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Elevated Ca^{2+} ATPase (SERCA2) activity in tuna hearts: Comparative aspects of temperature dependence

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Abstract

Tunas have an extraordinary physiology including elevated metabolic rates and high cardiac performance. In some species, retention of metabolic heat warms the slow oxidative swimming muscles and visceral tissues. In all tunas, the heart functions at ambient temperature. Enhanced rates of calcium transport in tuna myocytes are associated with increased expression of proteins involved in the contraction-relaxation cycle. The cardiac SR Ca^{2+}-ATPase (SERCA2) plays a major role during cardiac excitation–contraction (E-C) coupling. Measurements of oxalate-supported Ca^{2+}-uptake in atrial SR vesicles isolated from four species of tunas indicate that bluefin have at least two fold higher Ca^{2+}-uptake than all other tunas examined between 5 and 30 °C. The highest atrial Ca^{2+}-uptake was measured in bluefin tuna at 30 °C (23.32±1.58 nmol Ca^{2+}/mg/min). Differences among tunas in the temperature dependency of Ca^{2+}-uptake were similar for ATP hydrolysis. Western blot analysis revealed a significant increase in SERCA2 content associated with higher Ca^{2+} uptake rates in the atrial tissues of bluefin tuna and similar RyR expression across species. We propose that the expression of EC coupling proteins in cardiac myocytes, and the higher rates of SERCA2 activity are an important evolutionary step for the maintenance of higher heart rates and endothermy in bluefin tuna.

Keywords: Ca^{2+}-ATPase; SERCA2; Tuna; Temperature; Endothermy; Fish; Atrium

1. Introduction

Endothermy in teleost fish and sharks is rare. However, representatives from two families: Lamnidae and Scombridae, have genera that maintain elevated tissue temperatures in their brain, eyes, viscera and swimming muscles (Carey and Teal, 1969; Carey et al., 1985; Block et al., 1993; Bernal et al., 2001). Tunas are active epipelagic predators that display evolutionary convergence with lamnid sharks for a number of morphological and physiological traits that limit the thermal conductance between the body and the sea. Lamnid sharks and tunas possess an elaborate vasculature, composed of counter-current heat exchangers (*rete mirabilia*) located in the circulation of the viscera, brain and body musculature that allow the retention of metabolic heat (Carey and Teal, 1969; Block, 1991; Block and Carey, 1985; Carey et al., 1985). Studies employing electronic tags in these fish have shown that Atlantic and Pacific bluefin tunas can maintain high thermal excess in the muscle and peritoneal cavity (Block et al., 2001; Marcinek et al., 2001; Kitagawa et al., in press). Similarly, lamnid sharks such as the salmon shark (*Lamna ditropis*) maintain significant gradients between ambient water and tissue temperatures. Due to the anatomical constraints of the counter-current heat exchangers, the hearts of these endothermic tunas and sharks function at ambient water temperatures. The active lifestyle of these fish requires cardio-respiratory adaptations that ensure high rates of oxygen delivery to their warm and highly aerobic tissues. How the hearts operates across a wide range of ambient temperatures all the while maintaining an adequate supply to tissues often significantly warmer than the heart remains an open question.

Several studies have highlighted the elevated metabolic and cardiovascular performance in the tuna lineage. For example, the tuna heart maintains higher cardiac outputs, higher ventricular

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pressures and higher heart rates than other teleosts (Brill and Bushnell, 2001; Korsmeyer et al., 1997a,b; Blank et al., 2004). In addition, bluefin tuna hearts have elevated aerobic capacity with high levels of citrate synthase (Driedzic and Gesser, 1994; Moyes et al., 1992; Blank et al., 2004; Swimmer et al., 2004), high mitochondrial oxygen consumption (Moyes et al., 1992), high myoglobin content (Driedzic, 1983; Giovane et al., 1980) as well as a highly developed coronary circulation (Tota, 1983; Farrell et al., 1992). A large gill surface area and thin gill epithelia ensure sufficient oxygen uptake from the water (Muir and Hughes, 1969). Together these traits ensure adequate oxygen supply to the cardiac system and underlie the unique capacity of tunas to sustain elevated cardiac outputs, high metabolic rates and exceptional swimming performance.

Myocyte contraction in fish, as in all vertebrates, is dependent on the intracellular free Ca^2 concentration. In most fish species, extracellular Ca^{2+} is hypothesized to be the primary source of Ca^{2+} for cardiac muscle activation (Tibbits et al., 1992). The L-type calcium channel and the Na^+/Ca^{2+} exchanger have been shown to deliver a large proportion of the Ca^{2+} used during cardiac contraction in several fish species (Hove-Madsen and Tort, 2001; Woo and Morad, 2001; Shiels et al., 2000; Vornanen, 1997, 1999). Alternatively in more active fishes such as trouts, and tunas, it has become increasingly clear that cardiac contraction is dependent on intracellular sarcoplasmic reticulum (SR) Ca^{2+}stores (Brill and Bushnell, 2001; Vornanen et al., 2002; Landeira-Fernandez et al., 2004; Shiels et al., 1999). The role of SR in tunas has been obtained using ryanodine, a specific and potent inhibitor of SR Ca^{2+}release. Ryanodine application to muscle strip preparations from the hearts of tuna and trout has resulted in a decrease in tension development (Keen et al., 1995; Shiels et al., 1998, 2000). Furthermore, recent functional studies have shown significantly higher ventricular SR Ca^{2+} uptake rates in tunas when compared to mackerel (Landeira-Fernandez et al., 2004).

The cardiac cycle is characterized by the sequential contraction of the atrium followed by the ventricle. In mammals, the duration of the contraction is shorter in the atrium than in the ventricle. The basis for this difference stems from the physiological properties of the myocytes themselves, and thus, can be demonstrated *in vivo* as well as in isolated cellular preparations (Aho and Vornanen, 1999; Blank et al., 2004). The faster contraction of the atrial cells requires the myocytes to cycle Ca^{2+} more quickly. Measurements of sarcoplasmic reticulum (SR) function, an intracellular sink for Ca^{2+}, indicate higher calcium uptake rates in rat atrium *vs.* rat ventricle (Minajeva et al., 1997). This is accompanied by an increase in the expression of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) mRNA in rat atrium (Luss et al., 1999).

In this study, we investigated the Ca^{2+}-ATPase activity and expression pattern of SERCA2 and RyR2 in the atrium of tunas of the family Scombridae in response to different temperatures. The SERCA enzymes are a family of membrane-bound ATPases able to translocate Ca^{2+} ions across the membrane using chemical energy derived from ATP hydrolysis. The sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) genes are expressed in different muscle tissues. The SERCA1 gene is expressed in white muscle or fast-twitch skeletal muscle, while the SERCA2a isoform is expressed in cardiac and red or slow-twitch skeletal muscle (Lytton et al., 1992; MacLennan et al., 1985). We hypothesized here that cold tolerant tunas with higher heart rates, elevated metabolism and a higher level of heat conservation in their swimming muscle and visceral tissues, would display higher SERCA2 Ca^{2+}-uptake activities.

2. Materials and methods

2.1. Animals

Pacific bluefin (*Thunnus orientalis*), albacore (*Thunnus alalunga*), and yellowfin tunas (*Thunnus albacares*) with masses between 10 and 15 kg, and bigeye tuna (*Thunnus obesus*) with masses of 30–35 kg were caught on hook-and-line in the California Current off the southwestern coast of North America. All fish were pithed prior to heart removal in accordance with the Stanford University animal use protocol. The fish heart was removed within minutes of capture and in most cases was still beating just prior to freezing. The atrium was separated, cut into small pieces and freeze-clamped with copper tongs cooled in liquid nitrogen. Tissues were frozen in liquid nitrogen and stored on board the vessel and transported to the TRCC (Tuna Research and Conservation Center) in specialized liquid N_2 transport containers (Dry shippers, Taylor-Wharton CX100). Hearts were stored at −80 °C until they were used for the preparations.

2.2. Sarcoplasmic reticulum isolation

Atrial microsomes enriched in sarcoplasmic reticulum vesicles were prepared as previously described (Harigaya and Schwatz, 1969) and modified by Landeira-Fernandez et al. (2004). Briefly, 5 g of frozen heart tissue was homogenized in 10 vol. of buffer containing 20 mM Mops-Tris pH 7.0; 100 mM KCl; 100 mM NaCl, 1 mM $MgCl_2$ and a cocktail of protease inhibitors (1 mM PMSF, 1 μM Pepstatin A, 1 mM Iodocetamide, 1 μM Leupeptin, 1 mM Benzamidine and 0.1 μM Aprotin) using a Teflon pestle and 50–100 ml homogenizer. The homogenate was centrifuged at 10,000*g* twice for 20 min. in a Sorval SS34 rotor at 4 °C. The supernatant was filtered through 2 layers of cheesecloth and then centrifuged at 37,000*g* for 30 min. in a Beckman Ti50.2 at 4 °C. The pellet was resuspended in a high ionic strength medium containing 20 mM Mops-Tris pH 7.0 and 0.6 M KCL using Teflon pestle and then centrifuged at 100,000*g* for 30 min. at 4 °C. The pelleted SR microsomes were resuspended in a small volume of cold buffer containing 50 mM Mops-Tris pH 7.0, 50 mM KCL and 0.32 M sucrose, and stored in liquid nitrogen until use. The protein concentration was quantified by the Bradford (1976) method.

2.3. Ca^{2+} uptake

Unless otherwise specified, 0.6 mg/ml of cardiac SR vesicles was added to a cuvette containing 1.5 ml of 50 mM Mops-Tris pH 7.0, 100 mM KCL, 1 mM $MgCl_2$, 10 mM Sodium Azide, 10 mM

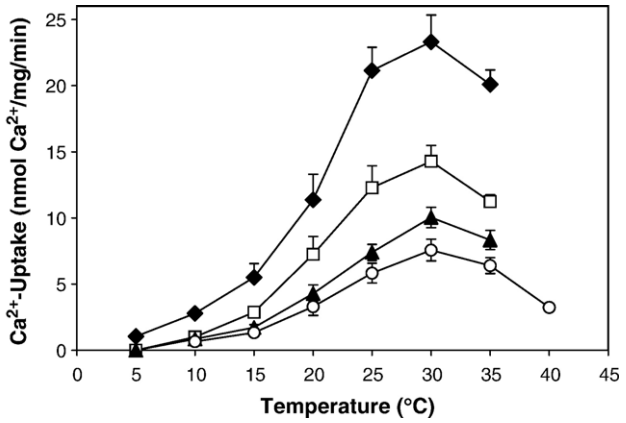

Fig. 1. Ca^{2+} uptake catalyzed by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) in atrial sarcoplasmic reticulum (SR) microsomes. Temperature dependence from (♦) bluefin tuna; (□) albacore tuna; (▲) yellowfin tuna (○) bigeye tuna: Values represent mean±SE of experiments performed with atrial preparations from at least 4 individuals. Absence of error bars indicates that the error bars are smaller than symbol.

potassium-oxalate, 5 mM creatine phosphate and 10 μg/ml creatine phosphokinase (as a ATP regenerating system) and 1.5 μM of the sensitive fluorescent dye fura-2. The cuvette was placed into a Shimadzu RF5301 spectrofluorophotometer and the vesicles and buffer were allowed to equilibrate to temperature for 2 min. The Ca^{2+} uptake reaction was started by the addition of 1.5 mM MgATP. After the steady-state was reached, 10 μM of Ca^{2+} was added and the reaction was allowed to reach steady-state again. The reaction was terminated with the addition of 3 μL of triton x-100 or 1.5 μM of the Ca^{2+} ionophore A_{23187}. Initial rates of Ca^{2+} uptake were calculated from the time it took for the uptake of half the added Ca^{2+} following the method described in Kargacin et al. (1988). The concentration of free Ca^{2+} and the dissociation constant (K_d) for the Fura2/Ca^{2+} complex were measured for each experimental temperature using MaxQuelator (Chris Patton, Stanford University; http://www.stanford.edu/~cpatton).

2.4. ATPase activity

The rate of ATP hydrolysis was measured using the colorimetric method previously described by Fiske and Subbarow (1925). Mg^{2+}-dependent activity was measured in a medium with no added Ca^{2+} and in the presence of 2 mM of EGTA. Ca^{2+}-ATPase activity was determined by subtracting Mg^{2+}-dependent activity from the activity measured in the presence of both Mg^{2+} and 10 μM Ca^{2+}. The reaction medium was composed of 50 mM Mops-Tris pH 7.0, 100 mM KCL, 4 mM $MgCl_2$, 10 mM sodium azide, 3 mM ATP, and 10 μM Ca^{2+} or 2 mM EGTA. At the desired temperature, the reaction was started by the addition of 0.1 mg/ml SR vesicles and stopped by the addition of ice cold 20% TCA. At set times, aliquots from the reaction medium were sampled and the rate of ATP hydrolysis per minute was calculated.

2.5. SDS-PAGE and Western blot analyses

The SR vesicle proteins were separated by electrophoresis on 4–20% pre-cast Tris–Hepes-SDS polyacrylamide mini-gels (Pierce Biotechnology Inc.) or on 3–12% polyacrylamide full size gels according to Laemmli (1970). Gels were silver stained or blotted onto PVDF membranes and probed with either a polyclonal antibody specific to SERCA2 (Morrissette et al., 2003) at 1:750 dilution or a polyclonal antibody specific to the ryanodine receptor (Chugun et al., 2003) at a 1:1000 dilution. Western blots were developed using alkaline phosphatase conjugated goat anti-rabbit secondary antibody (Sigma). SERCA2 and RyR protein expression was assessed densitometrically using both the silver-stained gels and Western blots using NIHimage software.

2.6. Statistical analysis

One-way ANOVA analysis was used to identify significant statistical differences on SERCA2 activity and Western blot analysis, applying the Newman–Keuls test for *a posteriori* analysis. In all statistical analysis, the significant level adopted was 95%.

3. Results

3.1. Temperature dependence of SERCA2 Ca^{2+}-uptake

Atrial SR vesicles from Pacific bluefin, albacore, yellofin and bigeye tuna species were able to sequester Ca^{2+} ions at the expense of ATP hydrolysis. Controlled experiments were done in the presence of 1 μM thapsigargin, a specific inhibitor of SERCA pumps, but ineffective at blocking plasma membrane Ca^{2+} pumps (PMCA) or Na^+/Ca^{2+} exchangers. Thapsigargin completely inhibited Ca^{2+} uptake by atrial SR vesicles. The addition of the detergent, Triton X100, or a specific Ca^{2+} ionophore A_{23187}, resulted in the release of the entire store of sequestered Ca^{2+} from the vesicles (data not shown).

The rates of atrial SR vesicle Ca^{2+}-uptake from different tuna species were measured over a range of temperatures (5–35 °C). Fig. 1 demonstrates that SERCA2 activities in all four tuna species were highly temperature dependent. The rate of Ca^{2+} uptake in tunas increased as the temperature of the medium increased until uptake rates reached maximal activity at 25–30 °C. Temperatures above 30 °C caused a decrease in the rate of Ca^{2+} uptake, possibly due to the partial inactivation of the enzyme (Fig. 1). The SERCA2 SR vesicles from bluefin tuna atrium displayed the highest rate of Ca^{2+} uptake at all temperatures tested being ~2 fold higher than albacore and ~3 fold higher than yellowfin and bigeye tunas (Fig. 1 and Table 1). Among the tunas studied, the atrial SR vesicles from

Table 1
SR-Ca^{2+}-uptake rates in atrium of different Scombrid species

Fish	Rate of Ca^{2+} uptake (nmol Ca^{2+} mg^{-1} min^{-1})
Pacific bluefin	21.15±1.51[a]
albacore	12.28±1.65[b]
yellowfin	7.40±0.60[c]
bigeye	5.83±0.23[c]

Values represent mean±SE of experiments performed with preparations from at least 4 individual fish. The temperature of the assay medium was 25 °C. Different letters are values statistically different ($p<0.05$). The assay medium and experimental conditions were described in Materials and methods.

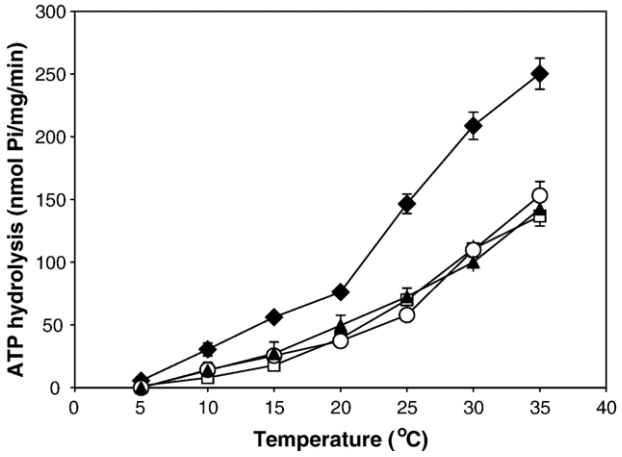

Fig. 2. Temperature dependence of ATP hydrolysis rate catalyzed by the SERCA2 enzyme from (♦) bluefin tuna; (□) albacore tuna; (▲) yellowfin tuna (○) bigeye tuna. Values represent mean±SE of experiments performed with atrial preparations from at least 4 individuals. Absence of error bars indicates that the error bars are smaller than symbol.

bigeye tuna and yellowfin tuna showed the lowest rates of Ca^{2+} uptake with the bigeye having a rate that was slightly below that of yellowfin tuna at all temperatures tested (Fig. 1).

3.2. Temperature dependence of ATP hydrolysis

The ATP hydrolysis measurements in all tuna atrial SR vesicles also showed a strong dependence on temperature The rate of SERCA2 ATP hydrolysis increased continuously with the raise in temperature.. At all temperatures tested, bluefin tuna atrium exhibited a 2–3 fold higher ATP hydrolysis rate than the other tunas (Fig. 2). At temperatures below 10 °C, Ca^{2+}-dependent ATP hydrolysis could only be accurately measured in bluefin tuna atrium SR vesicles. These data are consistent with the Ca^{2+} uptake measurements shown in Fig. 1. However, bigeye, yellowfin and albacore tuna displayed no difference in the rate of ATP hydrolysis (Fig. 2). This is distinct from the differences found in the atrial Ca^{2+} uptake rates in these species (Fig. 1), but this difference could be due to the low accuracy of the ATP hydrolysis methodology.

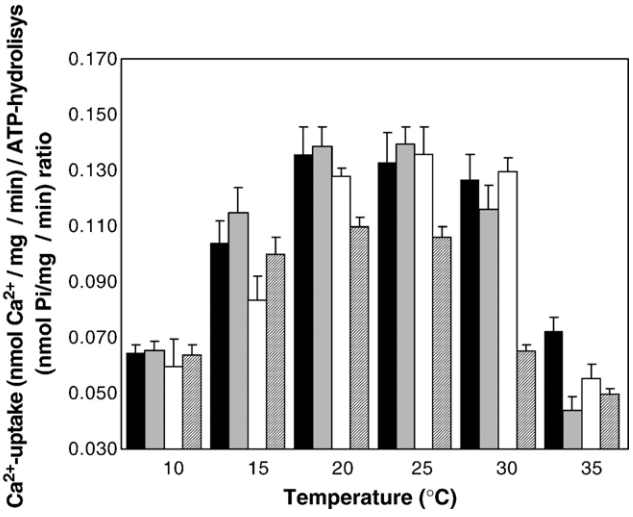

Fig. 3. Ca^{2+}uptake and ATP hydrolysis ratio catalyzed by the SERCA2 enzyme. (■) bluefin tuna; (■)albacore; (□)yellowfin tuna; (▒) bigeye tuna. Values represent mean±SE.

Table 2
Q_{10} from SR Ca^{2+}-uptake rates in atrium of different Scombrid species

	Temperature Interval
Fish	10–20 °C
Bluefin	5.39±0.35
Albacore	4.94±0.24
Yellowfin	4.08±0.38
Bigeye	4.44±0.48

Values represent mean±SE of experiments performed with preparations from at least 4 individual fish.

3.3. Coupling between Ca^{2+}-uptake and ATP hydrolysis

The ratio between Ca^{2+} transported to ATP hydrolyzed indicates how coupled the Ca^{2+} transported across the SR membrane is due to the ATP hydrolysis. The maximal coupling ratio was very similar for all tuna species, showing maximal efficiency at temperatures between 20 and 30 °C. The exception was in bigeye tuna, which showed a maximum coupling ratio at lower temperatures, between 15 and 25 °C (Fig. 3).

3.4. Q_{10}

The effects of temperature on the rate of SERCA2 activity were quantified by calculating the temperature coefficient (Q_{10}) at the linear portion of the Arrhenius plot. The increase of calcium uptake with rising temperature showed a break above 25 °C on the Arrhenius plot (data not shown). Calculated Q_{10s} for SERCA2 activity were ~4–5 over the linear portion of the Arrhenius plot and showed no significant differences among tuna species at a temperature interval of 10–20 °C (Table 2).

3.5. Gel electrophoresis and Western blot analysis

SDS-Gel electrophoresis and Western blot analysis using polyclonal antibodies specific to SERCA 2, showed an increase in SR protein expression among the Scombrid tunas and corroborate the Ca^{2+} uptake results above. A single 110 kD band reacts with the polyclonal antibody raised against a conserved cardiac SERCA2 protein sequence (Fig. 4). Analysis of protein band

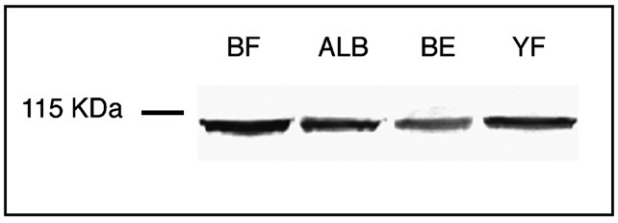

Fig. 4. Western blot analysis of microsomes with 15 μg of protein from atrium of different species of tunas. (MM) Molecular mass markers; BFT (bluefin tuna), ALT (albacore tuna), YFT (yellowfin tuna) and BYT (bigeye tuna). Molecular mass (110KDa) estimated based on the relative mobility of the protein indicates the SERCA2 pump.

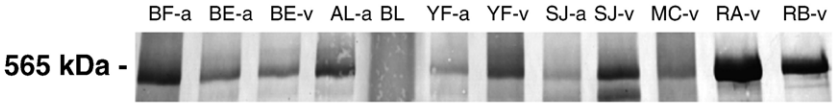

Fig. 5. Western blot analysis of polyclonal antibody specific to fish ryanodine receptor 1 (RyR) in microsomes from different species of tuna and mammals. (BF-a) bluefin atrium, (BE-a) bigeye atrium, (BE-v) bigeye ventricle, (AL-a) albacore atrium, (BL) blank, (YF-a) yellowfin atrium, (YF-v) yellowfin ventricle, (SJ-a) skipjack atrium, (SJ-v) skipjack ventricle, (MC-v) mackerel ventricle, (RA-v) rat ventricle, (RB-v) rabbit ventricle. Molecular mass (565 KDa) estimated based on the relative mobility of the protein indicates the RyR.

densities revealed that bluefin tuna had significantly higher expression of atrial SERCA2 when compared to other tuna species. Bluefin tuna SR vesicles have 1.58±0.15; 1.8±0.21; 2.07±0.19 (n=5) fold higher expression of atrial vesicle SERCA2 than albacore, yellowfin and bigeye tuna respectively.

Western blot analysis also revealed that an antibody prepared from carp heart ryanodine receptor (Chugun et al., 2003) exclusively reacts with a single band of 567 kD in SR microsomes prepared from tunas and mammals (Fig. 5). The analysis of RyR protein band densities revealed that all tunas displayed a greater expression of ventricular RyR protein than mackerel, but no significant differences were found between the tuna species. As expected, the rat and rabbit mammalian ventricular SR displayed much higher RyR expression levels than the fish species.

4. Discussion

In the present study, sarcoplasmic reticulum Ca^{2+}-uptake, Ca^{2+}-ATPase activity, and SERCA2, and RyR2 expression were examined in four species of tunas. The use of identical experimental conditions allowed a direct comparison of SR Ca^{2+}-handling in the tropical and cold-temperate tuna species. The Ca^{2+} cycling by SR in the heart of the tunas was investigated by comparing the temperature dependence of the Ca^{2+}-ATPase (SERCA2) in atrial microsomal vesicle preparations. The measurements of oxalate-supported Ca^{2+}-uptake in SR-enriched heart vesicles indicates that Pacific bluefin tunas are able to sustain the highest Ca^{2+}-uptake rate across a broad range of temperatures when compared to the other tuna species. The rates of Ca^{2+}-uptake and ATP hydrolysis in all four tuna preparations decreased significantly as temperature dropped (Figs. 1 and 2). Low SERCA2 activity would result in a slowing of the relaxation rates in the atrium and ventricle tissues. This decrease in SERCA2 may be a causal link to the reduction of cardiac function at cold temperatures observed *in vitro* and *in vivo*. Whole animal *in situ* preparations and *in vivo* recordings of heart function have shown that tuna hearts show a marked cold-induced bradycardia (Korsmeyer et al., 1997a; Blank et al., 2004). Low temperatures resulting in a decrease in SERCA2 activity were observed in carp, yellowfin tuna and Pacific bluefin tuna ventricular preparations providing further evidence for a molecular basis for a decrease in whole animal cardiac performance in the cold (Aho and Vornanen, 1998; Landeira-Fernandez et al., 2004). These observations are consistent with data obtained in this study on tuna atrial microsomal preparations indicating that reductions in temperature result in a significant decrease in the translocation of calcium by the SERCA2 pump.

The SERCA2 temperature dependence and coupling for SR atrial vesicles measured in this study, displayed similar patterns for bluefin, albacore, yellowfin and bigeye tuna (Figs. 1 and 2). This suggests similar rate-limiting steps in the catalytic cycle of this enzyme and similar kinetic characteristics (e.g. vMax) for the SERCA2 isoforms expressed in tunas. What appears to be varying at the cellular level is the absolute content of the enzyme in different species. The Q_{10} of the atrial tuna SERCA2 indicates that the enzyme is strongly influenced by changes in temperature. The Q_{10} calculated for all tuna species studied at the temperature interval between 10–20 °C do not differ statistically. The similarity of SERCA2 temperature sensitivities between tuna species suggests that a similar SERCA2 isoform is expressed in all tuna species studied. The Q_{10} values for the tuna species in the present study were similar to previously published reports on ventricular tissues (Landeira-Fernandez et al., 2004). The Q_{10} for SERCA2 isoforms expressed in the heart of different tuna species are indicative of similar conformational changes of the enzyme as a result of temperature changes (Hochachka and Somero, 2002).

The SERCA2 isoform is a member of the P-type ATPase family and uses the energy derived from ATP hydrolysis to transport ions across the membrane. The SERCA2 enzyme is responsible for the translocation of Ca^{2+} ions at the expense of ATP. The ratio between Ca^{2+}-uptake and ATP hydrolysis indicates the amount of Ca^{2+} transported across the membrane per ATP hydrolyzed. This corresponds to a coupled transport system where the chemical energy derived from ATP hydrolysis is converted into osmotic energy conserved as a Ca^{2+} gradient across the membrane. A high level of coupled transport indicates high efficiency of Ca^{2+} ion translocation at the expenses of ATP (Reis et al., 2001; De Meis et al., 1997). The SERCA2 Ca^{2+}/ATP coupling atrium ratios values in Fig. 3, varied from ~0.070 measured at 10 °C to ~0.150 measured at 20–25 °C for all species of tuna studied and are similar to previous values found for bluefin and albacore tuna ventricular SERCA2, using similar techniques (Landeira-Fernandez et al., 2004). A lower SERCA2 Ca^{2+}/ATP coupling ratio (~0.040) was found in two different mammal heart preparations (humans and dogs) measured at 35 °C using a different reaction medium composition, and $^{45}Ca^{2+}$ and ATP^{32} as radioactive isotopes to measure the SERCA2 activity (Schwinger et al., 1995; Engelender and De Meis, 1996).

This paper demonstrates that the tunas studied display a similar temperature-dependent profile. In all fish tested, the Ca^{2+}/ATP ratio displays a bell shape curve with a maximal ratio between 15 and 30 °C (Fig. 3). Importantly, there was an upper limit of enzyme function beyond which the capacity to transport calcium decrease. Apparently, the SERCA2 isoform found in tuna heart

SR decreases efficiency at high temperatures. The highest temperatures experienced by scombrids may compromise the heart rate and oxygen delivery due to the limitation of calcium sequestration in the sarcoplasmic reticulum.

The decreases in Ca^{2+}-uptake rates are not followed by the ATP hydrolysis at high temperatures, indicating that possibly SERCA2 conformational changes can drive the enzyme to use the energy of ATP hydrolysis towards a different outcome other than Ca^{2+} transport. In this case, part of the chemical energy derived from ATP cleavage is used for Ca^{2+} transport and part can be converted into heat. Evidence reported from different laboratories indicates that enzymes are able to handle the energy derived from the hydrolysis of phosphate compounds in such a way as to determine the parcel that is used for work and the fraction that is converted into heat (Chinet et al., 1992; De Meis, 1998, 2001; De Meis et al., 1997). The ability to modulate the conversion of energy into either heat or work varies depending on both the enzyme and the experimental conditions used.

In mammals, a SR 52 amino acid phosphoprotein called phospholamban (PLB) regulates the cardiac SERCA2 activity by its interactions with the intrinsic membrane protein (Simmerman and Jones, 1998; Tada and Katz, 1982). PLB inhibits the activity of SERCA2 by lowering its affinity for Ca^{2+}. Phosphorylation of PLB by protein kinase A (PKA) and by a membrane-associated Ca^{2+} and calmodulin-dependent protein kinase (CaM kinase) (Simmerman et al., 1986; Wegener et al., 1989) reverses the inhibitory interaction, presumably through dissociation of phosphorylated phospholamban from SERCA2 (Inui et al., 1986; Suzuki and Wang, 1986). Molecular studies have shown the presence of a phospholambam-like protein from different fish tissues, such as fundulus heart and liver and shark rectal glands (Mahmmoud et al., 2000**)**. We performed experiments of Ca^{2+} uptake in the presence of protein kinase A (PKA), but no effect was observed compared with control experiments (data not shown). It is possible that tuna heart SERCA2 activity can also be regulated by phospholambam but more experiments are required to confirm its role in fish hearts.

Recently laboratory studies of the Pacific bluefin tuna indicated that this species is tolerant to cold (Blank et al., 2004). The bluefin tuna heart performs at colder temperatures than the tropical yellowfin tuna. Both tunas show a pronounced cold-induced bradycardia, with the heart slowing as temperature cools. Arrythmias were observed at 8 °C in yellowfin hearts but the bluefin heart remained functional at temperature as low as 2 °C. Metabolic rate measurements also indicate that the bluefin tuna sustains metabolic performance over a cooler range of ambient temperatures than yellowfin tunas (Blank et al., 2007). Field studies using electronic tags on similar sized *Thunnus* species indicate that juvenile Pacific bluefin experience cooler mean water temperatures throughout their seasonal cycle in the California Current (Kitagawa et al., 2006; Schaefer et al., in press). Although the Pacific bluefin tuna exhibit a greater capacity to expand their niche into northern latitudes, both species exhibit a distinctive bounce diving behavior where they maintain only a short period at depth where ambient temperatures are often cooler. Juvenile yellowfin and Pacific bluefin tuna tagged with archival tags show this behavior regularly diving deep depths but maintaining only a short period at depths in cool waters (Kitagawa et al., 2006). This bounce diving behavior mostly likely is associated with the reduction in function of the SERCA2 enzyme. By ascending, the tuna re-warm the heart and increase the enzymatic machinery capacity and potentially increase oxygenation of the tissues.

We have shown here that correlated with the increased cold exposure in bluefin tuna is an increase in expression of cardiac EC coupling proteins when compared to yellowfin (Blank et al., 2004; Landeira-Fernandez et al., 2004). Recent studies of the ultrastructure of the heart confirm the presence of significant sarcoplasmic reticulum content in the tuna myocytes (Di Maio and Block, unpublished data). Furthermore, heart cells in cold-acclimated trout show an increased capacity for sarcoplasmic reticulum (SR) Ca^{2+} cycling (Aho and Vornanen, 1998), and burbot, a cold-active fish, shows a particularly large effect of ryanodine on ventricular force production (Tiitu and Vornanen, 2002). Together these studies point to an increased reliance on the sarcoplasmic reticulum during myocyte contraction in the cold. The major role for increased SR content most likely to provide surface area for incorporation of more SERCA2 pumps.

The increase in SR Ca^{2+} pool influence has been reported in the cardiac muscle contraction cycle during the development of vertebrate animals. Hamilton and Ianuzzo (1991) observed that Ca^{2+} uptake of the heart is faster in smaller mammals with high heart rates and it is associated with an increase in the amount of SR. Landeira-Fernandez et al. (2004) describe an increase on SERCA2 activity on ventricular tissues across the Scombridae family and hypothesize a relationship between the enhancement of EC coupling proteins and the increased metabolic rate of tunas. Western blot analysis showed an increase of SERCA2 binding across the atrial and ventricular tissues of the *Thunnus* members, indicative of an increase in the SR-Ca^{2+} pool associated with the development of higher metabolic rates and in some species, an endothermic capacity. The highest SERCA2 expression found in Pacific bluefin tuna atrial SR vesicles was an indicative of a higher contraction frequency in this muscle. The Western blot analysis indicated that the bluefin tuna has a significantly lower concentration of SERCA2 and RyR2 when compared to mammalian hearts.

The peak in Pacific bluefin tuna calcium handling capacity in the heart tissues coincides with evidence indicating higher metabolic rates in this species, increased cold tolerance in the lab studies (Blank et al., 2007) and prolonged exposures to cooler environmental temperatures as revealed in electronic tagging studies (Kitagawa et al., 2006; Boustany et al., in press; Block et al., 2001, 2005). The significant increased expression of SERCA2 may underlie the niche expansion of this lineage into higher latitudes and colder waters. Maintenance of performance is dependent upon the hearts ability to supply the tissues with oxygen and enhanced expression of cardiac SERCA2 would allow these fish to function at more northern latitudes where cooler sea surface temperatures prevail (Kitagawa et al., 2006). Similar niche expansion and cardiac link has been observed in Lamnid sharks (Weng et al., 2005).

The functional results presented here are consistent with several studies in fish that describe an increase in SR expression

involved in cold acclimation and the improvement of cardiac contractility at cold temperatures. Aho and Vornanen (1998) reported an increase of at least two folds in the rate of Ca^{2+} uptake in crude ventricular homogenates for cold acclimated rainbow trout and Hove-Madsen and Tort (2001) showed that in trout atrial myocytes, low temperatures did not inhibit the function of sarcoplasmic reticulum. The capacity to deliver a higher cardiac output in tunas is thus supported by cardiac muscle that has increased the Ca^{2+} transport capacity in both atrium and ventricular tissues.

The pattern of enzymatic function found for Pacific bluefin tuna and albacore tuna most likely reflects their similar patterns of geographic distribution. These two species are primarily found overlapping in latitudinal habitat preferences, occupying both warm and cold temperate oceanic regions (Kitagawa et al., 2006; Zainuddin et al., 2006). Yellowfin tuna that reside at lower latitudes, experience seasonally warmer temperatures and had atrial SERCA2 that performed best at temperatures above 20 °C. Thus, when tunas occupy warmer waters above 30 °C, which they do occasionally when breeding or temperatures lower than 15 °C, at depth or higher latitudes, there would be a decline of oxygen delivery to the whole animal, due to a decrease in the activity of the heart associated with reduced function of SERCA2 transport. This could potentially lead to a decrease in cardiac output and tissue oxygenation requiring the fish to seek out more optimal water temperatures for cardiac performance.

The influence of the sarcoplasmic reticulum in the regulation of excitation–contraction coupling (E-C coupling) in the heart of these fishes at low and high temperatures remains an open question. A strong SERCA2 temperature dependence was revealed in this study examining enzyme function in different tuna species indicating a decrease in functional transport capacity in the cold. The high levels of Ca^{2+} uptake that were found in bluefin tuna atrial microsomes appears to be a cellular response to the limitations imposed by cold temperatures on P-type ATPases. If a higher SERCA2 pump activity is a precondition for the development of higher metabolic rates and expression of endothermic traits, it suggests that a key step in the evolution of the *Thunnus* lineage was the increased expression of SERCA2 in the atrial and ventricular myocytes. By increasing the capacity to delivery a cardiac output across a wider range of ambient temperatures, this species could readily explore regions with colder seawater temperatures and higher productivity, and in doing so, extend its niche to high latitudes.

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