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Expression of calsequestrin in atrial and ventricular muscle of thermally acclimated rainbow trout

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SUMMARY

Calsequestrin (CASQ) is the main Ca^{2+} binding protein within the sarcoplasmic reticulum (SR) of the vertebrate heart. The contribution of SR Ca^{2+} stores to contractile activation is larger in atrial than ventricular muscle, and in ectothermic fish hearts acclimation to low temperatures increases the use of SR Ca^{2+} in excitation–contraction coupling. The hypotheses that chamber-specific and temperature-induced differences in SR function are due to the increased SR CASQ content were tested in rainbow trout (*Oncorhynchus mykiss*) acclimated at either 4°C (cold acclimation, CA) or 18°C (warm acclimation, WA). To this end, the trout cardiac CASQ (*omCASQ2*) was cloned and sequenced. The *omCASQ2* consists of 1275 nucleotides encoding a predicted protein of 425 amino acids (54 kDa in molecular mass, MM) with a high (75–87%) sequence similarity to other vertebrate cardiac CASQs. The transcript levels of the *omCASQ2* were 1.5–2 times higher in CA than WA fish and about 2.5 times higher in the atrium than ventricle ($P<0.001$). The omCASQ2 protein was measured from western blots using a polyclonal antibody against the amino acid sequence 174–315 of the omCASQ2. Unlike the *omCASQ2* transcripts, no differences were found in the abundance of the omCASQ2 protein between CA and WA fish, nor between the atrium and ventricle ($P>0.05$). However, a prominent qualitative difference appeared between the acclimation groups: two CASQ isoforms with apparent MMs of 54 and 59 kDa, respectively, were present in atrial and ventricular muscle of the WA trout whereas only the 54 kDa protein was clearly expressed in the CA heart. The 59 kDA isoform was a minor CASQ component representing 22% and 13% of the total CASQ proteins in the atrium and ventricle of the WA fish, respectively. In CA hearts, the 59 kDa protein was present in trace amounts (1.5–2.4%). Collectively, these findings indicate that temperature-related and chamber-specific differences in trout cardiac SR function are not related to the abundance of luminal Ca^{2+} buffering by cardiac CASQ.

Key words: sarcoplasmic reticulum, fish heart, excitation–contraction coupling, temperature.

INTRODUCTION

The force of cardiac contraction is regulated at the level of individual myocytes by modulating the free systolic Ca^{2+} concentration (Chapman, 1983). Transient rise of intracellular Ca^{2+} concentration is produced by sarcolemmal (SL) entry of extracellular Ca^{2+} and intracellular release of Ca^{2+} from the sarcoplasmic reticulum (SR) *via* the Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism (Fabiato and Fabiato, 1978). According to the current paradigm of vertebrate cardiac excitation–contraction (e–c) coupling, SL Ca^{2+} entry plays a much larger role in ectothermic vertebrates, e.g. frog and fish, than in endothermic hearts (Fabiato and Fabiato, 1978; Pizarro et al., 1985; Tibbits et al., 1992). Cardiac myocytes of ectothermic vertebrates are thinner and have more surface area for a unit volume than mammalian cardiac myocytes, and their myofilaments are superficially located immediately beneath the SL, making SL Ca^{2+} entry an effective alternative to the SR Ca^{2+} release as a source of activating Ca^{2+} (Santer, 1985). Indeed, both L-type Ca^{2+} current and Na^{+}-Ca^{2+} exchange (NCX) seem to make a significant contribution to intracellular Ca^{2+} in frog and fish hearts (Klizner and Morad, 1983; Vornanen, 1997; Vornanen, 1999; Hove-Madsen et al., 2000). Consistent with the current paradigm, the force of cardiac contraction in many ectothermic vertebrates is relatively resistant to ryanodine (Ry) and thapsigargin, specific inhibitors of SR Ca^{2+} release channel and SR Ca^{2+}-ATPase, respectively (Penefsky, 1974; Driedzic and Gesser, 1988; Vornanen, 1989; Bonnet et al., 1994; Aho and Vornanen, 1999; Costa et al., 2000; Harwood et al., 2000).

However, some recent findings are seemingly inconsistent with the hypothesis of the predominantly extracellular origin of activator Ca^{2+}. In pacemaker cells of the frog heart sizeable SR Ca^{2+} stores have been demonstrated (Ju and Allen, 1998), and in cardiac myocytes of some fish species massive SR Ca^{2+} stores seem to exist. For example, in atrial and ventricular myocytes of the rainbow trout heart, caffeine releasable Ca^{2+} stores are almost an order of magnitude larger than in mammalian cardiac myocytes (Hove-Madsen et al., 1998; Hove-Madsen et al., 1999; Shiels et al., 2002). Thus, there is a large unresolved gap in our knowledge of fish cardiac e–c coupling: fish cardiac myocytes can have massive SR Ca^{2+} stores whereas SR seems to make only a relatively small contribution to systolic Ca^{2+} (Moller-Nielsen and Gesser, 1992). Mechanistic explanation of this dichotomy and its physiological significance are unexplained and difficult to solve in intact myocytes because both SL Ca^{2+} influx and SR Ca^{2+} stores have enough capacity to cause a large systolic Ca^{2+} transient and may compensate each other when the other is specifically blocked (Harwood et al., 2000). One possible way of solving this problem is to approach it from bottom-up, i.e. to work out the capacities and kinetics of individual molecular entities of e–c coupling one by one and finally merge them together by computer simulation. Recently we showed that Ry receptors (RyRs) of the trout heart have low sensitivity to cytosolic Ca^{2+}, which may be one factor that limits the release of SR Ca^{2+} stores (Vornanen, 2006). In this study we examine the presence of a SR Ca^{2+} buffer, calsequestrin (CASQ), which is known to modulate

the SR Ca^{2+} load and regulate the activity of RyRs (Györke and Terentyev, 2008).

CICR occurs between L-type Ca^{2+} channels and RyRs, which are closely apposed in the narrow junctional cleft between SL and SR. Ca^{2+} release from the SR is regulated by cytosolic Ca^{2+} level through the Ca^{2+}-specific binding sites of the RyR (Meissner, 2004). A small Ca^{2+} influx through the L-type Ca^{2+} channels induces the opening of the release channel *via* a high affinity cytosolic Ca^{2+} binding site of the RyR whereas a low affinity cytosolic Ca^{2+} binding site is implicated in the inactivation of the release channel (Fabiato, 1985; Ashley and Williams, 1990; Laver et al., 1995; Xu and Meissner, 1998). Opening of the cardiac RyR is also sensitive to the luminal Ca^{2+} level, suggesting distinct Ca^{2+} regulatory sites on the luminal side of the channel or on associated proteins (Sitsapesan and Williams, 1994; Györke and Györke, 1998; Xu and Meissner, 1998; Laver, 2007). Luminal Ca^{2+} dependence of RyR activity makes the intraluminal Ca^{2+} buffering a significant variable for CICR, even though the molecular mechanisms of this regulation are still incompletely understood (Terentyev et al., 2003). CASQ2 is a major Ca^{2+} binding protein in the SR of cardiac muscle fibers and binds Ca^{2+} with a high capacity (about 60 mol of Ca^{2+} per mol of CASQ) and a moderate affinity (dissociation constant, K_d about 1 mmol l^{-1}) in a Ca^{2+}-dependent polymerization process of CASQ (Beard et al., 2004; Park et al., 2004). CASQ buffers the free Ca^{2+} concentration of the SR to a constant level, and the amount of CASQ within the SR plays a role in the Ca^{2+} load that SR is able to maintain. Furthermore, CASQ is suggested to modulate Ca^{2+} release from the SR *via* two regulatory proteins (Györke and Terentyev, 2008): in a Ca^{2+}-free mode CASQ inhibits RyR opening *via* triadin and junctin whereas Ca^{2+} binding to CASQ may relieve this inhibitory effect (Terentyev et al., 2003). This mechanism is probably a part of the load-dependence of Ca^{2+} release where increased SR Ca^{2+} content stimulates Ca^{2+} release whereas reduced SR Ca^{2+} content inhibits Ca^{2+} release from the SR of cardiac myocytes (Fabiato, 1985; Bassani et al., 1995; Lukyanenko et al., 1996). However, there is substantial evidence indicating that RyRs can sense luminal Ca^{2+} in the absence of CASQ; thus, suggesting that CASQ is not absolutely necessary for Ca^{2+}-dependent modulation of RyR gating (Jiang et al., 2007; Kong et al., 2008; Knollmann et al., 2006). Considering the suggested role of CASQ in the regulation of SR Ca^{2+} load and Ca^{2+} release, chamber-specific and temperature-related differences in SR function of the fish heart could be related to variation in the amount of CASQ in the SR. Therefore, the objective of the present study is to examine the expression of CASQ in the heart of thermally acclimated rainbow trout. As contraction of atrial muscle is more strongly inhibited by Ry than ventricular contraction and as acclimation to low temperatures seems to increase the participation of the SR in e–c coupling (Keen et al., 1994; Gesser, 1996; Aho and Vornanen, 1999), it was hypothesized that expression of CASQ is higher in CA than WA fish and higher in atrium than ventricle.

MATERIALS AND METHODS

Fish origin and care

Rainbow trout (*Oncorhynchus mykiss* Walbaum) (200–300 g in body mass) were obtained from a local fish farm (Kontiolahti, Finland). In the animal facilities of the University of Joensuu, the fish were kept in stainless steel tanks (500 or 1000 l) with a constant circulation of aerated tap water. For temperature acclimation, fish were randomly divided into two groups at 9°C and water temperature was gradually (3°C per day) cooled to 4°C (cold acclimation, CA) or warmed to 18°C (warm acclimation, WA). Fish were allowed to remain at the constant temperature for at least four weeks under a regime of 15 h:9 h light:dark cycle and were fed to satiation five times a week with nutrient pellets (Biomar, Brande, Denmark). All experiments were conducted with the consent of the national committee for animal experimentation (permission STH252A).

Cloning and sequencing

Fish were stunned with a blow to the head and killed by cutting the spine. The heart was cut into small pieces and homogenized in a sterile mortar in a small amount of liquid nitrogen. Total RNA was extracted from the pooled samples with TRIZOL Reagent (Invitrogen, Carslbad, CA, USA) and treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). RNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) followed by a second extraction step with chloroform–isoamyl alcohol (24:1). The RNA was precipitated with 3 mol l^{-1} sodium acetate and absolute ethanol, washed with RNase-free 70% ethanol, dried and dissolved in RNase-free water.

The total RNA was reverse transcribed to cDNA with the M-MuLV RT enzyme (Finnzymes, Espoo, Finland) using 0.5–1 μg RNA with oligo(dT)18 random primers. Partial cDNAs for rainbow trout cardiac CASQ were obtained by two PCR amplifications using degenerative primers designed to the corresponding vertebrate genes (Tables 1 and 2, Fig. 1). The DyNAzymeEXT (Finnzymes) polymerase was used in all reactions. The first amplification consisted of 3 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 30 s and extension at 72 for 1.5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52–55°C for 30 s, depending on the melting temperature of the primers, and extension at 72°C for 1 min. The second amplification consisted of 39 cycles of denaturation at 95°C for 30 s, annealing at 50–57°C for 30 s and extension at 72°C for 0.5–1 min, depending on the length of the product. The cDNA separated on a 0.8% agarose gel and purified using the QIAEXII Gel Extraction Kit (Qiagen, Hilden, Germany) was ligated to the pGEM-T Easy vector (Promega) and transformed to *Escherichia coli* DH5α cells for cloning. Plasmids were extracted using the E.Z.N.A. Plasmid Extraction Kit (Omega Bio-Tek, Norcross, GA, USA) and sequenced with an automatic sequencer using M13 primers and the Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Cloning of the 5′ end of the cDNA was performed with 5′ RACE System Kit (Invitrogen). Only the CASQ2 mRNA was reverse transcribed to cDNA using a gene-specific primer GSP1 (Table 1, Fig. 1). A gene-specific primer GSP2 was used in the first PCR amplification and a gene-specific primer GSP3 in the second PCR. Cloning of the 3′ end was performed with the 3′ RACE System Kit (Invitrogen) using a gene-specific primer GSP5 (Table 1, Fig. 1). In both RACEs PCR amplifications consisted of a hot start at 95°C for 4 min before adding the polymerase, 33 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension at 72°C for 2 min. The cloning and sequencing of the PCR products were performed as described above.

Primers specific to the beginning (F12) and the end (R10) of the coding area (Table 1, Fig. 1) were used for cloning the trout cardiac omCASQ2 in one piece. Reagents and PCR program of the amplification were the same as in 5′ RACE and 3′ RACE. PCR products were separated on agarose gels and ligated to the pGEM-T Easy vector and cloned in *E. coli* DH5α cells for sequencing.

Sequence comparisons

Statistical analysis of the amino acid composition of cardiac CASQ of seven vertebrate species was made using EMBOSS Pepstats

Table 1. Description of the primers used in this study, their melting temperatures (T_m) and suppliers

Primer name	Primer	T_m	Description	Supplier
F1	5′-TTCCCCACRTAYGAYGGGAARGAC-3′	64.4	Cloning	TAG Copenhagen, Denmark
F2	5′-GATGTCYTRGTGGARTTYCTCTTGGA-3′	63.2	Cloning	TAG Copenhagen, Denmark
F4	5′-TATGARCCMTTYATGGAKGARCC-3′	59.8	Cloning	TAG Copenhagen, Denmark
F6	5′-CCGACAAAGACACCAAGGTT-3′	60.2	Cloning/antigen preparation	TAG Copenhagen, Denmark
F12	5′-ATGCTTGCCCTGTGGCTGCT-3′	61.4	Cloning	TAG Copenhagen, Denmark
R2	5′-ATCTCCATCCAGTCYTCCAGCTCCTC-3′	64.8	Cloning	TAG Copenhagen, Denmark
R3	5′-CTTGAARGTCWTCTCCCARTARG-3′	59.8	Cloning	TAG Copenhagen, Denmark
R5	5′-AGCAGTGGGAAGTCATCAGG-3′	60.3	Cloning/antigen preparation	TAG Copenhagen, Denmark
R7	5′-YTAKTCATCRTCMTCRTCRYYRTC-3′	60.2	Cloning	TAG Copenhagen, Denmark
R10	5′-TTATTCATCATCGTCATCCTCGTCT-3′	59.7	Cloning	TAG Copenhagen, Denmark
AAP	5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′	77.4	5′ RACE	Invitrogen, UK
AUAP	5′-GGCCACGCGTCGACTAGTAC-3′	63.4	3′ RACE	Invitrogen, UK
GSP1	5′-CTCCTTTTCCTCCATAAC-3′	51.4	5′ RACE	TAG Copenhagen, Denmark
GSP2	5′-CTGAGCAGCCAGCTCCAGCACCAT-3′	67.8	5′ RACE	TAG Copenhagen, Denmark
GSP3	5′-GTTGTTTCTGCAGCTCCTTCCCATCAG-3′	66.5	5′ RACE	TAG Copenhagen, Denmark
GSP5	5′-TACTGACGCTGACAGCATCTGGCTGGA-3′	68.0	3′ RACE	TAG Copenhagen, Denmark
QF1	5′-GATAGGAAATGCTCTGGAGCTG-3′	60.3	RT-qPCR	TAG Copenhagen, Denmark
QF2	5′-GAGAATCACTGAAATGGTTCATAGC-3′	59.7	RT-qPCR	TAG Copenhagen, Denmark
QF3	5′-GGATCAGCTTACTGTTATGTTATGCTAT-3′	60.7	RT-qPCR	TAG Copenhagen, Denmark
QR1	5′-CACTGTAGTGTTCTGAGTCCTCGT-3′	59.9	RT-qPCR	TAG Copenhagen, Denmark
QR2	5′-TGTTGTCTCTAGCCACCTCCTT-3′	60.3	RT-qPCR	TAG Copenhagen, Denmark
M13 universe	5′-GTAAAACGACGGCCAGT-3′	52.8	pGEM-T Easy	TAG Copenhagen, Denmark
M13 reverse	5′-CAGGAAACAGCTATGAC-3′	50.4	pGEM-T Easy	TAG Copenhagen, Denmark
T7 promoter	5′-TAATACGACTCACTATAGGG-3′	53.2	pET-15b	TAG Copenhagen, Denmark
T7 terminator	5′-GCTAGTTATTGCTCAGCGG-3′	56.7	pET-15b	TAG Copenhagen, Denmark

(https://hotpage.csc.fi/appl/molbio/Pise/5.a/pepstats.phtml, 10 November 2008). The following sequences were obtained from GenBank. *Danio rerio*: NM_001002682.1 and NP_001002682.1 (CASQ2); *Xenopus tropicalis*: NM_203805.1 and NP_989136.1 (CASQ2), NP_988894.1 (CASQ1); *Mus musculus*: NM_009814.1 and NP_033944.1 (CASQ2), NP_033943.1 (CASQ1); *Homo sapiens*: NM_001232.2 and NP_001223.2 (CASQ2), NP_001222 (CASQ1); *Oryctolagus cuniculus*: NM_001101691.1 and NP_001095161.1 (CASQ2), NP_001075737.1 (CASQ1); *Bos taurus*: NM_001035374.1 and NP_001030451.1 (CASQ2), NP_001071345.1 (CASQ1). Identities of cDNAs and similarities of amino acid sequences were calculated in BioEdit Sequence Alignment Editor 7.0.5.3 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) using the matrix files 'IDENTIFY' and 'BLOSUM62' for cDNA and amino acid sequences, respectively. Sequence alignments were made with ClustalX 2.0.10 (Conway Institute, UCD, Dublin, Ireland).

A phylogenetic tree of the teleost CASQs was constructed with ClustalX 2.0.10 using the Neighbor Joining method and viewed with TreeView 1.6.6 (University of Glasgow, UK). Amino acid sequences of skeletal and cardiac CASQs of five teleost species, obtained *via* Ensembl Genome Browser, were included in the analysis (Table 3). *CASQ1* of the nematode *Caenorhabditis elegans* was used as an out-group member of the phylogenetic tree.

Real-time quantitative PCR

Expression of the *omCASQ2* gene was measured by quantitative RT-PCR (qRT-PCR) using the DyNAmo SYBR Green 2-Step qRT-PCR Kit (Finnzymes). RNA isolation was performed using the TRIZOL Reagent (Invitrogen), and DNase treatment with 0.1 U µl RQ1 RNase-Free DNase using 2 µg of RNA in a total reaction volume of 20 µl. Expression levels were measured using three primer pairs: QF1/QR1, QF2/QR2 and QF3/QR1 (Table 1; Fig. 2A). In the SYBR Green qPCR, 1 µl of the cDNA was used as a template and 10 pmol of the primers were applied. *DnaJ* (Hsp40) homolog, subfamily A, member 2 (*DnaJA2*) was used as a reference gene to which the expression levels of the *omCASQ2* were normalized (Hassinen et al., 2008). The SYBR Green fluorescence was red by the real-time PCR instrument (Peltier Thermal Cycler 200 with Chromo 4 Continuous Fluorescence Detector, MJ Research Inc., Waltham, MA, USA) after every 40 cycles of denaturing at 94°C for 10 s, annealing at 56°C for 20 s and extension at 72°C for 30 s.

Table 2. Primers used in cloning of the omCASQ2 cDNA from the heart of cold acclimated and warm acclimated rainbow trout

	Area of the product (nucleotide positions)	Length of the product (bp)	First amplification		Second amplification	
			Forward primer	Reverse primer	Forward primer	Reverse primer
1	–78–211	289	AAP	GSP2	AUAP	GSP3
2	76–972	897	F1	R2	F1	R3
3	397–1040	644	F1	R2	F2	R2
4	651–1263	613	F4	R7	F4	R7
5	1011–1881	871	GSP5	AUAP	–	–

CASQ, casquestrin.

Table 3. Protein sequences from five fish species used in construction of the phylogenetic tree for teleost calsequestrins (CASQs)

Species	Gene and its paralogs	Protein ID of the transcript
Caenorhabditis elegans	*CASQ1*	F40E10.3.1
Danio rerio	*CASQ2*	ENSDARP00000020399
	zgc:154027	ENSDARP00000011545
		ENSDARP00000070377
	zgc:100957	ENSDARP00000056539
Gasterosteus aculeatus	*CASQ2,* 1 of 2	ENSGACP00000004905
		ENSGACP00000004907
	CASQ2, 2 of 2	ENSGACP00000018543
	CASQ1, 1 of 2	ENSGACP00000000240
	CASQ1, 2 of 2	ENSGACP00000018651
Oryzias latipes	*CASQ2*	ENSORLP00000022393
	CASQ1, 1 of 2	ENSORLP00000010862
	CASQ1, 2 of 2	ENSORLP00000015085
Takifugu rubripes	*CASQ2*, 1 of 2	ENSTRUP00000027964
		ENSTRUP00000027965
		ENSTRUP00000027966
		ENSTRUP00000027967
		ENSTRUP00000027968
		ENSTRUP00000027969
		ENSTRUP00000027970
	CASQ2, 2 of 2	ENSTRUP00000009515
		ENSTRUP00000009516
		ENSTRUP00000009517
		ENSTRUP00000009518
		ENSTRUP00000009519
	CASQ1, 1 of 2	ENSTRUP00000013081
		ENSTRUP00000013082
	CASQ1, 2 of 2	ENSTRUP00000018278
Tetraodon nigroviridis	*CASQ2*, 1 of 2	ENSTNIP00000016949
	CASQ2, 2 of 2	ENSTNIP00000013238
	CASQ1, 1 of 2	ENSTNIP00000010220
	CASQ1, 2 of 2	ENSTNIP00000010750

The last 2–5 numbers of the protein ID (marked in bold) appear in the tree after the gene name and identify the various transcripts of the gene. CASQ1 of the nematode C. elegans was used as an out-group member of the phylogenetic tree.

The melting curve was measured from 72°C to 95°C in every 0.5°C for 1 s. The results were analyzed using Opticon Monitor version 2.03.5 (MJ Research) and Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA, USA). Each PCR amplification was repeated 3–4 times and the final results are the mean of the repeats from 3–5 samples.

Production of polyclonal antibody to the omCASQ2

EMBOSS Antigenic software (Kolaskar and Tongaonkar, 1990) and Antigen Design Tool (http://www.genscript.com, 28 December 2005) were used in designing the region for the omCASQ2 antigen. The cDNA in the area of 516–944, cloned in *E. coli* DH5α cells, was isolated and purified from the plasmid using restriction endonucleases *Tru9 I* and *Mae I* (Roche Diagnostics, Basel, Switzerland), 0.8% agarose gel electrophoresis and QIAEXII Gel Extraction Kit. The antigen-coding region was ligated to the pET-15b vector (Novagen Merck KGaA, Darmstadt, Germany) cut with the restriction endonuclease *Nde I* (Finnzymes). After ensuring the accuracy of the product by cloning in *E. coli* JM109 cells (Promega) and sequencing using T7 promoter and terminator primers (Table 1), the vector with the proper ligand was transformed to an expression host BL21(DE3) cells of *E. coli* for antigen production. A total of 2.4 mg of 30 kDa antigenic product was purified and lyophilized from the BL21(DE3) cells and used for polyclonal antibody production in rabbits (Inbio Ltd, Tallinn, Estonia). The protein-A purified and ELISA-tested rabbit anti-omCASQ2 antibody (IgG) was used for detection of the trout cardiac CASQs in western blots.

Western blotting

Hearts were frozen in a liquid nitrogen, cut into small pieces and homogenized with a glass–teflon homogenizer in 5 volumes of 10 mmol l^{-1} Tris-HCl buffer containing 1 mmol l^{-1} EDTA and EGTA, 0.5 mmol l^{-1} phenylmethylsulphonyl fluoride and 1% Protease Inhibitor Cocktail (Sigma Chemical Co., St Louis, MO, USA). Protein concentration was measured according to Lowry et al. (Lowry et al., 1951), and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970). The samples were diluted in Laemmli sample buffer to a protein concentration of 2.5 mg ml^{-1} and heated at 95°C for 5 min. Forty μg of proteins was separated on a 12% gel and stained with Coomassie Brilliant Blue R-250 or transferred to a nitrocellulose membrane for the omCASQ2 detection with polyclonal antibodies (Towbin et al., 1979). The membrane was balanced in Tris-buffered saline (TBS) for 15 min before blocking the non-specific binding sites in 5% non-fat milk powder (Valio, Helsinki, Finland) in TBS for one hour. Next it was rinsed three times in TBS including 0.05% Tween 20 (TBST) and incubated with gentle shake in primary antibody for 2 h. The antibodies were diluted in TBST at 1:3000 and 1:1000 for the anti-omCASQ2 antibody and the ab3516 anti-canine cardiac CASQ antibody (Abcam, Cambridge, UK), respectively. Unbound antibody was washed away by shaking the membrane three times for 5 min in TBST, and thereafter it was incubated in secondary antibody (Goat anti-Rabbit IgG-h&l alk phos, Novus Biologicals Inc., Littleton, CO, USA) diluted at 1:5000 in TBST for 2 h, washed two times in TBST for 5 min and rinsed with deionized water. The membrane was dried and incubated in 0.1 mol l^{-1} Tris, 0.5 mmol l^{-1} $MgCl_2$, 0.03% nitroblue tetrazolium and 0.015% 5-bromo-4-chloro-3-indolylphosphate for 4 min, after which the reaction was stopped by incubation in 20 mmol l^{-1} Tris and 5 mmol l^{-1} EDTA for 15 min. All incubations were done at room temperature. Acrylamide was from Merck (Darmstadt, Germany), glycerol from JT Baker (Deventer, Holland), TEMED, N,N′-methylene-bis-acrylamide and SDS from Bio-Rad (Hercules, CA, USA) and other reagents were from Sigma. The intensity of the bands was measured using Kodak 1D 3.5 software (Eastman Kodak Company, NY, USA). Broad Range Molecular Weight Standards or Kaleidoscope Prestained Standards (Bio-Rad) were run on the gels for molecular mass (MM) determination.

Statistics

Statistical comparisons between mean values were made with the Student's non-paired *t*-test using the SPSS software package (SPSS Inc., Chicago, IL, USA). Kolmogorov–Smirnov test was used for studying the normality of the samples and Levene's test for the equality of variances. Data are presented as means ± s.e.m.

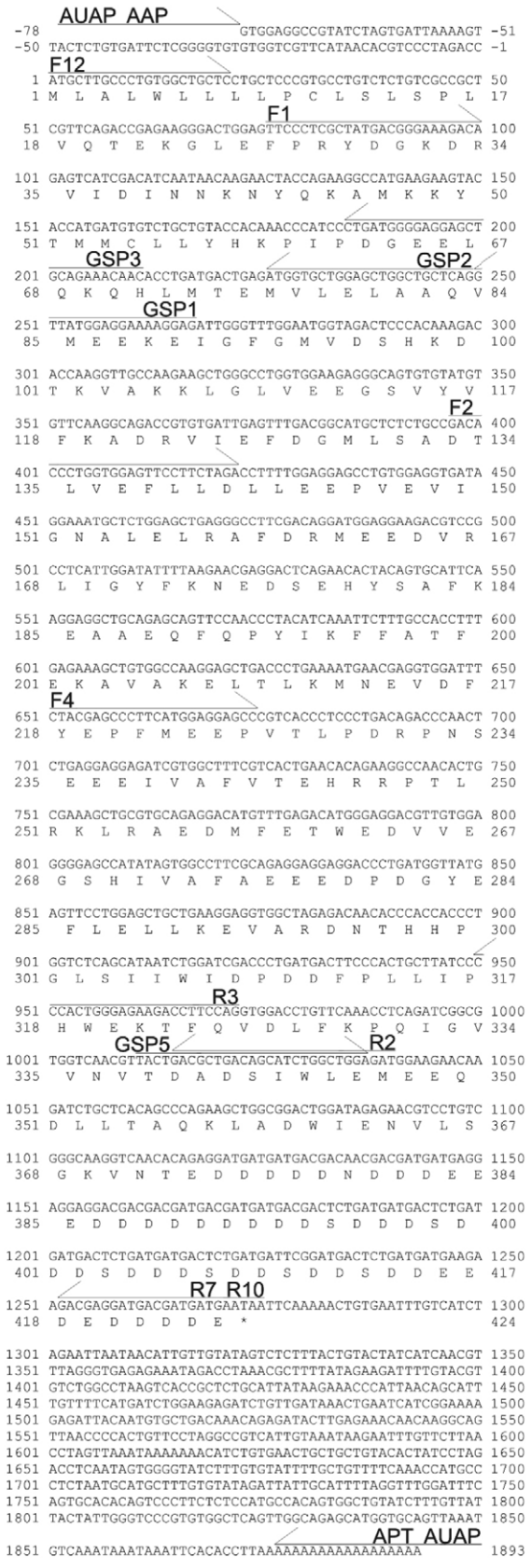


Fig. 1. Nucleotide and deduced amino acid sequences of the rainbow trout cardiac calsequestrin (CASQ) (GenBank accession number FJ626740), including the 5′- and 3′-untranslated regions. Positions of the primers used for cloning are marked with arrows.

RESULTS

Cloning and sequencing of trout cardiac *CASQ2*

Initially CASQ mRNA of the rainbow trout heart (*omCASQ2*) was obtained in five parts (Tables 1 and 2; Fig. 1). To obtain the whole coding sequence of the trout cardiac *omCASQ2* in one piece, primers specific to the beginning (F12) and the end (R10) of the coding area were used (Table 1). As a result of PCR amplification, a large variety of cDNA products with different lengths were obtained from both CA and WA fish (Fig. 2A). Variant W3 of the WA fish and variants C1 and C2 of the CA fish contained all of the 11 exons of the vertebrate *CASQ* (Zarain-Herzberg et al., 1988) and no introns. These variants differ most in the 3′ tail, where a short sequence of 12 base pairs is repeated 2–4 times. This cDNA sequence, 5′-GACTCTGATGAT-3′, constitutes a deduced sequence of four amino acids, DSDD. In addition, two longer (W1, W2) and seven shorter variants (W4, W5, C3, C4, C5, C6, C7) of the *omCASQ2* were found (Fig. 2A). Variants W1 and W2 include introns 2–3 and 3–4, variant W4 excludes exon 3 and includes introns 7–8 and 8–9, variant C3 excludes exons 7 and 8 and parts of exons 6 and 9, and variant W5 excludes exons 5, 6 and 7 and parts of exons 4 and 8. Variants C4–C7 included only parts of the first and the last exon. The single nucleotide substitutions in different *omCASQ2* variants probably represent allelic differences between fish individuals as pooled samples were used for cloning.

When the nucleic acid sequences were translated to amino acid sequences, premature stop codons appeared in W1, W2, W4 and C3 (Fig. 2B). In the WA fish, the full length variant W3 constitutes a possible active omCASQ2 protein whereas the shorter variant W5 (in which the missing part does not change the reading frame) lacks 115 amino acids in the middle of the sequence. In the full length variants of the CA fish, C1 and C2, five to eight nucleotides are missing at the 5′ end, possibly due to degradation of the cDNA or failures in PCR amplification. Ignoring these imperfections, C1 and C2 could also encode the full length omCASQ2 proteins. Furthermore, two of the four extremely short variants of the CA fish also maintain the correct reading frame. Translation of C4 would produce a polypeptide chain of 62 amino acids, and translation of C6 produces a polypeptide of 114 amino acids (Fig. 2B). The reading frame shifted in the middle of C5 and C7, resulting in polypeptides that do not have stop codons inside the cloned area. These findings indicate that the *omCASQ2* gene produces at least three different full length nucleotide sequences, W3 probably encoding a functional omCASQ2 protein. The W3 variant is 1275 nucleotides long with 78 and 618 long 5′ and 3′ UTR areas, respectively. When translated to an amino acid sequence, W3 includes 11 putative phosphorylation sites and one N-linked glycosylation site (Fig. 2B). The names and GenBank accession numbers of the variants that encode a putative protein are as follows: CASQ2 W3 (FJ626740), CASQ2 W5 (FJ626743), CASQ2 C1 (FJ626741) and CASQ2 C2 (FJ626742).

Sequence comparisons and deduced amino acid composition of the omCASQ2

The sequence of the omCASQ2 was compared with CASQ2 sequences of six other vertebrate species (Tables 4 and 5). The identity at the nucleotide level varied between 61% (cow, *B. taurus*) and 70% (zebra fish, *D. rerio*), and similarity at the amino acid level between 75% (cow) and 87% (zebra fish). The omCASQ2 is one of the largest cardiac CASQs with a calculated MM of 49 kDa and an amino acid chain length of 424 residues (Table 4). Only the CASQ2 of the cow is larger with 431 amino acid residues and a MM of about 50 kDa. The MM of other five species varies between 46 and 48 kDa, and the proteins are 8–25 amino acids shorter than the omCASQ2. The large size of the omCASQ2 is mainly due to

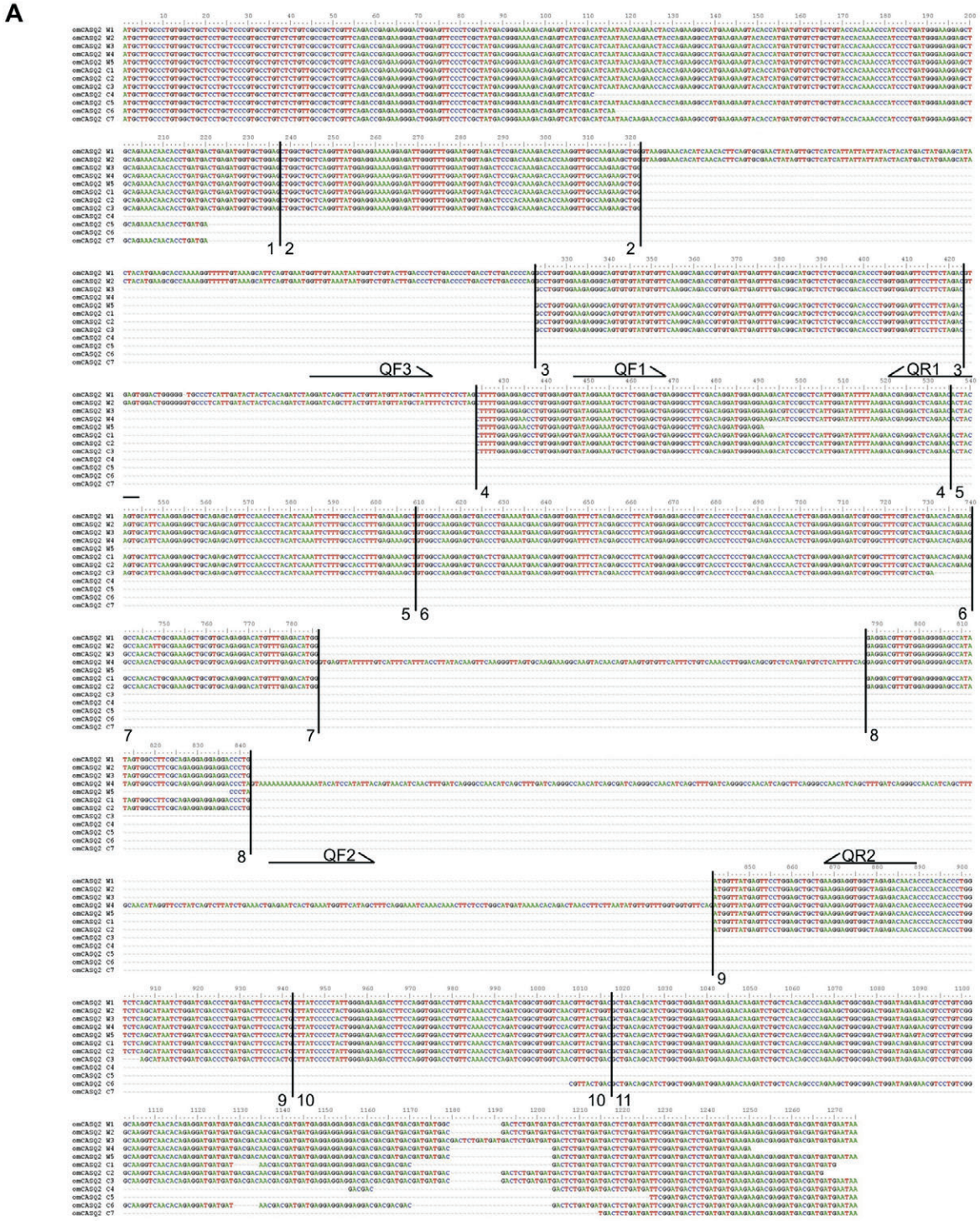
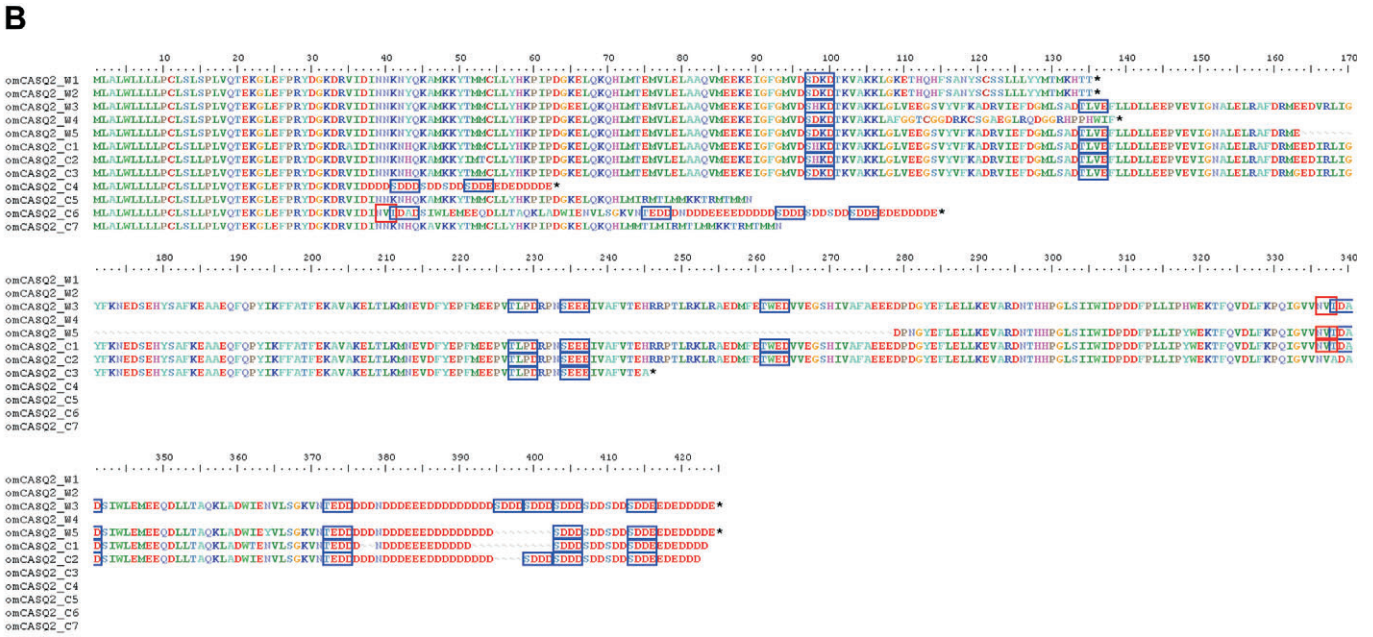


Fig. 2. See next page for legend.

the long unstructured C-terminal tail consisting of acidic (Asp and Glu) and uncharged (Ser) polar amino acids (Fig. 2C). The percentage of acidic Asp and Glu residues was 34% in W3 and W5 (Table 4), despite differences in their sequence lengths. The percentage of acidic residues in the omCASQ2 is similar as in zebra fish (34%) and frog (35%) CASQ but higher than in CASQs of endothermic vertebrates (29–31%).

A phylogenetic tree was constructed using amino acid sequences of skeletal and cardiac CASQs of five teleost species and rooted with the CASQ1 of the nematode *Caenorhabditis elegans* (F40E10.3.1) (Table 3; Fig. 2D). In the tree, cardiac CASQs formed a separate group from skeletal CASQs. The four sequences of the omCASQ2 (W3, W5, C1 and C2) were placed next to each other in the group of cardiac CASQ2; thus, indicating that they are products of the same gene and not sequences of different paralogs. Rainbow trout, zebra fish and medaka have only one *CASQ2* gene whereas fugu, tetraodon and stickleback have two *CASQ2* paralogs. The three transcript products of the zebra fish paralogs, *zgc:154027* and



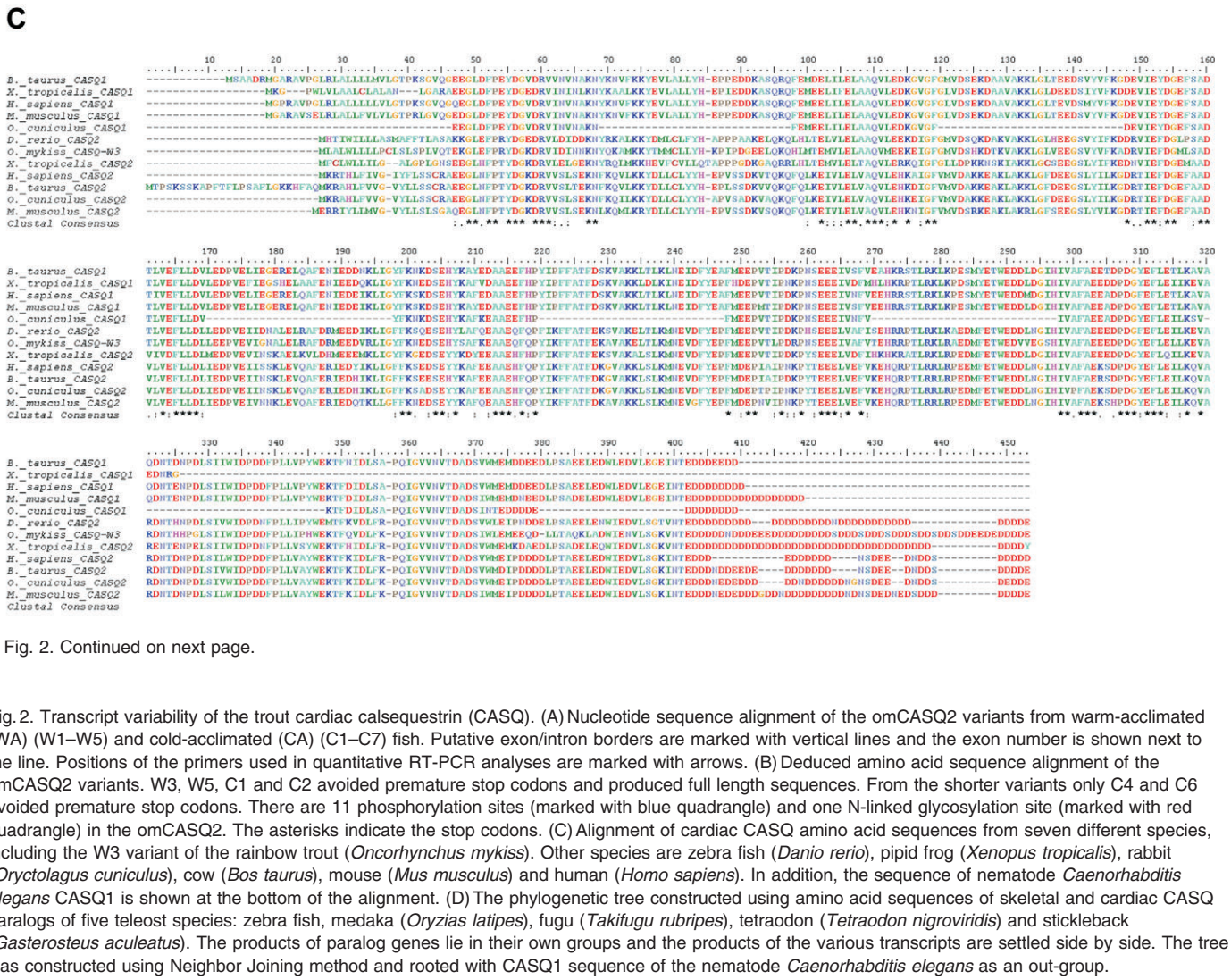


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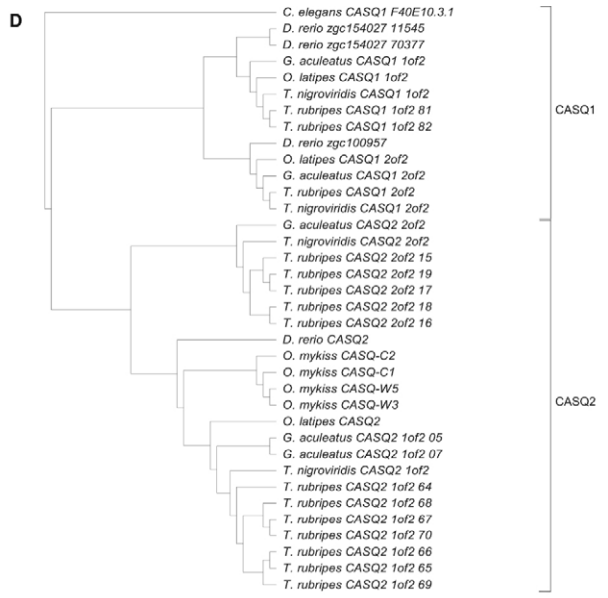
Fig. 2. Transcript variability of the trout cardiac calsequestrin (CASQ). (A) Nucleotide sequence alignment of the omCASQ2 variants from warm-acclimated (WA) (W1–W5) and cold-acclimated (CA) (C1–C7) fish. Putative exon/intron borders are marked with vertical lines and the exon number is shown next to the line. Positions of the primers used in quantitative RT-PCR analyses are marked with arrows. (B) Deduced amino acid sequence alignment of the omCASQ2 variants. W3, W5, C1 and C2 avoided premature stop codons and produced full length sequences. From the shorter variants only C4 and C6 avoided premature stop codons. There are 11 phosphorylation sites (marked with blue quadrangle) and one N-linked glycosylation site (marked with red quadrangle) in the omCASQ2. The asterisks indicate the stop codons. (C) Alignment of cardiac CASQ amino acid sequences from seven different species, including the W3 variant of the rainbow trout (*Oncorhynchus mykiss*). Other species are zebra fish (*Danio rerio*), pipid frog (*Xenopus tropicalis*), rabbit (*Oryctolagus cuniculus*), cow (*Bos taurus*), mouse (*Mus musculus*) and human (*Homo sapiens*). In addition, the sequence of nematode *Caenorhabditis elegans* CASQ1 is shown at the bottom of the alignment. (D) The phylogenetic tree constructed using amino acid sequences of skeletal and cardiac CASQ paralogs of five teleost species: zebra fish, medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*) and stickleback (*Gasterosteus aculeatus*). The products of paralog genes lie in their own groups and the products of the various transcripts are settled side by side. The tree was constructed using Neighbor Joining method and rooted with CASQ1 sequence of the nematode *Caenorhabditis elegans* as an out-group.



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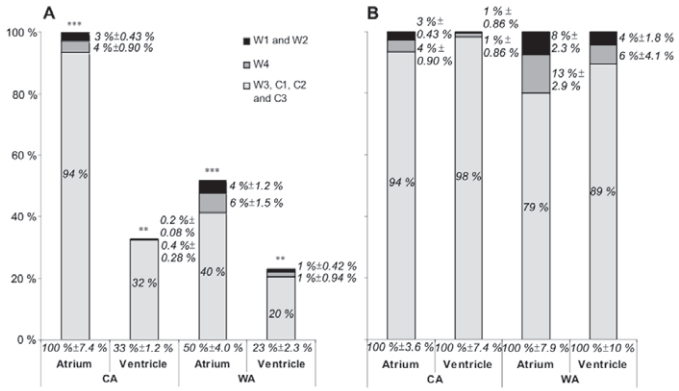

Fig. 3. The transcript expressions of cardiac calsequestrin (CASQ) in atrium and ventricle of warm-acclimated (WA) and cold-acclimated (CA) rainbow trout using primers QF1/QR1 (all the full-length variants, values shown under the category axis), QF2/QR2 (W4 and other variants that include the intron 8–9, values shown on the right side of the columns) and QF3/QR1 (W1, W2 and other variants that included the intron 3–4, values shown on the right side of the columns). The relative expressions of variants W3, C1–C3 (value shown inside the columns) were calculated by subtracting the expression of variants W1, W2 and W4 from the expression of all full length variants. The expression levels are relative to the expression levels of the mRNA of the *DnaJA2* gene. (A) All results are normalized to the expression of obtained with the primer pair QF1/QR1 in CA atrium. $^{**}P<0.005$; $^{***}P<0.001$. (B) The QF1/QR1 relative expression in each organ and acclimation group is set as 100% and the QF2/QR2 and QF3/QR1 results are normalized to this value. Values are means ± s.e.m. (N=5).

zgc:100957, settled in the group of skeletal CASQs and therefore represent *CASQ1* genes. Taken together, the phylogenetic sequence analyses indicate that the omCASQ2 belongs to the family of teleost cardiac CASQs and is clearly different from the skeletal CASQs.

Real-time quantitative PCR

Consistent with the cloning results three different PCR products were present in mRNA analyses. The primer pair QF1/QR1 can pick up four cloned variants of the WA fish (W1–W4) and three cloned variants of the CA fish (C1–C3) but excludes variants W5 and C4–C7 (Fig. 2A). When the expression level of the *CASQ2* QF1/QR1 transcript of the CA atrium (N=5) is set as 1, the relative expression levels of the *omCASQ2* in WA atrium (N=5), CA ventricle and WA ventricle are 0.504, 0.330 and 0.228, respectively (Fig. 3A). Thus, the expression level of the *omCASQ2* is about 2 ($P<0.001$) and 1.5 times ($P<0.005$) as high in CA fish than in WA fish for atrial and ventricular muscle, respectively. Furthermore, the expression level of the *omCASQ2* is 2–3 times higher in atrium than in ventricle ($P<0.001$).

Table 4. Identities of cardiac calsequestrin (CASQ) cDNA sequences of seven vertebrate species

cDNA identity % (IDENTIFY)	*Oncorhynchus mykiss*	*Danio rerio*	*Xenopus tropicalis*	*Mus musculus*	*Homo sapiens*	*Oryctolagus cuniculus*	*Bos taurus*
Oncorhynchus mykiss	ID	70	67	66	62	66	61
Danio rerio	70	ID	69	67	67	68	65
Xenopus tropicalis	67	69	ID	68	66	68	64
Mus musculus	66	67	68	ID	82	83	78
Homo sapiens	62	67	66	82	ID	87	84
Oryctolagus cuniculus	66	68	68	83	87	ID	82
Bos taurus	61	65	64	78	84	82	ID

The sequence of the W3 variant of the rainbow trout omCASQ2 was compared with the CASQ2 sequences of other vertebrate species obtained from GenBank.

Table 5. Similarities of cardiac calsequestrin (CASQ) amino acid sequences of seven vertebrate species

Amino acid similarity % (BLOSUM62)	*Oncorhynchus mykiss*	*Danio rerio*	*Xenopus tropicalis*	*Mus musculus*	*Homo sapiens*	*Oryctolagus cuniculus*	*Bos taurus*
Oncorhynchus mykiss	ID	87	81	80	78	80	75
Danio rerio	87	ID	85	85	84	86	80
Xenopus tropicalis	81	85	ID	84	81	83	78
Mus musculus	80	85	84	ID	91	93	88
Homo sapiens	78	84	81	91	ID	95	91
Oryctolagus cuniculus	80	86	83	93	95	ID	92
Bos taurus	75	80	78	88	91	92	ID

The W3 variant of the trout omCASQ2 was compared with the CASQ2 sequences of other vertebrate species obtained from GenBank.

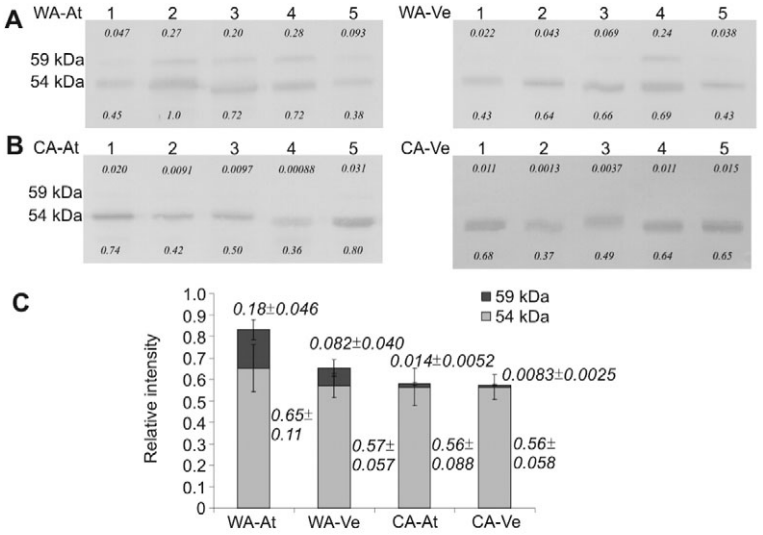

Fig. 4. Identification and quantification of the trout cardiac calsequestrins (CASQs) with a polyclonal antibody to the omCASQ2. Representative western plots comparing CASQ content in atrium and ventricle of warm-acclimated (WA) (A) and cold-acclimated (CA) (B) trout. Numbers on the bottom and on the top of the western plots show staining intensities of the 54 kDa and 59 kDa proteins, respectively, relative to the strongest band of the 54 kDa protein in each run. (C) A bar graph showing the mean expression (± s.e.m.) of 54 kDa and 59 kDa proteins (N=5). Forty μg of atrial and ventricular homogenate was applied to each lane. WA–At, atrium of WA fish; CA–At, atrium of CA fish; WA–Ve, ventricle of WA fish; CA–Ve, ventricle of CA fish.

The difference observed in the amount of the *CASQ2* QF1/QR1 transcripts between CA and WA fish did not exist in the *CASQ2* QF2/QR2 or *CASQ2* QF3/QR1 transcripts (N=5) (Fig. 3B). Furthermore, the expression levels of the *CASQ2* QF2/QR2 and *CASQ2* QF3/QR1 transcripts were only 3–13% of the expression levels of *CASQ2* QF1/QR1 transcripts in the atrium and 1–6% in the ventricle of CA and WA fish, respectively. In PCR amplification of the *omCASQ2* QF3/QR1, the measured expression level consists of two cloned variants in WA fish (W1, W2) and putative variants of the CA fish that were not found in cloning. In PCR amplification of the *CASQ2* QF2/QR2 the measured expression levels represent only the W4 and putative uncloned variants of the CA fish. Collectively, these results indicate that transcript abundance of the *omCASQ2* is higher in the atrium than ventricle and upregulated in both cardiac chambers during acclimation to the cold.

Expression of CASQ protein in the trout heart

A polyclonal antibody raised to the omCASQ2 sequence between amino acids 174 and 315 was used for identifying and quantifying the omCASQ2 protein in western blots. Surprisingly, in the atrium and ventricle of WA and CA trout hearts, the antibody clearly recognized two proteins with an apparent MM of 54 and 59 kDa, respectively (Fig. 4). The 59 kDa protein was a minor component representing 22% and 13% of the total anti-omCASQ2 sensitive proteins in the atrium and ventricle of WA fish, respectively (P>0.05) (Fig. 4A). In contrast to WA trout, the 59 kDa protein was practically non-extant in the atrium and ventricle of CA fish (Fig. 4B).

The staining intensity of the 54 kDa protein was similar for the samples of CA and WA fish (P>0.05) in both cardiac chambers (Fig. 4C), indicating stability of the omCASQ2 expression in thermally acclimated trout. When compared with the amount of CASQ (MM about 70 kDa) in trout skeletal muscle (MM about 70 kDa), the intensity of the cardiac 54 kDa protein was 60–80% in the atrium and ventricle of WA fish (N=3) and CA fish (N=3) (data not shown) in comparison with the CASQ of trout myotomal muscle.

We compared the results of our fish-specific antibody western blots with those probed with a commercially available polyclonal canine cardiac CASQ antibody. The anti-canine CASQ, ab3516, detected both the 54 kDa and the 59 kDa protein of the CA trout, but contrary to the anti-omCASQ2 antibody, ab3516 seemed to recognize the 59 kDa protein better than the 54 kDa protein (Fig. 5A,B,D,E). In general agreement with the results of the fish specific antibody, the intensity of the 59 kDa protein was 46 and 19 times stronger in WA than CA trout for atrium and ventricle, respectively (Fig. 5B,E). Similar differences in the expression of 59 kDa protein are also evident in Coomassie stained SDS-PAGE gels (Fig. 5C,F): larger amounts of the 59 kDa protein are present in WA than CA fish, and the 59 kDa protein is slightly more strongly expressed in the atrium than ventricle.

Taken together, these results indicate that CA trout expresses mainly one CASQ isoform (54 kDa) whereas WA trout expresses two CASQ isoforms. The omCASQ2 content of atrial and ventricular muscle of the trout heart is similar and there are no temperature-related differences in omCASQ2 abundance in either the atrium or ventricle.

DISCUSSION

Atrial and ventricular myocytes of the rainbow trout heart have massive SR Ca^{2+} stores, exceeding those of the mammalian heart 6–10-fold (Hove-Madsen et al., 1998; Hove-Madsen et al., 1999; Shiels et al., 2002). Furthermore, acclimation to low temperature increases contribution of SR Ca^{2+} stores to cardiac e–c coupling as evidenced by the increased sensitivity of contraction force to Ry in CA trout (Keen et al., 1994; Aho and Vornanen, 1999). Mechanistic basis of the large SR Ca^{2+} storing capacity and molecular mechanisms responsible for the increased SR Ca^{2+} release in CA hearts are,

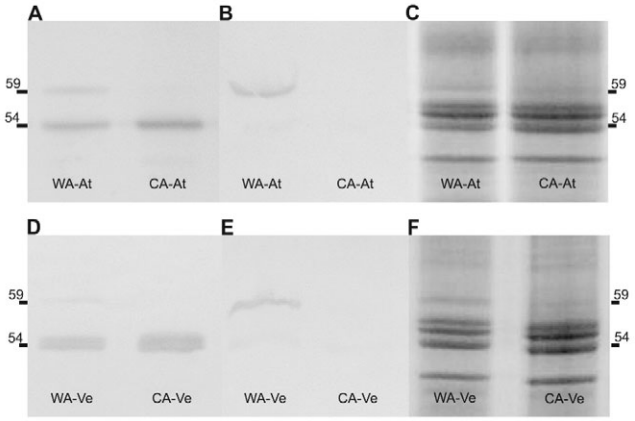

Fig. 5. Identification of trout cardiac calsequestrins (CASQs) with a polyclonal antibody to canine cardiac CASQ (ab3516). Comparison of CASQ staining in atrial and ventricular homogenates with the anti-omCASQ2 antibody (A,D) and the ab3516 (B,E). Note the strong staining of the 59 kDa protein with ab3516. C and F show corresponding SDS-PAGE gels stained with Coomassie Brilliant Blue R-250. For labeling see the caption of Fig. 4.

Table 6. Amino acid composition of W3 and W5 variants of the *omCASQ2* gene and *CASQ2* sequences of six other vertebrate species from GenBank

	MM	Residues	Aliphatic	Aromatic	Non-polar	Polar	Charged	Basic	Acidic
Oncorhynchus mykiss W3	49033	424	113	46	199	225	168	47	121
Oncorhynchus mykiss W5	34606	301	84	27	145	156	116	30	86
Danio rerio	47330	409	89	50	199	210	161	45	116
Xenopus tropicalis	47951	414	106	50	199	215	170	49	121
Mus musculus	48354	416	92	49	196	220	158	48	110
Homo sapiens	46436	399	90	52	196	203	151	49	102
Bos taurus	49915	431	118	56	209	222	165	56	109
Oryctolagus cuniculus	47356	409	113	51	199	210	157	50	107

MM, molecular mass.

however, largely unknown. Although CASQ is not absolutely necessary for SR Ca^{2+} storage and release (Knollmann et al., 2006), it is generally expressed in vertebrate heart and regulates cardiac e–c coupling. The present study examines, for the first time, the expression of CASQ2 protein in the fish heart. A 54 kDa isoform of cardiac CASQ (omCASQ2) was the main CASQ component in the atrium and ventricle of the trout heart in both CA and WA fish. Notably, thermal acclimation did not affect the abundance of the 54 kDa CASQ2 proteins, suggesting that the effect of chronic thermal changes on SR Ca^{2+} release are not related to the amount of this omCASQ2 isoform. However, it is shown that thermal acclimation is associated with a qualitative change in CASQ2 protein expression: acclimation of trout to 18°C induces the expression of 59 kDa CASQ2 in addition to the 54 kDa omCASQ2. However, the identity of this gene product could not be resolved and its physiological function remains to be shown.

Comparison of the omCASQ2 with other vertebrate CASQ

In mammals, two CASQ isoforms exist, one specific for the skeletal muscle (CASQ1) and another for the cardiac muscle (CASQ2), the latter being the sole CASQ isoform in the heart (MacLennan and Wong, 1971; Cala and Jones, 1983; Campbell et al., 1983). The apparent MM of the trout omCASQ2 in the alkaline SDS gels (Laemmli, 1970) was 54 kDa, which corresponds well the MM of the mammalian CASQ2 (53 kDa) (Cala and Jones, 1983; Campbell et al., 1983). The sequence comparisons indicate that the omCASQ2 belongs to the family of the vertebrate cardiac CASQs and is clearly distinct from the skeletal CASQs. Furthermore, it is clear that the different variants of the omCASQ2 are products of the same gene. Multiple transcript variants are unlikely to ensue from cloning artifacts, because several of the products are outcomes of the splicing process on exon/intron borders and because they constitute significant amounts (0.6–21.0%) of the total transcripts. For example W1 and W2 result from incomplete splicing and include two introns, which introduce premature stop codons in the transcripts. Similarly, W4 is a splicing product missing one exon and including two introns but would have a premature stop codon even after removal of the introns. Due to the premature stop codons these transcripts will not produce true splice variants but they may still have regulative effects on Ca^{2+} binding and polymerization of the omCASQ2 or interaction of the omCASQ2 with other proteins. The C-terminal tail is important in forming Ca^{2+} binding pockets in the polymerized chains of CASQ and includes binding motifs for junctin and triadin (Beard et al., 2009). Therefore, the products of apparently incomplete splicing, and even the shortest omCASQ2 variants having the C-terminal tail, could interfere with the function of the full length omCASQ2. It is well known that incomplete protein from alternative splicing can interfere with the function of catalytically active isoforms by competing for substrate molecules or by direct inhibition/interaction of the full-length protein (Ross et al., 1997; Nagano et al., 1999).

The trout omCASQ2 is, in several respects, similar to cardiac CASQs of other vertebrates but also displays some differences. The trout omCASQ2 is 8–25 residues longer than the cardiac CASQs of endothermic vertebrates (with the exception of the cow CASQ2 which is 7 residues longer than the omCASQ2) and 15 resides longer than the zebra fish CASQ2. CASQ2 is a highly acidic protein with about one third of the amino acids being either Glu or Asp. The number of negatively charged Glu and Asp residues of the trout omCASQ2 is similar (34%) to CASQ of zebra fish (*D. rerio*) (34%) and clawed frog (*Xenopus laevis*) (35%) but higher than those of mammalian and avian CASQs (29–31%), mainly due to the longer C-terminal tail. Although the long C-terminal tail increases the number of negative charges of the omCASQ2 molecule, it probably does not have any major effect on Ca^{2+} binding, which is mainly dependent on polymerization of CASQ into linear needle-shaped crystals (Park et al., 2004). The negative charges of the C-terminal tail are needed to form back-to-back (C-terminal) dimers of CASQ, which starts the polymerization process at relatively low Ca^{2+} concentrations (Wang et al., 1998). The long C-terminal tail of ectothermic CASQ2s might regulate the Ca^{2+}-sensitivity of the polymerization.

Identity of the 59 kDa protein of the trout heart is presently unresolved. The 59 kDa protein was recognized by two polyclonal CASQ antibodies, the fish-specific anti-omCASQ2 antibody and the anti-canine cardiac CASQ antibody, strongly suggesting that the 59 kDa protein is CASQ or a CASQ-like protein. Although, its MM is similar to mammalian skeletal CASQ (60 kDa), it is probably not a skeletal CASQ, because the MM of the rainbow trout skeletal CASQ (70 kDa), like that of the common carp (68 kDa) (*Cyprinus carpio*) (Watabe et al., 1991), is much higher than the MM of mammalian skeletal CASQ. Even though the 59 kDa protein was regularly expressed in atrial and ventricular muscle of the WA fish, *omCASQ2* transcripts of the corresponding length were not present in the *omCASQ2* clones of the WA trout heart. CASQ is a glycoprotein, which contains mannose and N-acetylglucosamine sugars (Jorgensen et al., 1977), and therefore the 59 kDa protein could be a glycolysated form of the 54 kDa protein. Similar to canine CASQ (Scott et al., 1988; Cala and Jones, 1991), trout CASQ2 variant W3 amino acid sequence includes several putative casein kinase II phosphorylation sites and one N-linked glycosylation site. However, enzymatic removal of the carbohydrate moiety of the cardiac CASQ usually reduces its MM only by 1–2 kDa (Milner et al., 1991), which is too little to explain the 5 kDa difference in MM between the trout cardiac CASQs. Further studies are needed to solve the identity and physiological function of the 59 kDa protein whose expression is induced by exposure of the trout to high environmental temperatures.

Expression of the omCASQ2

Expression of both mRNA and protein levels of the omCASQ2 were measured. Although transcript expression of the full length

omCASQ2 was elevated 1.5–2-fold in atrial and ventricular muscle of the CA trout heart, the amount of the omCASQ2 protein did not differ between the acclimation groups. The divergence between transcript and protein levels may be partly due to the 59 kDa isoform, which was almost exclusively expressed in the WA heart. It is possible that transcripts of the 59 kDa protein were not picked up by the primers designed to the *omCASQ2*. Considering that the 59 kDa isoform formed only 13–22% of the total CASQs in the WA heart, the 1.5–2-fold difference in *omCASQ2* transcript levels between WA and CA fish, does not however match with the protein expression. Deviation between transcript and protein levels suggest that the abundance of the omCASQ2 protein is regulated by the translation process, and therefore transcript levels cannot be used as a measure for the amount of the omCASQ2 protein.

In mammalian heart, SR Ca^{2+} release is enhanced at high SR Ca^{2+} loads when Ca^{2+} binds to CASQ and increases the opening probability of the RyRs (Györke and Terentyev, 2008). Because SR Ca^{2+} load of trout atrial myocytes is higher than in ventricular myocytes (Haverinen and Vornanen, 2009), it was anticipated that this would be expressed as a higher omCASQ2 content in atrial tissue and therefore might also explain the higher Ry sensitivity of atrial contraction in comparison with ventricle (Gesser, 1996; Aho and Vornanen, 1999). Contrary to this hypothesis, the amount of omCASQ2 was similar in both cardiac chambers. Similar to the trout heart, atrial and ventricular muscle of the rat heart have similar CASQ contents, even though SR Ca^{2+} content is about 3 times larger in atrial than ventricular myocytes (Walden et al., 2009). With regards to chamber-related differences in SR Ca^{2+} load and CASQ content, fish and mammalian hearts seem to be qualitatively similar. It can be concluded that atrio-ventricular differences in SR Ca^{2+} content of the vertebrate heart are not dependent on CASQ concentration and therefore must be associated with other properties or molecular entities of the SR, like SR volume or additional SR Ca^{2+} buffers.

In rainbow trout, acclimation to cold increases Ry sensitivity of cardiac contraction, indicating thermal plasticity of the SR function (Keen et al., 1994; Aho and Vornanen, 1999). However, the omCASQ2 content of the trout heart was resistant to thermal acclimation in both cardiac chambers, suggesting that changes in the amount of CASQ are not responsible for the altered SR function. In different pathophysiological states of the mammalian heart, CASQ content remains quite constant unlike many other molecules of the e–c coupling machinery (Lehnart et al., 1998). Evidently, cardiac CASQ is not a particularly plastic entity in vertebrate heart and tends to be conserved at the characteristic level of the tissue. It should be noted however that the trout heart differs from mammalian hearts in that the WA trout heart expresses two CASQ isoforms, i.e. there is a qualitative change in cardiac CASQ composition.

Quantitative aspects

Because SR Ca^{2+} stores of the trout cardiac myocytes are much larger than those of the mammalian cardiac myocytes, it would be interesting to know whether this is associated with the higher omCASQ2 content. Our objective was to compare CASQ content in mouse and trout hearts using CASQ antibodies but those studies were not feasible, because antibodies to fish and mammalian cardiac CASQs differed in their affinity to heterologous CASQs. It was, however, shown that CASQ content is 20–40% less in the heart than in the myotomal muscle of the trout. We could not find similar tissue comparisons for other vertebrates, and overall surprisingly little quantitative data exist for the amount of CASQ in vertebrate muscles (Volpe and Simon, 1991; Murphy et al., 2009). Data on absolute amounts of CASQ in cardiac muscle are totally lacking but it was recently shown that rat skeletal muscles composed primarily of fast type II or slow type I fibers contain 32 and 11 μmol CASQ per liter of fiber volume, respectively (Murphy et al., 2009). Considering that the trout heart contains 60–80% of the CASQ content of the myotomal muscle and using the value of 11 $\mu mol\,l^{-1}$ measured for slow fibers of the rat skeletal muscle, CASQ content of the trout heart would be 6.6–8.8 $\mu mol\,l^{-1}$ myocyte volume. Further, assuming that fish SR volume is 4% of the myocyte volume (Bowler and Tirri, 1990), concentration of the omCASQ2 in trout heart SR would be 0.165–0.220 $mmol\,l^{-1}$. Cardiac CASQ can bind about 60 Ca^{2+} ions per molecule (Park et al., 2004), which gives a total Ca^{2+} buffering capacity of 15.8–21.12 $mmol\,l^{-1}$ within the SR (396–528 $\mu mol\,l^{-1}$ myocyte volume) for the trout heart. This is slightly more than in mammalian heart (Shannon and Bers, 1997) and in reasonable agreement with the maximum SR Ca^{2+} content of 18.4 and 26.88 $mmol\,l^{-1}$ (462 and 672 $\mu mol\,l^{-1}$ myocyte volume) in the trout ventricular and atrial cells, respectively (Haverinen and Vornanen, 2009). It should be noted, however, that this calculation is based on lager SR volume (4%) in fish cardiac myocytes in comparison with mammalian cardiac myocytes (1.5%) (Page, 1978), and assumption of a similar SR volume in the two vertebrate groups would mean substantially larger SR Ca^{2+} buffering by CASQ in fish hearts.

Conclusions

The present results suggest that adequate amounts of the omCASQ2 are present in trout atrial and ventricular muscle to buffer the large caffeine releasable Ca^{2+} stores of trout atrial and ventricular myocytes. We did not manage to make direct comparisons on CASQ content between trout and mouse hearts, and therefore the question on larger Ca^{2+} buffering capacity of trout cardiac SR in comparison with mammalian heart still remains unanswered. Purified cardiac CASQs as standards in Western blots and species-specific antibodies are needed to make quantitative comparisons of CASQ content between vertebrate groups and between different fish species and to better understand the physiological significance of CASQ content in cardiac e–c coupling. Ca^{2+} binding capacity of CASQ might be temperature-dependent (Watabe et al., 1991), and it is important to examine the temperature-dependent properties of Ca^{2+} binding of fish cardiac CASQs in order to elucidate its role in SR Ca^{2+} buffering and regulation of RyR function in ectothermic vertebrates.

LIST OF ABBREVIATIONS

CA	cold acclimation
CASQ	calsequestrin
CICR	Ca^{2+}-induced Ca^{2+}-release
e–c	excitation–contraction coupling
MM	molecular mass
Ry	ryanodine
RyRs	ryanodine receptors
SL	sarcolemmal
SR	sarcoplasmic reticulum
TBS	tris-buffered saline
WA	warm acclimation

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