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RESEARCH ARTICLE

Expression of SERCA and phospholamban in rainbow trout (*Oncorhynchus mykiss*) heart: comparison of atrial and ventricular tissue and effects of thermal acclimation

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SUMMARY

In the heart of rainbow trout (*Oncorhynchus mykiss*), the rate of contraction and Ca^{2+} uptake into the sarcoplasmic reticulum (SR) are faster in atrial than ventricular muscle, and contraction force relies more on SR Ca^{2+} stores after acclimation to cold. This study tested the hypothesis that differences in contractile properties and Ca^{2+} regulation between atrial and ventricular muscle, and between warm- (WA) and cold-acclimated (CA) trout hearts, are associated with differences in expression of sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) and/or phospholamban (PLN), an inhibitor of the cardiac SERCA. Quantitative PCR (SERCA only) and antibodies raised against SERCA and PLN were used to determine abundances of SERCA2 transcripts and SERCA and PLN proteins, respectively, in atrium and ventricle of trout acclimated to cold (+4°C, CA) and warm (+18°C, WA) temperatures. Expression of SERCA2 transcripts was 1.6 and 2.1 times higher in atrium than ventricle of WA and CA trout, respectively ($P<0.05$). At the protein level, differences in SERCA expression between atrium and ventricle were 6.1- and 23-fold for WA and CA trout, respectively ($P<0.001$). Acclimation to cold increased SERCA2 transcripts 2.6- and 2.0-fold in atrial and ventricular muscle, respectively ($P<0.05$). At the protein level, cold-induced elevation of SERCA (4.6-fold) was noted only in atrial ($P<0.05$) but not in ventricular tissue ($P>0.05$). The expression pattern of PLN was similar to that of the SERCA protein, but chamber-specific and temperature-induced differences were much smaller than in the case of SERCA. In the ventricle, PLN/SERCA ratio was 2.1 and 7.0 times higher than in the atrium for WA and CA fish, respectively. These findings are consistent with the hypothesis that low PLN/SERCA ratio in atrial tissue enables faster SR Ca^{2+} reuptake and thus contributes to faster kinetics of contraction in comparison with ventricular muscle. Similarly, cold-induced decrease in PLN/SERCA ratio may be associated with faster contraction kinetics of the CA trout heart, in particular in the atrial muscle.

Key words: cardiac contraction, sarcoplasmic reticulum, calcium uptake, fish, excitation–contraction coupling.

INTRODUCTION

Contraction of the cardiac muscle cell is initiated by a transient rise of intracellular Ca^{2+} concentration. In the vertebrate heart, the activating Ca^{2+} is of dual origin: (1) influx from extracellular space *via* sarcolemmal L-type Ca^{2+} channels and Na^+/Ca^{2+} exchanger (NCX) and (2) release from the sarcoplasmic reticulum (SR) *via* SR Ca^{2+} release channels (ryanodine receptors, RyR) (Fabiato, 1983). The trigger for relaxation is the lowering of the cytosolic Ca^{2+} concentration by the combined activity of the sarco(endo)plasmic reticulum Ca^{2+} pump (SERCA), the sarcolemmal Ca^{2+} pump and NCX, which replenish SR and extracellular Ca^{2+} stores (Bers, 2002).

There are large phylogenetic differences in the relative contribution of the two Ca^{2+} stores to contractile activation of vertebrate cardiac myocytes. In endothermic vertebrates, sarcolemmal Ca^{2+} influx functions mainly as a trigger for Ca^{2+} release from the SR, the latter contributing 70–96% of the contractile Ca^{2+} in different species (Fabiato, 1983; Bers, 2002). The situation is opposite in ectothermic vertebrates, where sarcolemmal Ca^{2+} influx through L-type Ca^{2+} channels and/or NCX provides a major part of the contractile Ca^{2+}, while the SR plays a smaller and somewhat variable role in the activation of contraction (Fabiato, 1983; Klizner and Morad, 1983; Tibbits et al., 1992; Hove-Madsen et al., 1999; Vornanen et al., 2002; Shiels and White, 2005). In cardiac muscle of different fish species, SR Ca^{2+} release contributes 0–50% of the contractile Ca^{2+} (Driedzic and Gesser, 1988; Hove-Madsen, 1992; Shiels et al., 1999; Rocha et al., 2007; Galli et al., 2009), and in general atrial myocardium is more strongly dependent on SR Ca^{2+} stores than ventricular myocardium (Gesser, 1996; Aho and Vornanen, 1999; Tiitu and Vornanen, 2003).

In fish hearts, contribution of the SR to cytosolic Ca^{2+} management varies depending on temperature. Acute increases in temperature usually increase SR Ca^{2+} release (Hove-Madsen, 1992; Shiels and Farrell, 1997). However, the thermal history of the fish also affects SR function in the way that acclimation to cold enhances the function of SR in contractile activation. Acclimation to cold appears as a greater inhibitory action of ryanodine (Ry), a blocker of SR Ca^{2+} release channels, on the force of cardiac contraction (Keen et al., 1994; Aho and Vornanen, 1999). This is associated with an increased rate of thapsigargin-sensitive Ca^{2+} uptake in cardiac homogenates of the cold-acclimated (CA) trout (Aho and Vornanen, 1998; Aho and Vornanen, 1999). These findings strongly suggest that temperature-induced changes in cardiac contractility in the CA trout are partly due to increases in the activity of the SR Ca^{2+} pump. However, the molecular basis of these changes is still poorly elucidated.

The interplay between cardiac SERCA2 and phospholamban (PLN) is crucial for Ca^{2+} cycling through the SR and therefore for

relaxation and contraction of the cardiac muscle (Kadambi et al., 1998). The SERCA pump is formed by a single polypeptide with a molecular mass of about 110 kDa (MacLennan et al., 1985) and it transports two Ca^{2+} ions for each ATP molecule hydrolysed. In mammals, several SERCA isoforms are encoded by a family of three genes (*ATP2A1–3*) *via* alternative splicing. The mammalian cardiac SERCA is encoded by the *ATP2A2* gene with three known protein products (SERCA2a–c), from which SERCA2a is the dominant cardiac isoform (Periasamy and Kalyanasundaram, 2007). Two skeletal SERCA isoforms (SERCA1a and SERCA1b) have been cloned and sequenced from fish (Londraville et al., 2000; Zhang et al., 2011), but the total number of SERCA-encoding genes in fish, and their expression in the fish heart, is still unknown. Activity of the cardiac SERCA is regulated by PLN, a small integral membrane protein of 52 amino acids (Tada and Kadoma, 1989). In its dephosphorylated state, PLN binds to SERCA and inhibits SR Ca^{2+} pump activity. Inhibition is relieved under β-adrenergic activation, when protein kinase A (PKA) and/or Ca^{2+}/calmodulin-dependent protein kinases phosphorylate PLN, resulting in increased Ca^{2+} affinity of the SERCA and faster SR Ca^{2+} uptake under non-saturating Ca^{2+} concentrations (Periasamy and Kalyanasundaram, 2007).

We hypothesized that chamber-specific differences and temperature-induced changes in SERCA2 and PLN expression contribute to contractile differences between atrial and ventricular muscle of the trout heart, and between CA and warm-acclimated (WA) trout hearts. Theoretically, the enhanced SR contribution to contractility (atrium *vs* ventricle and CA *vs* WA heart) could occur as a result of either increased expression of the SERCA pump and/or decreased expression of the inhibitory SERCA modulator, PLN. The results of this study are consistent with the hypotheses that expression of the SERCA is higher in atrium than ventricle of the trout heart and that a lower PLN/SERCA ratio of atrial tissue in comparison with ventricle and in CA hearts in comparison with WA hearts contributes to chamber- and temperature-related differences in SR Ca^{2+} cycling.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (Kontiolahti, Finland) and, in the laboratory, initially placed in a 1000 liter stainless steel storage tank at the water temperature of the fish farm pond (~10°C). For temperature acclimation, the fish were randomly divided into two groups [warm acclimation (WA), 239–507 g in body mass, *N*=5; cold acclimation (CA) 140–606 g in body mass, *N*=5] and the temperature of the acclimation tanks (500 liters) was set at the temperature of the storage tank. Temperature was then adjusted by 3°C per day, up or down, until the temperature was 18°C (WA) or 4°C (CA). Fish were maintained at the constant (±1°C) acclimation temperatures under oxygen-saturated conditions for a minimum of 4 weeks before experiments. Trout were fed commercial nutrient pellets (Biomar, Brande, Denmark) five times a week and the photoperiod was 12 h:12 h light:dark. All experiments were conducted with the permission of the national committee for animal experimentation (STH252A).

Cloning of SERCA fragment and sequencing of partial gene

Fish were stunned with a blow to the head and killed by cutting the spine. Isolated atria and ventricles were frozen in liquid nitrogen and stored at –80°C until use. Frozen tissues were thoroughly ground in a mortar in a small volume of liquid nitrogen and dissolved in ice-cold TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated according to the instructions of the manufacturer and stored in 75% ethanol (in RNase-free water) at –80°C until use. RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA), and extracted with phenol–chloroform–isoamyl alcohol (25:24:1) followed by a second extraction with chloroform–isoamyl alcohol (24:1). RNA was precipitated from the aqueous phase with absolute ethanol (2.5 × vol) and 3 mol l^{-1} sodium acetate (pH 7) (0.1 × vol) at –20°C for 30 min, washed in 70% ethanol, dried and dissolved in RNase-free water. One to 10 μg of the total RNA was used as a template in cDNA synthesis by 400 U of RevertAid Reverse Transcriptase (Fermentas, MD, USA). One microliter of the reaction mix was used as a template in the PCR amplification reactions by DyNAzymeEXT DNA Polymerase (Finnzymes, Espoo, Finland) including 5 pmol of both degenerative primers (SERCA2Fw and SERCA2Rv) (TAG Copenhagen A/S, Copenhagen, Denmark) (Table 1). The primers were designed according to the sequences of the SERCA2 gene of human (*Homo sapiens*, GenBank accession number NM_001681.3) and rabbit (*Oryctolagus cuniculus*, NM_001089321.1), zebra fish (*Danio rerio*) cardiac/slow twitch Ca^{2+} ATPase paralogs, *atp2a2a* (SERCA2a, NM_200965.1) and *atp2a2b* (SERCA2b, NM_001030277.1), and the corresponding EST sequence of the killifish (*Fundulus heteroclitus*, CN958843.1). The amplification procedure consisted of four degenerative cycles of denaturation at 94°C for 30 s, annealing at 40°C for 30 s and extension at 72°C for 1 min, and 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 30 s. PCR products were separated on an agarose gel and the product of the expected size was purified with a QIAEXII Gel Extraction Kit (Qiagen, Hilden, Germany) and ligated to pGEM-T Easy vector (Promega). Vectors including the insert were cloned in *Escherichia coli* DH5α cells, extracted with a E.Z.N.A. Plasmid Extraction Kit (Omega Bio-Tek, Norcross, GA, USA) and sequenced with an automatic sequencer using a Terminator Sequencing Kit (Applied Biosystems, Foster City, CA,

Table 1. Primers used in the PCR amplification reactions of the trout SERCA2

Primer name	Primer sequence	Primer length (bp)	T_m (°C)
SERCA2Fw	5′-GTGCTCGTCACSATMGAGAT-3′	20	57.3
SERCA2Rv	5′-GAGGRGTGATCTGGAAGATG-3′	20	57.3
M13 univ	5′-GTAAAACGACGGCCAGT-3′	17	52.8
M13 rev	5′-CAGGAAACAGCTATGAC-3′	17	50.4
omSERCA2QFw	5′-GTGCAATGCCCTTAACAGCCT-3′	21	59.8
omSERCA2QRv	5′-ACGGGCAGTGGCTCCACATA-3′	20	61.4
omDnaJA2QFw	5′-TTGTAATGGAGAAGGTGAGG-3′	20	55.3
omDnaJA2QRv	5′-TGGGCCGCTCTCTTGTATGT-3′	20	59.4

All primers were synthesized by TAG Copenhagen A/S. T_m, melting temperature.

USA) and vector-specific M13 primers (TAG Copenhagen A/S) (Table 1).

Quantitative RT-PCR

SYBR Green fluorescence was used for quantification of transcript levels. To this end, 2 µg of the purified RNA was dissolved in RNase-free water. Half of the RNA was used as a template in cDNA synthesis according to the DyNAmo SYBR Green 2-step qRT-PCR Kit (Finnzymes), while the other half was used in the control reaction lacking the reverse transcriptase. One microliter of the cDNA reaction mix was then used as a template in the real-time quantitative PCR (qRT-PCR) amplification consisting of 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. SYBR Green fluorescence was read after every cycle and the melting curve was measured from 72 to 95°C (every 0.5°C for 1 s) using the real-time PCR instrument (Peltier Thermal Cycler 200 with Chromo 4 Continuous Fluorescence Detector, MJ Research Inc., Waltham, MA, USA). Primers (omSERCA2QFw and omSERCA2QRv) and their sequence positions are shown in Table 1 and Fig. 1, respectively. Results were analysed using Opticon Monitor version 3.1.32 (Bio-Rad Laboratories, Hercules, CA, USA). Expression levels of the SERCA2 were first divided by the expression level of the reference gene in the same animal. Following this internal normalization, the mean SERCA2 expressions in both tissues of both acclimation groups were divided by the mean SERCA2 expression of the atrial tissue of the WA trout. The reference gene, a homolog of member 2 of the DnaJ (Hsp40) subfamily A (DnaJA2) (Hassinen et al., 2007), was chosen because it is found to have a transcription rate less dependent on thermal acclimation than the other commonly used reference genes, e.g. β-actin or ribosomal proteins (Vornanen et al., 2005). The primers of the reference gene, omDnaJA2QFw and omDnaJA2QRv, are shown in Table 1.

Western blotting

Immunoblot analysis was performed using rabbit anti-zebra fish SERCA (anti-drSERCA) or anti-zebra fish PLN (anti-drPLN) antibody as the primary polyclonal antibody, and the ImmunoPure peroxidase-conjugated goat anti-rabbit IgG (H&L) (Thermo Fisher Scientific, Rockford, IL, USA) as the secondary antibody. Primary antibodies for SERCA and PLN were raised against the peptides NH_2-KYGFNELPAEEGKS-COOH and NH_2-CHMTRSAIRRASNIE-COOH, respectively, based on the amino acid sequences 35–48 of SERCA1 (atp2a1, GenBank accession number NP_001007030) and 6–19 of PLN (NP_001188490) of the zebra fish (*Danio rerio*). Both the peptide synthesis and the antibody production were conducted by CovalAb (Villeurbanne, France).

Western blots of SERCA and PLN were made from the same gel (N=5 for each tissue). A small piece (60–190 mg) of atrial or ventricular tissue was homogenized with a glass homogenizer in five volumes of Tris-buffered saline (TBS) including 1% Protease Inhibitor Cocktail (Sigma Chemical Co., St Louis, MO, USA). Total protein content was measured in triplicate from 20 µl of the sample homogenate according to Lowry et al. (Lowry et al., 1951). Samples were diluted in Laemmli sample buffer (Laemmli, 1970), 1:5 (atrial tissue) and 1:7 (ventricular tissue), boiled for 4 min and stored at –80°C. Protein (40 µg) was separated on a 12% polyacrylamide gel (or 7.5% gel in the anti-drSERCA antibody specificity tests) with PageRuler Plus Prestained Protein Ladder (Bio-Rad Laboratories)

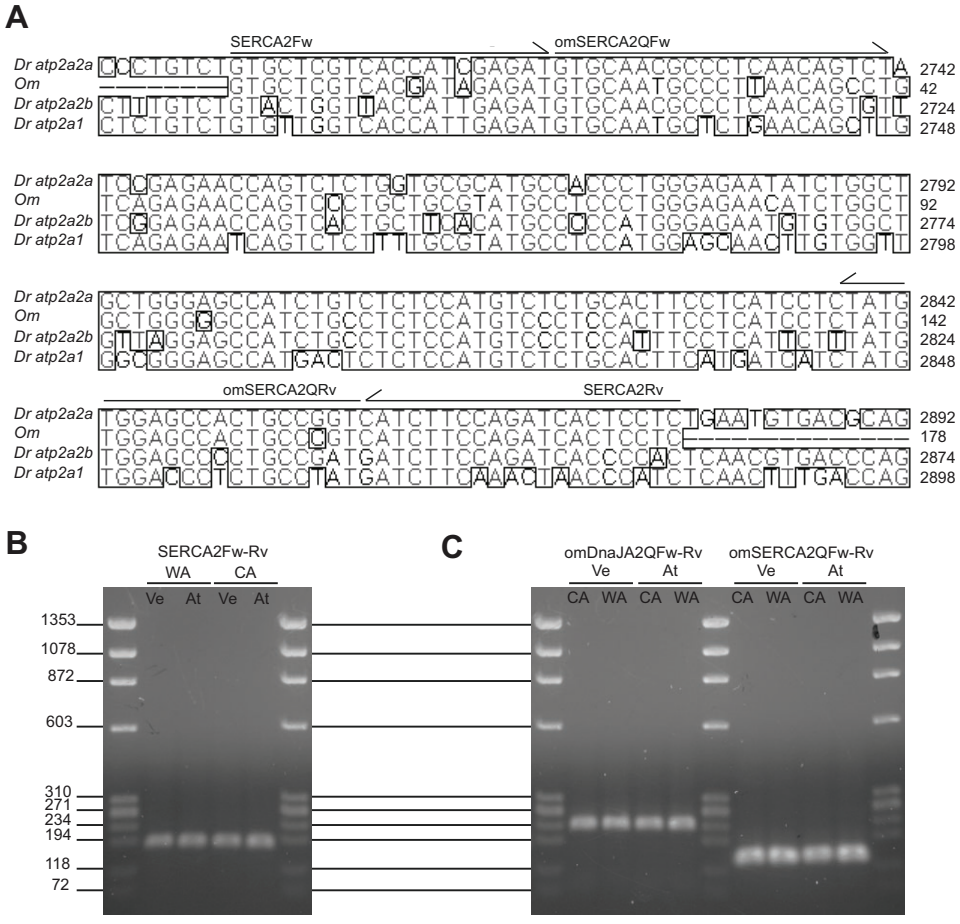

Fig. 1. (A) A sequence of SERCA2 mRNA segment (178 bp) cloned from the atrial muscle of the cold-acclimated rainbow trout (*Oncorhynchus mykiss*, Om) in comparison with the corresponding areas of SERCA2a (*atp2a2a*), SERCA2b (*atp2a2b*) and SERCA1 (*atp2a1*) of the zebra fish (*Danio rerio*, Dr). The pairing sites of the primers used in the cloning (SERCA2Fw and SERCA2Rv) and real-time quantitative PCR (omSERCA2QFw and omSERCA2QRv) are indicated by horizontal arrows. (B) Electrophoretic separation of the products of PCR amplification using degenerative primers (SERCA2Fw-Rv) reveals a single product of approximately 194 base pairs in length. (C) Electrophoretic separation of the products of qRT-PCR amplifications using primers specific to the reference gene (omDnaJA2QFw-Rv) and SERCA2 gene (omSERCA2QFw-Rv) reveal products of approximately 240 and 140 base pairs, respectively. The sizes of markers are given as number of base pairs. WA, warm acclimation; CA, cold acclimation; Ve, ventricle; At, atrium.

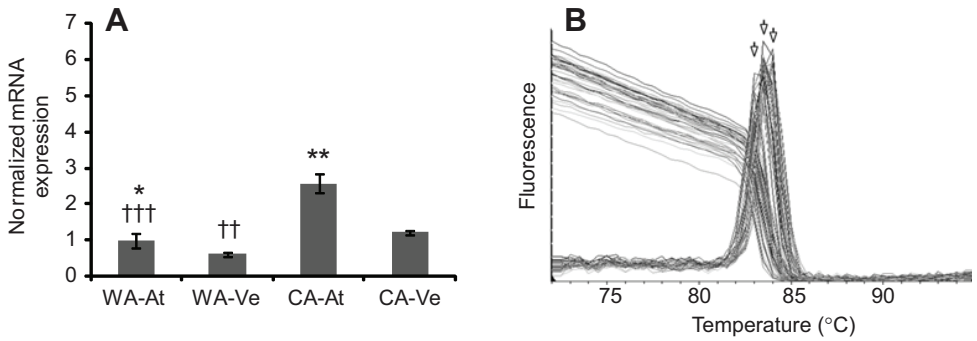

Fig. 2. (A) Expression of SERCA mRNA in atrium (At) and ventricle (Ve) of the rainbow trout (*Oncorhynchus mykiss*) acclimated to either +4°C (CA) or +18°C (WA). Expression levels are normalized to the SERCA expression in the atrium of the WA fish. Asterisks indicate statistically significant differences (**P*<0.05; ***P*<0.01) between atrium and ventricle within the acclimation group and daggers (††*P*<0.01; †††*P*<0.001) between the two acclimation groups. The results are means ± s.e.m. of five fish (*N*=5) and three technical replicates in the real-time quantitative PCR for each tissue. (B) Melting curves of SERCA qPCR indicating 1–3 PCR products with melting temperatures of 83.0, 83.5 and 84.0°C (arrows), respectively.

and, from the same gel lane, transferred to 0.45 µm and 0.2 µm nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad Laboratories) for the detection of SERCA and PLN, respectively. Proteins from the same PAGE run were stained with Coomassie Brilliant Blue R-250 to verify equal sample loading. The mean optical densities (OD) between the 250 and 36 kDa bands in each lane were measured from the photographed SDS-PAGE gel.

Detection was performed using a Pierce SuperSignal West Pico Rabbit IgG Detection Kit (Thermo Fisher Scientific). Five per cent non-fat milk powder (Valio, Helsinki, Finland) in TBS, including 0.05% Tween 20 (TBST), was used as a blocking reagent for 1 h at room temperature (RT, 20°C). Membranes were incubated with the primary antibody overnight at +4°C and with the secondary antibody for 1 h at RT. The primary antibody solution included either anti-drSERCA (1.3 µg ml^{-1}) or anti-drPLN (0.59 µg ml^{-1}) antibody, and the secondary antibody solution contained peroxidase conjugated ImmunoPure Goat Anti-Rabbit IgG (H+L) (40 ng ml^{-1}) diluted with 5% non-fat milk powder in TBST. Membranes were incubated in Pierce SuperSignal West Pico Working Solution (Thermo Fisher Scientific) and exposed to Kodak BioMax MS film (Eastman Kodak Company, Rochester, NY, USA) for 90 s (SERCA) and 30 min (PLN). Films were developed in Kodak LX24 X-Ray Developer (Kodak) and fixed in Ilford Rapid Fixer (Harman Technology Limited, Mobberley, UK). Films were scanned, and ODs of the bands were measured using ImageJ software (version 1.40g, National Institutes of Health, Bethesda, MD, USA).

Statistics

The results are means ± s.e.m. of at least five fish. The data were analysed with one-way ANOVA followed by Tukey's *post-hoc* test or, when comparing the results from atrial and ventricular samples within the acclimation group, with paired samples *t*-test (SPSS Statistics version 17.0; SPSS Inc., Chicago, IL, USA). Before the statistical analysis, the normality of distribution was examined with the Kolmogorov–Smirnov test, and if the criteria of normality were not fulfilled, a logarithmic transformation of the data was performed. A *P* value of 0.05 was regarded as the limit for statistical significance.

RESULTS

Expression of SERCA2 transcripts

The SERCA2 gene (*atp2a2*) of the rainbow trout has not been cloned and its sequence is unknown. In order to design species-specific primers for measuring *atp2a2* gene expression in the heart of rainbow trout, a short stretch of 178 base pairs of the trout *atp2a2* mRNA was cloned and sequenced using degenerative primers (SERCA2Fw and SERCA2Rv; Table 1, Fig. 1). The primers were designed to clone a highly conserved domain near the 3′-end of the *atp2a2* mRNA. The cloned sequence of the trout SERCA2 was 90% identical with the *atp2a2a* of the zebra fish between nucleotides 2701 and 2878, and 79% identical with the corresponding area of the zebra fish *atp2a2b* (nucleotides 2683–2860). In addition, the trout SERCA2 sequence was 71% identical with the zebra fish *atp2a1* (SERCA1, GenBank accession number NM_001007029.1) between nucleotides 2707 and 2884. These comparisons strongly suggest that the cloned trout sequence was indeed a product of the *atp2a2a* gene. Accordingly, the qRT-PCR primers (omSERCA2QFw and omSERCA2QRv; Table 1) were designed based on the cloned area and were assumed to be specific for the *atp2a2a* mRNA of the rainbow trout. All PCR products were separated on an agarose gel to check the specific binding of the primers (Fig. 1B). It should be noted, however, that although the agarose gel electrophoresis showed only one band for all primer pairs, melting curve analysis of the trout *atp2a2* samples revealed three potentially different melting curves, with melting temperatures of 83, 83.5 and 84°C (Fig. 2B). This may indicate that there were more than one *atp2a2* transcripts of approximately the same size.

Expression of the SERCA2 mRNA was measured from atrium and ventricle of CA and WA rainbow trout by qRT-PCR. After setting the mean SERCA2 expression of the atrial tissue of the WA trout as 1.0 (±0.12), the normalized expression levels were 2.6±0.15, 1.2±0.039 and 0.6±0.042 for atrium of the CA fish, ventricle of the CA fish and ventricle of the WA fish, respectively (Fig. 2A). In both acclimation groups, the relative expression of SERCA2 mRNA was higher in atrium than ventricle (P<0.05 for WA and P<0.01 for CA fish). Furthermore, acclimation to +4°C increased SERCA2 mRNA expression 2.6 times in atrium (P<0.001) and 2.0 times in ventricle (P<0.01) in comparison with trout acclimated to +18°C.

Expression of SERCA2 and PLN proteins

Specificity of the fish anti-drSERCA antibody for different SERCA isoforms was characterized by immunoblotting samples from trout atrium, ventricle, brain and white myotomal muscle (Fig. 3A). In the myotomal muscle, the antibody recognized two distinct bands with molecular masses of about 110 and 95 kDa, respectively, while only one band was found in atrial and ventricular muscle. The apparent molecular mass of the trout cardiac SERCA2 was 110 kDa, which is close to the molecular mass of the zebra fish and

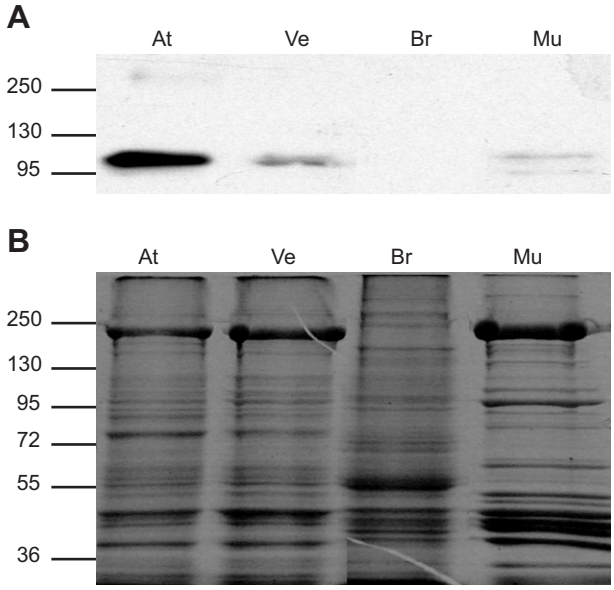

Fig. 3. (A) Binding of the anti-drSERCA1 antibody to the proteins from different tissues of the (CA) rainbow trout (*Oncorhynchus mykiss*) separated in 7.5% polyacrylamide gel. In both atrial and ventricular tissue, the antibody recognized one major band of about 110 kDa molecular mass, while in the white myotomal muscle two equally stained bands with approximate molecular masses of 110 and 95 kDa, respectively, appeared. In the homogenate of the whole trout brain, the antibody did not recognize any protein. (B) A 7.5% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250 shows similar protein loading of the samples. At, atrial tissue; Ve, ventricular tissue; Br, brain tissue; Mu, white myotomal muscle.

mammalian SERCA2. Obviously the antibody recognizes the fast skeletal muscle isoform SERCA1, in addition to the cardiac/slow-twitch muscle isoform SERCA2. Despite similar sample loading (Fig. 3B), no bands were detected in brain tissue, suggesting that either the antibody does not recognize the non-muscle isoform (SERCA3) or the amount of the SERCA in the trout brain is below the detection limit of the method used.

Chamber- and temperature-related differences were detected in the amount of SERCA proteins of the trout heart (Fig. 4A). After setting the SERCA expression of the WA atrium as 1.0 (±0.37), the normalized expression levels for atrium of the CA fish, ventricle of the CA fish and ventricle of the WA fish were 4.6±1.2, 0.20±0.039 and 0.16±0.018, respectively. In both acclimation groups, the expression of SERCA was higher in atrium than ventricle (P<0.05 for WA and P<0.001 for CA fish). Interestingly, although acclimation to cold raised the expression of SERCA almost 5-fold in the atrium (P<0.05), it did not alter the amount of SERCA in the ventricle (P>0.05).

In the heart of rainbow trout, the fish anti-drPLN antibody recognized one band with a molecular mass slightly less than 10 kDa (Fig. 4B), representing the PLN monomer. When PLN expressions were normalized to the PLN level of the WA atrium (1.0±0.15), the expression levels for atrium of the CA fish, ventricle of the CA fish and ventricle of the WA fish were 2.2±0.43, 0.72±0.23 and 0.58±0.054, respectively. Abundance of PLN was over three times higher in atrial than ventricular muscle of the rainbow trout heart (P<0.01). Acclimation to cold raised the expression of atrial PLN over 2-fold (P<0.05) but, similar to the SERCA expression, thermal acclimation did not have any effect on the amount of PLN in the ventricular muscle (P>0.05).

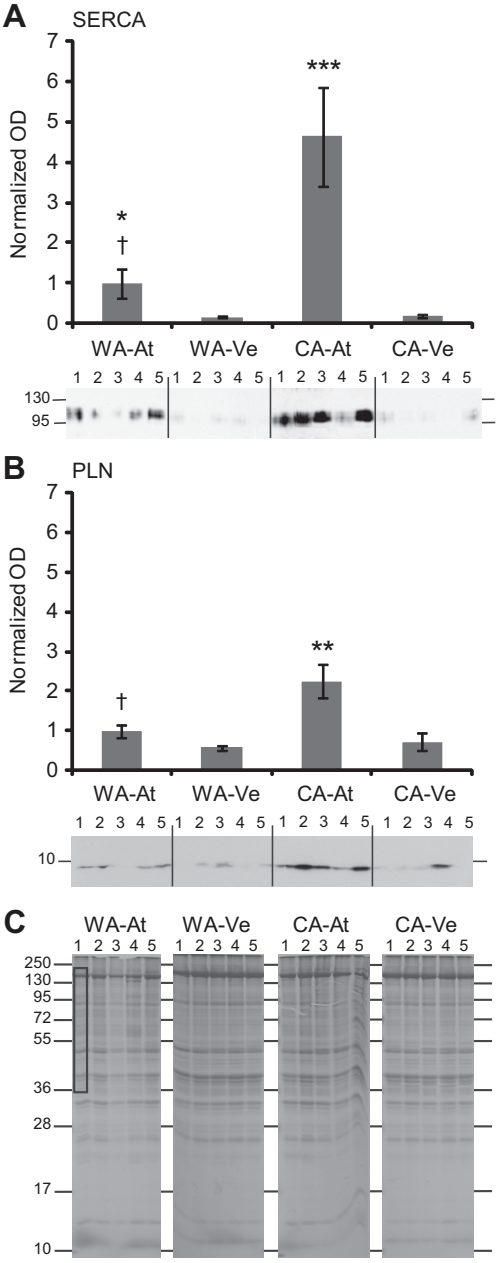

Fig. 4. Expression of SERCA2 and PLN in atrium (At) and ventricle (Ve) of the rainbow trout (*Oncorhynchus mykiss*) acclimated to either +4 (CA) or +18°C (WA). Bar graphs indicate mean (± s.e.m.; N=5) optical densities from western blots after separating the proteins by SDS-PAGE on 12% polyacrylamide gel and immunoblotting with (A) the anti-drSERCA and (B) the anti-drPLN primary antibody. The values are normalized to the mean optical density of the atrial samples from the WA fish. Asterisks indicate statistically significant differences (*P<0.05; **P<0.01; ***P<0.001) between atrium and ventricle within the acclimation group and the dagger (†P<0.05) between the two acclimation groups. Western blots from five fish (1–5) for each tissue are shown below the bar graphs. (C) A 12% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250 was used as loading control. The mean optical densities were measured from the same area between 36 and 250 kDa of every lane (area indicated by a box on the lane WA-At 1).

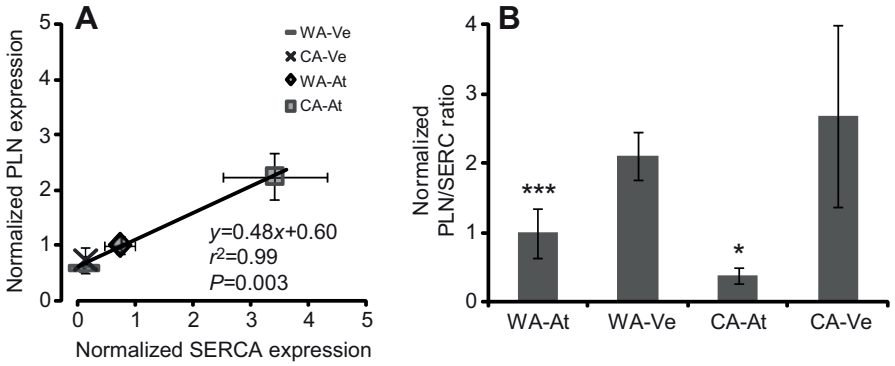

Fig. 5. Comparison of SERCA and PLN expression in the atrium (At) and ventricle (Ve) of the rainbow trout (*Oncorhynchus mykiss*) acclimated to either +4 (CA) or +18°C (WA). (A) Correlation between the protein expression of SERCA and PLN. All values are normalized to the mean optical density of the atrial samples from the WA fish. (B) PLN/SERCA ratio. Asterisks indicate statistically significant differences (*P<0.05; ***P<0.001) between atrial and ventricular samples within the acclimation group.

PLN/SERCA ratio is important for the activity of the SERCA pump, and it was determined by immunoblotting PLN and SERCA from the same gel lane for each sample. A close correlation (r^2=0.99, P=0.003) existed between the expression of SERCA and PLN (Fig. 5A), but the expression of PLN seemed to be less dependent on acclimation temperature, and the difference in PLN expression between atrial and ventricular tissue was less than in the case of SERCA. PLN/SERCA ratio was 2.1 (P<0.001) and 7.0 (P<0.05) times higher in ventricle than atrium for WA and CA fish, respectively (Fig. 5B). Temperature acclimation did not alter PLN/SERCA ratio in either atrial or ventricular tissue (P>0.05), despite the increasing trend from CA to WA atrium. It should be noted that the observed differences were not due to variability in protein loading of the gels (Fig. 4C). The mean ODs were measured from each lane between the 250 and 36 kDa bands. Within the groups (WA-At, WA-Ve, CA-At, CA-Ve), the ODs differed no more than 2.2% from the group mean. When comparing the groups, the mean ODs of CA-At and WA-Ve samples (0.312±0.00719 and 0.309±0.00474) were equal (P>0.05), and only slightly higher (P<0.05) than the mean ODs of WA-At and CA-Ve samples (0.265±0.00678 and 0.271±0.00264), which were equal (P>0.05) to each other. The small difference could be due to the 'edge-effect', i.e. more intensive staining of the proteins in the lanes nearest to the gel edge (Aldridge et al., 2008). The loading of any sample did not exceed the 15% standard deviation of the mean of all samples. Thus, we believe that the differences in the expression of SERCA and PLN and the lower PLN/SERCA ratio in the atrium of the CA fish are not a consequence of uneven protein loading.

DISCUSSION

The present results show that expression of two important SR molecules, SERCA and PLN, is higher in atrial than ventricular muscle of the rainbow trout heart. Notably, acclimation to cold promotes the expression of atrial SERCA and PLN, while abundances of ventricular SERCA and PLN remain unchanged. These findings provide molecular level explanations for the previously noted differences in contraction kinetics between atrial and ventricular muscle, and the functional changes in contractility of the trout heart following thermal acclimation.

Correlation between functional properties of trout heart and SERCA expression

The rate of isometric contraction is much faster in atrial than ventricular tissue of the trout heart, and acclimation to cold (+4°C) increases the rate of contraction in both cardiac compartments (Aho and Vornanen, 1999). Rapid contraction kinetics of the atrial myocardium is associated with a faster Ca^{2+} uptake rate of atrial SR in comparison with ventricular SR (Aho and Vornanen, 1998; Aho and Vornanen, 1999). Consistent with this, cell-type-specific differences in SR Ca^{2+} uptake rate have been detected in intact atrial and ventricular myocytes of the fish heart. In voltage-clamped myocytes of rainbow trout and burbot (*Lota lota*) hearts, the rate of caffeine-sensitive Ca^{2+} uptake was 60–80% faster in atrial than ventricular myocytes (Haverinen and Vornanen, 2009a). Similar to the chamber-related differences, the faster kinetics of contraction following cold acclimation can be partially attributed to the cold-induced enhancement of SR function, which is expressed as faster rate of SR Ca^{2+} uptake in crude homogenates of the trout heart (Aho and Vornanen, 1998; Aho and Vornanen, 1999). The present findings on the expression of SERCA are in general agreement with the functional findings, in that higher expression of the SERCA pump is expected to provide a faster rate of Ca^{2+} sequestration in atrial *vs* ventricular muscle and in CA *vs* WA atrial tissue. The higher SERCA2 expression should appear as a faster removal of cytosolic Ca^{2+}, faster relaxation and therefore a shorter duration of isometric contraction (He et al., 1997; Baker et al., 1998). The present findings are also consistent with the cold-induced proliferation of the fish cardiac SR, as noted in the perch (*Perca fluviatilis*) and bluefin tuna (*Thunnus orientalis*) (Bowler and Tirri, 1990; Shiels et al., 2011).

Chamber-specific and temperature-related differences in contraction kinetics cannot be attributed simply to the differences in SERCA expression, since the activity of SERCA is tightly regulated by PLN. Expression of PLN in different cardiac tissues was qualitatively reminiscent of the expression pattern of the SERCA protein; in particular, expression of PLN was 2.5-fold higher in atrium than ventricle. Differences in SERCA expression would probably have no functional consequences if the expression of PLN closely matches the SERCA expression. However, the differences in PLN expression between atrium and ventricle on the one hand, and between CA and WA fish on the other, are much less than those of SERCA. Therefore, it seems that PLN/SERCA ratio is favorable for high activity of SR Ca^{2+} pump in atrium in comparison with ventricle.

Aho and Vornanen reported that SR Ca^{2+} uptake of the CA trout ventricle was faster than that of the WA trout (Aho and Vornanen, 1998; Aho and Vornanen, 1999). However, in the present study, no differences in SERCA (or PLN) expression could be resolved between WA and CA trout ventricles. This apparent discrepancy may be explained by the high adrenergic tone of the CA trout heart (Graham and Farrell, 1989; Keen et al., 1993). Since the inhibitory effect of PLN on SERCA is relieved by activation of the β-adrenergic cascade (Periasamy and Kalyanasundaram, 2007), the higher adrenergic tone of the CA trout heart in comparison with the WA heart is expected to result in a faster SR Ca^{2+} uptake rate, even if the expression of PLN and SERCA were the same in CA and WA trout ventricles. In atrial muscle, the enhanced β-adrenergic signaling of the CA trout heart (Graham and Farrell, 1989; Keen et

al., 1993; Aho and Vornanen, 2001) would act synergistically with the observed temperature-related changes in the expression of atrial SERCA to specifically enhance atrial function of the CA trout (see 'Physiological significance of SERCA expression'). In fish hearts, the role of PLN-dependent regulation of SERCA may be particularly important, because β-adrenergic stimulation does not decrease Ca^{2+} sensitivity of the contractile element and consequently does not promote relaxation at the myofilament level (Gillis and Klaiman, 2011). However, to solve these issues, the phosphorylation level of the PLN needs to be determined directly in the hearts of thermally acclimated fish.

Physiological significance of SERCA expression

The present results indicate that expression of the SERCA–PLN system is markedly higher in atrial than ventricular muscle of the trout heart and that acclimation to cold specifically enhances the expression of atrial SERCA and PLN. This is consistent with the previous findings that atrial contraction is more strongly dependent on SR Ca^{2+} stores in comparison with ventricular contraction and that acclimation to cold enhances participation of SR in contractile activation more strongly in atrial than ventricular tissue of the trout heart (Aho and Vornanen, 1999). It is also known that atrial muscle has faster myofibrils than ventricular muscle and that acclimation to cold increases the activity of myofibrillar ATPase in the trout heart (Degn and Gesser, 1997; Aho and Vornanen, 1999; Yang et al., 2000). Furthermore, atrial action potentials (AP) are shorter than ventricular APs, and acclimation to cold decreases the duration of cardiac AP in both atrial and ventricular muscle of fish, including the rainbow trout (Haverinen and Vornanen, 2009b). Therefore, the higher SERCA content of atrium in comparison with ventricle and the higher expression of SERCA in CA than in WA trout heart seem to be tuned to myofibrillar ATPase activity and AP duration to produce a well-organized contractile entity. Synergistically, the three factors contribute to faster contraction in atrial muscle and in CA fish heart in comparison with ventricular tissue and WA fish hearts, respectively. In contrast, temperature has no effect on the expression of RyR (Tiitu and Vornanen, 2003), nor are there any chamber-related differences in RyR expression of the trout heart (Birkedal et al., 2009). Similarly, the amount of main SR luminal Ca^{2+} store protein, calsequestrin (Korajoki and Vornanen, 2009), and the size of the SR Ca^{2+} stores of the rainbow trout atrial and ventricular myocytes remain unaltered in thermal acclimation (Haverinen and Vornanen, 2009a). Evidently, expression level and activity of the SERCA pump are the main means to produce chamber- and temperature-specific changes in the rate of Ca^{2+} cycling through the fish cardiac SR.

The higher SERCA2 content and lower PLN/SERCA2 ratio of atrial muscle in comparison with ventricular myocardium are common findings for trout and mammalian hearts (Koss et al., 1995; Minajeva et al., 1997; Walden et al., 2009) (present study). The same applies to atrio-ventricular differences in myofibrillar ATPase activity, AP duration and contraction kinetics (Piroddi et al., 2007; Walden et al., 2009). These functional and molecular similarities are well in line with the similar functional role of atrial systole in ventricular filling of fish and mammalian hearts (Lai et al., 1998). It is currently thought that filling of the ventricles is a biphasic process in all vertebrates; in the early diastolic filling phase atria are passive conduits for venous return, while in the late phase of ventricular diastole atrial contraction completes the ventricular filling (Lai et al., 1998; Stehle and Iorga, 2010).

If the task of atrial contraction is to provide a 'final kick' for ventricular filling, why then is the atrial SERCA–PLN system specifically strengthened in the heart of CA rainbow trout? The fast atrial contraction is probably critical for the correct timing of the atrial systole just before the ventricular systole, in order to have an impact on the force of ventricular contraction and therefore on the stroke volume. However, fast recycling of cytosolic Ca^{2+} back into the SR is important for forceful atrial contraction and the strength of the final kick. In fish, acute temperature drop decreases heart rate, while stroke volume remains largely unchanged (Graham and Farrell, 1989). Although possible effects of temperature on early and late phases of diastolic filling of the fish ventricle remain unexplored, thermal compensation in the atrial SERCA–PLN system may be part of the mechanism that enables maintenance of stroke volume in the CA fish heart. Thermal compensation of the atrial SERCA function is needed to maintain the integrated functional entity in the face of cold-induced increases in heart rate and myofibrillar ATPase activity of the trout atrial muscle (Aho and Vornanen, 1999; Yang et al., 2000; Haverinen and Vornanen, 2009b).

Perspectives

The present findings are largely based on the use of a polyclonal antibody produced against a highly antigenic and evolutionally conserved area of the zebra fish SERCA1. The amino acid sequence against which it was produced is very similar for SERCA1 and SERCA2, and therefore the antibody is able to recognize several gene products. In cardiac muscle, the anti-drSERCA antibody produced only one major band with a molecular weight of 110 kDa, while in the white myotomal muscle it also recognized a 95 kDa protein. As SERCA1 is not known to be expressed in the heart of mammals or fish (Londraville et al., 2000), the trout cardiac SERCA – recognized by the antibody – is likely to represent SERCA2. In mammals, the *ATP2A2* gene produces three alternatively spliced isoforms (SERCA2a–c), from which SERCA2a is the main cardiac isoform (Periasamy et al., 2008). Because of the whole-genome duplication that occurred in the teleost fish lineage subsequent to its divergence from mammals (Jaillon et al., 2004), fish may possess paralogs to the *atp2a2* gene and have more variability in cardiac SERCA2 transcripts and proteins. Indeed, two paralogs of the *atp2a2* gene, with 1–3 protein coding transcripts, have been cloned from medaka (*Oryzias latipes*, ENSORLG00000010772 and ENSORLG00000015888 in Ensebl Genome Browser), stickleback (*Gasterosteus aculeatus*, ENSGACG00000017543 and ENSGACG00000013436) and zebra fish (ENSDARG00000029439 and ENSDARG00000005122). Melting curves of the trout *atp2a2* transcripts showed three closely clustered peaks, suggesting that trout heart may express several SERCA2 products. Therefore, future studies should examine putative qualitative (isoform) differences in trout atrial and ventricular SERCA2 and their changes under different thermal conditions. Cloning, sequencing and heterologous expression of different SERCA2 isoforms are needed to fully clarify SERCA protein function and its physiological significance in thermally acclimated fish hearts.

LIST OF ABBREVIATIONS

CA	cold-acclimated/cold acclimation
NCX	Na^+/Ca^{2+} exchanger
PKA	protein kinase A
PLN	phospholamban
RyR	ryanodine receptor
SERCA	sarco(endo)plasmic reticulum Ca^{2+} ATPase
SR	sarcoplasmic reticulum
WA	warm-acclimated/warm acclimation

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