatase (AP)-conjugated antirabbit IgG (Pierce, Rockford, IL, USA; diluted 1:1000) at room temperature. After two 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 sacrificed 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 mmol l⁻¹ EDTA; Canadian Life Technologies, Burlington, ON, Canada). Filaments were cut into small pieces and the tube was placed on an orbital shaker (300 r.p.m.) 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 s 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 resuspended and washed three times with PBS. Cells utilised for immunostaining were fixed and treated as described for tissue sections, except that isolated cells were maintained in suspension and were washed through centrifugation (300 g 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 (Goss et al., 2001; Galvez et al., 2002).

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 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 μ m mesh and washed (2× 5 min) with 0.1× PBS. Fixed gill cells were labelled using primary (alone or in combination) and secondary antibodies as described above with the following exception. Alexa Fluor 633 goat anti-mouse (1:400 dilution) was used instead of Alexa Fluor 546 to detect Na⁺/K⁺- ATPase. Cells were re-suspended in 0.5 ml of 0.1× 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 Fluor 488 fluorescence the FL1 channel was used, which detects a wavelength of 525 ± 25 nm. To measure Alexa Fluor 633, the FL4 channel was used, which detects a wavelength of 675 ± 25 nm. Samples were run at a medium flow rate (30 µl 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 $<5 \ \mu m$ and $>25 \ \mu m$.

Cell culture

Gill cultures were derived from adult rainbow trout held in McMaster University, Hamilton, Canada. Gill cell isolations were performed using sterile techniques as outlined (Part et al., 1993). Gill epithelia were subsequently grown on semipermeable membrane inserts using methods for single-seeded inserts (SSI), composed 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 2 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 three times in PBS and fixed in 4% PFA for 2 h (see above). Immunostaining 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, USA). One way analysis of variance (oneway ANOVA) was used when comparing relative ECaC mRNA expression in the enriched cell fractions using Sigma Stat (v2.03, SPSS Inc, Chicago, USA). 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 (Fig. 1; GenBank Accession no., 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 (Fig. 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 Fig. 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 (Fig. 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 (Fig. 3) and 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 (Fig. 4) through bootstrapped (100 pseudo data sets) NJ analysis showed that *rtECaC* is most closely related to *TRPV* subfamily but does not group with either *TPV5* or *TRPV6*.

Based on standard RT–PCR (35 cycles) and visualization using ethidium bromide staining, the gill had the highest expression of ECaC (Fig. 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 (Fig. 5B). Except for spleen, all extra-branchial tissues that were examined (kidney, intestine, heart, white muscle, liver, brain and blood) displayed detectable, but low levels of ECaC mRNA (Fig. 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 (Fig. 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 colocalisation of ECaC and Na⁺/K⁺-ATPase, second, there were cells expressing only ECaC and third, there were cells expressing only Na⁺/K⁺-ATPase (Fig. 6A). Fig. 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 (Fig. 6B) or omission of the ECaC primary antibody (Fig. 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 Fig. 6E; the immunoreactive band was eliminated after preabsorbing the primary antibody with immunizing peptide (A. Shahsavarani and S. F. Perry, manuscript submitted).

Results of *in situ* hybridization experiments confirmed the presence of ECaC mRNA along lamellar and filament surfaces (Fig. 7A). Furthermore, there appeared to be a higher proportion of ECaC mRNA-positive cells towards the tip of each lamella (Fig. 7A,B). Although identification of colocalising ECaC and Na⁺/K⁺-ATPase mRNA was not possible using *in situ* hybridization, a comparison of Fig. 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. Fig. 8 illustrates representative micrographs showing ECaC and Na⁺/K⁺-ATPase distribution in (Fig. 8A,B) crude cell suspensions, (Fig. 8C) enriched populations of PVCs and (Fig. 8D,E) enriched populations of PNA-negative MRCs. Despite the varying levels of purification, each fraction displayed a mosaic of four cell types

0.mykiss ECaC	ATGCCCCGGCCTTGGCAAGATCTGCTCCAGGTGAGCCCATTGGTGGAGCCAGTTTAGGTTCCCCCCAGAACAGG	ł
AAGGGGTGGAAAGAAATGG K G W K E M	LTGGATGAAACTITITTGCTGCAGAACAAAAGGACGAATGGCGTCCTTTTTTTTGCCGCCAAAGAGAGCAGTGCAGGTT 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	!
GCATTAAGAAACTTCTGGA CIKKLLI	ACTGTGCATCCACTAACATCTTTGAAAGAAGGGCTCTGGGGGGGG	۱
AGCTGCTTTAGCTCTGAT	3GACGGAGCACCTGAACTCATCAATGAGCCCATGACCTCTGAACTGTCCTTGGCATGAAACCTCTCCACATTGCCGTGGTG 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	;
AATCAGAACTTTAACCTAC N Q N F N L	FTCCGCAGTCTGATTGGTAAAGGGGCGGATGTAGCCACGCCCAGATCACGGCCTGTACTTCAGGAAGAGAAGAGGAGGG 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	:
TGCTCTACTATGGTGAGC L L Y Y G E H	ACATCCTGGCATTTGCGGCCTGTGTGGGGAATCAGGACATCATCTTCATGGTGATCAACGTAGGAGCCAGCACCAGGGCCCA 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 (د ک
GGACTCCATTGGTAACACI D S I G N T	AGTGCTCCACATCCTGGTCCTGCAGCCCAATAAGACTATAGCATGCTGGTGTGGGATCTGGTGTGGCACGTGACATTGAC 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	J
TGGACCAGGCTGTGCCAC	IGGACATGGTGCCCAACTACCATGGCTTAACACCCTTCAAACTGGTGCCAAGGAGGGCAACCTTGTGGCCTTCCAGCACC 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	
TGGTCAATCGGAGGCGAA	ICAACCAGTGGAACCTGGGACCCTTGACCTCTAACCTCTATGACCTACAGGGATCGACTCCTTGGTTGCCGACGACGACGAC INQWNLGPLTSNLYDLTGIDSLVADDDC	1
SVLEHI	COTOGOCAGCAAGAGAGAGAGGGCAAAGAGGATACTGGAAGTGGACCTCTGTTAGGCAATTGGTCAGTTTCAAGTGGAACCTC 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	:
TATGGAAAACACTACTTT Y G K H Y F	AGGTTGTTGCTGCTGTACCTCCTGTACATTGGGACCTTCACATGTGTGTG	;
AGAATTACACTGTATCTGA E N Y T V S I	ACATGGACAAAACCATCCGCGTGCAGAAAACTCTGAAGGAGAGTT#GTGACCTATGGGGACAACTTGCGTCTGGCAGGAGA 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 F	L 2
GATGATCAGCGTCCTGGG	IGCCCTGGTTATTCTGCTACTGGAGGTCCCAGATATGCTGAGAGTEGGGCCAAGCATTACTTTGGACAAACGGCCCTGGGG 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	;
GGGCCCTTCCATGTCATTC G P F H V I	CTTATTGCCTATGCGTTCCTGGTGGGTGCGGTGGGGGGGG	;
TGTGTCTGGTGCTGGGCTC	3GAGCAATGTTATGTTCTTCGCCCGAGGCTTTCAGATGCTGGGGCCTTACGTCATCATGATACAGAAGATTATATTTGGAGA N 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 I	L)
CCTGACCAAGTTCATGTGG L T K F M W	CTGAGCTTCATCGTGCTCATAGGGTTTTCCACCTCCCGGGGGGGG	;
Y R S F P I	ACGCTGTTCTCCCAGTTTGAGCTGAGTGTGGGTCTGATAGACCTGCTAGTGGACCACCACCATCACAACGCCCCCTATTGTCC 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	:
ATGTGCTGCACTGCACCT	ICTCTGTGGGTCTCCTACATACTGCTGACCACGCCACATGAGTGATACACAATGGAGAGTTGCCCAGGAGAG 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 F	; ?
GGACGAGCTCTGGAGGAC D E L W R T	ACAGGTGGTGGCCACTACCCTGATGTTGGAGAGAAGGTTGCCCCGTGCCTGGGCCCCGGCTGGGGGTGTGGGACTGCTC 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	:
TACGGCCTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	IGGTACCTCCGGGTTGAGGATCGCAACGACCCACTGGTGCAAAAGRGCGTCGCTACGTGCAAGCCTTCTCTAAGGATGAGG 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	;
ACCAGAGCAAGGAGCGGGA D Q S K E R I	AGGAGATGGAGAACACTGACATGTCAAAAGGACCTGGAAGCCCTCTGATCAGAACCAAACACAGGGGTGGGATAGATGGAAA 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	1
R K S L A C	TTGGCAGATGATTCGCCACAGCGCTCTGGGTTTAGATGTGGAACAGAAGAGCCTGAGGATGACCAGGAAGTAAGATACGTC 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 *		

Fig. 1. Nucleotide and predicted amino acid sequence of rainbow trout (Oncorhynchus mykiss) gill epithelial calcium channel (ECaC). The sequence shown represents the predicted coding region including the start (underlined) and stop codons. The (asterisk) sequence was obtained by RACE and verified by fulllength PCR and cloning.

based on immunostaining: 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-negative fraction exhibiting co-localisation of ECaC and Na⁺/K⁺-ATPase. These qualitative observations were confirmed by quantitative flow cytometry (Fig. 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-negative fractions (Fig. 8F). However, the cells derived from PNA-negative fractions exhibited significantly greater co-localisation of ECaC and Na⁺/K⁺-ATPase compared with cells obtained from PVC fractions ($13.1\pm0.7\%$ versus $3.1\pm0.3\%$; P=0.029, rank-sum test). It was not possible to obtain great enough numbers of PNA-positive cells to analyze this population by flow cytometry. An analysis of the relative levels of ECaC mRNA in the three cell populations (PVCs, PNA negative and PNA positive) demonstrated that ECaC expression was similar among these fractions (Fig. 9).

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1	MAPALARSAPGELNHWWSQFRFRLQNRKGW <u>KEMLDETFLLQNKRTNGVPLFFAAKESSAG</u>
61	* IKKLIDCASTNIFERRALGETALHVAVMNDNMEAALAINDGAPELINEPHTSELFIGHK
121	* <u>Plhiavanonfnlarsligkgadvatpratglyfrkrrggllyygehilafaacygnodi</u>
181	* * <u>ISHVINVGASTRAQD</u> SIGNTVLHILVLQPNKTIACLVLDLLLARDIELDQAVPLDNVPNY
241	* * HGLTPFKLAAKEGNLVAFOHLVNRRRINQWNLGPLTSNLVDLTGIDSLVADDDCSVLEHI
301	* * VGSKRREAKRILEVTPVRQLVSFKWNLYGKHYFRLLLLLYLLYIGTFTLCCVYRPLKDAP
361	* * * * * * * * ENYTVSDMDKTIRVQKTIKESYVTYGDNIRLAGEMISVIGALVIILLEVPDMLRVGAKHY
421	* FGQTALGGPFHVILIAVAFLVVLLCVFRVSGVQGETVVMAVCLVLGVSNVMFFARGFQMI
481	* * * GPYVINIQKIIFGDLTKFNWLSFIVLIGFSTSLWVVYN <mark>TODPDSLPAYRSFPITLFSOFE</mark>
541	* * ISYGLIDLPYDHTITTPPIYHYIHCTFSVVSYILLINLITAMM <mark>SDTQWRVAQERDELWRT</mark>
601	* QVVATTLMLERRLPRCLWPRLGVCGLLYGLGERWYLRVEDRNDPLVQKMRRYVQAFSKDE
661	* * * DQSKEREEMENTDMSKGPGSPLIRTKHRGGIDGNRKSLACWQMIRHSALGLDVEQEEPED
721	DOEABAA *

Fig. 2. Predicted rainbow trout (*Oncorhynchus mykiss*) epithelial calcium channel (ECaC) protein functional domains. Bold-underline indicates possible ankyrin repeats; boxed sequence, ion transport regions; bold sequence within the box, pore-forming region; Asterisks (*), predicted phosphorylation sites: serine (S)=16, threonine (T)=8, tyrosine (Y)=5.

Single seeded cell cultures containing only PVCs displayed both ECaC-positive and ECaC-negative cells (Fig. 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 four cell types based on immunostaining; ECaC-positive cells, Na⁺/K⁺-ATPase-positive cells, cells expressing both ECaC and Na⁺/K⁺-ATPase, as well as cells displaying no immunoreactivity (Fig. 11). As was observed on whole gill sections (Fig. 5) and crude cell suspension (Fig. 8), the doubleseeded cell cultures clearly contained two distinct types of Na⁺/K⁺-ATPase-positive cells, those expressing ECaC and those not expressing ECaC. Similarly, using MitoTrackerTM, two distinct populations of MRCs were identified: those exhibiting ECaC and those not exhibiting ECaC (Fig. 11D).

Discussion

The results of the present study demonstrate that the epithelial calcium channel found in fish, although similar in nucleotide and amino acid 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.



Fig. 3. Phylogenetic analysis of rainbow trout (*Oncorhynchus mykiss*) epithelial calcium channel (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; the scale bar represents replacement of 5% of the amino acids in the protein alignment. See Table 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.

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 L: indicative of gene duplication (Muller et al., 2000a; Muller et al., 2000b). 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 (Fig. 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 the TRPC, TRPM and TRPV families suggest that fish ECaC and TRPV subfamilies diverged prior to a possible gene duplication giving rise to TRPV5 and TRPV6 (Fig. 4). This





Fig. 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 bar represents replacement of 5% of the amino acid in the protein alignment. See Table 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.

Fig. 5. (A) Representative and (B) mean tissue distributions of epithelial calcium channel (ECaC) in rainbow trout (*Oncorhynchus mykiss*) as determined through RT–PCR and real-time RT–PCR, respectively. ECaC-QPCR1 and ECaC-QPCR2 primers were used for PCR detection of ECaC, and β -actin-FWD and β -actin-REV primers were used for the detection of β -actin (see Table 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 β -actin and standardised to ECaC expression in the gill (*N*=4).

conclusion is slightly different from the scheme presented by Qiu and Hogstrand (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* probably occurred recently in mammalian evolution.

In mammals, evidence suggests that there may be a significant tissue-dependent distribution of TRPV5 and TRPV6 (Muller et al., 2000a; Hoenderop et al., 2001; Nijenhuis et al., 2003; Song et al., 2003; van Abel et al., 2003; Nijenhuis et al., 2005). 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 (Perry and Flik, 1988; Flik and Verbost, 1993; Perry, 1997; Baldisserotto et al., 2005). 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

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becomes limiting (Rodgers, 1984). Therefore, mammals are not only faced with having to possess an optimal Ca^{2+} absorption mechanism in 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 the gill of pufferfish has also been reported (Qiu and Hogstrand, 2004). In contrast to mammals, in which ECaC expression is high in vitamin D₃-sensitive tissues (Van Ball et al., 1996; Hoenderop et al., 1999; Hoenderop et al., 2000; Wood et al., 2001; den Dekker et al., 2003), ECaC expression was low in rainbow trout intestine and kidney (Fig. 5). Previous studies on the effect of vitamin D₃ in fish have suggested a possible regulatory role for this hormone in Ca^{2+} uptake by the intestine



Fig. 6. Localisation of epithelial calcium channel (ECaC), Na+K+-ATPase and nuclei in rainbow trout (Oncorhynchus mykiss) gill epithelia. The cell nuclei appear blue, ECaC appears green and Na+/K+-ATPase appears red; colocalization of ECaC with Na+/K+-ATPase appears yellow. (A) 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. (B) A representative image of a gill section pre-absorbed with the ECaC peptide antigen. (C) A representative image of a gill section on which the ECaC primary antibody was omitted. (D) A higher magnification image showing two Na⁺/K⁺-ATPase-positive cells with one displaying no ECaC immunoreactivity (1) and the other exhibiting colocalization (2). (F) 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.

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(Fenwick et al., 1984; Sundell and Bjornsson, 1990). Therefore it is surprising that ECaC expression is extremely low (Fig. 5) in a tissue that appears to be physiologically sensitive to vitamin D_3 . Further studies are required to determine how



Fig. 7. Localisation of epithelial calcium channel (ECaC) and Na⁺/K⁺-ATPase mRNA and protein in rainbow trout (*Oncorhynchus mykiss*) gill epithelia. (A) A representative image of a gill section probed with a homologous ECaC oligonucleotide probe: arrows labelled a indicate cells expressing detectable levels of ECaC mRNA; arrows labelled b indicate MRCs expressing detectable levels of ECaC mRNA and those labelled c indicate MRCs that do not appear to express high levels of ECaC mRNA. (B) A representative image of a gill section treated with primary antibodies against ECaC and Na⁺/K⁺-ATPase; the cell nuclei appear blue, ECaC appears green and Na⁺/K⁺-ATPase appears red; co-localization of ECaC with Na⁺/K⁺-ATPase appears yellow; asterisks indicate areas of intense ECaC mRNA or protein at the tips of lamellae where MRCs are rarely found. (C) A representative image of a gill section probed with a homologous Na⁺/K⁺-ATPase oligonucleotide probe.

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 (Ishihara and Mugiya, 1987; Perry and Flik, 1988; Fenwick, 1989; Perry et al., 1992; McCormick et al., 1992; Marshall et al., 1992; Flik et al., 1995; Flik et al., 1996; Li et al., 1997; Moron et al., 2003). 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, which is thought to represent the initial step in Ca²⁺ absorption across the gill.

Homologous polyclonal antibodies raised against rtECaC were used in conjunction with a heterologous mouse monoclonal Na⁺/K⁺-ATPase antibody (α 5) to examine the cellular distribution of rtECaC in the gill epithelium. Previous studies have established that $\alpha 5$ 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 size could easily be explained by post-translational modification(s). 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. However, in addition to the single immunoreactive band of approximately the predicted molecular mass 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 (at least qualitatively) that conditions known to increase Ca²⁺ uptake (elevated cortisol, softwater exposure and hypercapnia) caused a marked increase in the intensity of immunoreactivity (A. Shahsavarani and S. F. Perry, unpublished observations).

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-localised with a subset of MRCs, there were numerous lamellar PVCs exhibiting ECaC immunoreactivity. The results of *in situ* hybridisation support a broad cellular distribution of ECaC



Fig. 8. Localisation and quantitative distribution of epithelial calcium channel (ECaC) and Na⁺/K⁺-ATPase protein in suspensions of rainbow trout (*Oncorhynchus mykiss*) gill epithelial cells using (A–E) immunocytochemistry and (F) flow cytometry. In A–E, the cell nuclei appear blue, ECaC appears green and Na⁺/K⁺-ATPase appears red; co-localization of ECaC with Na⁺/K⁺-ATPase appears yellow. (A,B) Representative images of crude cell suspension prior to separation of different cell types: (1) ECaC-positive cells, (2) Na⁺/K⁺-ATPase-positive cells and (3) cells co-expressing ECaC and Na⁺/K⁺-ATPase. (C) A representative image from a cell suspension enriched with pavement cells (PVCs). (D,E) Representative images from purified cell suspensions enriched with peanut lectin agglutinin-negative (PNA⁻) mitochondria rich (MR) cells. (F) 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 s.e.m.; *significant difference between the two cell populations (rank-sum test, *P*<0.05).



Fig. 9. Relative epithelial calcium channel (ECaC) mRNA expression in enriched populations of pavement cells (PVCs), peanut lectin agglutinin-negative (PNA⁻) mitochondria rich (MR) cells and PNA⁺ MR cells. Data are shown as means ± 1 s.e.m. (*N*=6).

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 or absence of peanut lectin agglutinin (PNA) binding sites (Goss et al., 2001; Galvez et al., 2002). The PNA-positive (PNA⁺) cells appeared to resemble the typical MRC (chloride cell) of the freshwater fish gill (Perry, 1997) whereas the PNAnegative (PNA⁻) cells exhibited characteristic similar to PVCs and were probably identical to the MR PVCs described in

earlier literature (Goss et al., 1992; 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: (1) ECaC positive, (2) Na⁺/K⁺-ATPase positive, (3) ECaC and Na⁺/K⁺-ATPase positive or (4) ECaC and Na⁺/K⁺-ATPase negative. Further examination of enriched population of PVCs, PNA- cells and PNA⁺ (mRNA analysis only) cells confirmed the broad distribution of ECaC amongst the various cell types. Interestingly, the majority (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., who demonstrated several unidentified cell types in addition to the MR PVCs in the PNA⁻ fraction (Galvez et al., 2002).

Fletcher et al. developed a technique to co-culture rainbow trout PVCs and MRCs on permeable inserts (referred to as double-seeded inserts or DSIs) (Fletcher et al., 2000). These preparations differed from the single seeded insert (SSIs) that contained only PVCs. Although both preparations exhibited equivalent rates of Ca2+ uptake, only the DSIs 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 Ca²⁺ 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+ MRCs and PNA- MRCs (with or without enrichment of Na⁺/K⁺-ATPase). However, because PVCs contain apical membrane ECaC and the capacity for epithelial active transport of Ca^{2+} is unlikely to reflect the presence or absence of ECaC, alone, it is probably another component of the overall Ca²⁺



Fig. 10. (A,B) Immunocytochemical localisation of epithelial calcium channel (ECaC) in single-seeded rainbow trout gill epithelial cell culture. The cell nuclei appear blue and ECaC appears green. Note the presence of both ECaC-positive and ECaC-negative (denoted by asterisk) cells. (C) Omission of primary antibody eliminated all fluorescence.

transport system (perhaps NCX or PMCA) that 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⁺/Ca²⁺ 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 MRCs are 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 PVCs have a



Fig. 11. Immunocytochemical localisation of epithelial calcium channel (ECaC) and Na⁺/K⁺-ATPase in double-seeded rainbow trout gill epithelial cell cultures using (A) confocal or (B–D) epifluorescence microscopy. In B–D, the cell nuclei appear blue, ECaC appears green and Na⁺/K⁺-ATPase appears red; co-localization of ECaC with Na⁺/K⁺-ATPase appears yellow. (D) MitoTrackerTM was used to localize mitochondria-rich cells (stained green) and ECaC (red). A is a reconstruction of 22 optical sections showing the presence of ECaC-positive mitochondria rich cells (MRCs, arrows) lying on top of a confluent layer of ECaC-positive and ECaC-negative (asterisks) pavement cells. In B and C the cell types are: (1) ECaC-positive cells; (2) Na⁺/K⁺-ATPase-positive cells; (3) cells co-expressing ECaC and Na⁺/K⁺-ATPase; (4) cells positive for neither ECaC nor Na⁺/K⁺-ATPase. In D, the cell types are: (2) MRCs; (3) MRCs co-expressing ECaC.

lower intrinsic rate of Ca^{2+} uptake compared to 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 and 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 Ca²⁺ balance in fish. The hormonal regulation of ECaC is likely to involve both post-translational and transcriptional control mechanisms. For example, rapid adjustments of Ca²⁺ uptake across the apical membrane largely reflect post-translational modifications of ECaC leading to rapid changes in Ca²⁺ conductance. The anti-hypercalcemic hormone, stanniocalcin, is arguably the most important hormone regulating the Ca²⁺ conductance of ECaC and plays a crucial role in reducing the rate of Ca²⁺ 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 Ca²⁺ transporting proteins (NXC, PMCA) on the basolateral membrane.

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