

## Temperature dependence of the $\text{Ca}^{2+}$ -ATPase (SERCA2) in the ventricles of tuna and mackerel

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**Landeira-Fernandez, Ana M., Jeffery M. Morrisette, Jason M. Blank, and Barbara A. Block.** Temperature dependence of the  $\text{Ca}^{2+}$ -ATPase (SERCA2) in the ventricles of tuna and mackerel. *Am J Physiol Regul Integr Comp Physiol* 286: R398–R404, 2004. First published November 6, 2003; 10.1152/ajpregu.00392.2003.—Recent physiological studies on the cardiovascular performance of tunas suggest that the elevated heart rates of these fish may rely on increased use of intracellular sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  stores. In this study, we compare the cellular cardiac performance in endothermic tunas (bluefin, albacore, yellowfin) and their ectothermic sister taxa (mackerel) in response to acute temperature change. The cardiac sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA2) plays a major role during cardiac excitation-contraction (E-C) coupling, transporting  $\text{Ca}^{2+}$  from the cytosol into the lumen of the SR and thus promoting the relaxation of the muscle. Measurements of oxalate-supported  $\text{Ca}^{2+}$  uptake in SR-enriched ventricular vesicles indicated that tunas were capable of sustaining a rate of  $\text{Ca}^{2+}$  uptake that was significantly higher than the mackerel. Among tunas, the cold-tolerant bluefin had the highest rates of SR  $\text{Ca}^{2+}$  uptake and ATPase activity. The differences among  $\text{Ca}^{2+}$  uptake and ATP hydrolysis rates do not seem to result from intrinsic differences between the SERCA2 present in the different tunas, as shown by their similar temperature sensitivities and similar values for activation energy. Western blots reveal that increased SERCA2 protein content is associated with the higher  $\text{Ca}^{2+}$  uptake and ATPase activities seen in bluefin ventricles compared with albacore, yellowfin, and mackerel. We hypothesize that a key step in the evolution of high heart rate and high metabolic rate in tunas is increased activity of the SERCA2 enzyme. We also suggest that high levels of SERCA2 in bluefin tuna hearts may be important for retaining cardiac function at cold temperatures.

cardiac sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -adenosinetriphosphatase; scombrid fish; Arrhenius plot; excitation-contraction coupling

THE FAMILY SCOMBRIDAE is composed of wide-ranging pelagic fishes including the tunas, bonitos, and mackerels. Tunas are well known for exceptional physiological and morphological specializations that set this group apart from all other teleosts (12). Tunas are swift, powerful swimmers that have morphological and physiological specializations for a highly migratory oceanic lifestyle. Among the Scombridae, tunas are unique in having high metabolic rates and systemic endothermy (12, 17–19). To support their elevated metabolic rates, tunas maintain high heart rates and high cardiac outputs compared with other teleost fish (15). Maximal heart rates of  $>200$  beats/min have been measured in skipjack tunas, exceeding heart rates of most teleosts (35). To produce high cardiac output, tunas have evolved hearts that are large, thick walled, and pyramidal (55). These morphological characteristics help create high ventricu-

lar pressures and high cardiac outputs, typical of high-energy-demand hearts in other taxa (15, 16).

Tunas have a high rate of metabolic heat production (19, 27) and an elaborate system of countercurrent heat exchangers, called *retia mirabilia*, that minimize convective heat loss through the circulatory system (17, 26). Archival tags placed in the peritoneal cavity indicate species such as Atlantic bluefin experience a wide range of environmental temperatures (2.8–31°C) and maintain elevated peritoneal temperatures (~17–33°C) (8). In addition to warming the swimming muscles, temperate tunas have countercurrent heat exchangers in the viscera, brain, and eyes (12, 17, 42). Collectively, the heat exchangers allow tunas to maintain the highly aerobic tissues of the brain, eyes, skeletal muscle, and viscera above ambient water temperature. Consequently, the body is largely defended from external temperature fluctuations that could compromise function. Importantly, the hearts of all tunas operate at ambient temperature, as they receive blood that has been cooled by the countercurrent heat exchangers and the coronary circulation is directly from the gills. Furthermore, the hearts of tunas are located close to the ventral body wall where heat loss via conduction is high. Thus the temperature of the heart in tuna reflects changes in ambient temperature regardless of body size or regional endothermy (16). Direct temperature measurements on freshly caught tunas indicate that while the slow-oxidative muscle is significantly warmer than ambient temperature, the heart remains at ambient temperature (D. J. Marcinek and B. A. Block, unpublished observations).

Tunas range widely across oceanic habitats with their preferred water temperatures varying depending on species. Recent electronic tagging studies have helped to delineate the tuna's habitat (28). Atlantic bluefin tunas (*T. thynnus*) encounter ambient water temperatures from 2.8 to 31°C, and mature fish can spend extended time in cold water masses during yearly migrations or when foraging beneath the thermocline (10, 12). Juvenile Pacific bluefin tuna tagged in the eastern Pacific also show a high thermal tolerance, encountering ambient waters from 4 to 26°C (7, 44). By contrast, yellowfin tuna (*T. albacares*), a tropical and warm temperate species, prefer temperatures that range from 17 to 30°C and only make brief dives to colder waters (7–11°C) below the thermocline (11, 14). Albacore tunas (*T. alalunga*) are temperate fish that appear to prefer water temperatures from about 8 to 20°C (28).

How the cardiovascular system of tunas is capable of maintaining function across the wide range of temperatures potentially encountered during horizontal and vertical movements in the ocean remains unknown (15). Studies of tuna cardiac

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function indicate that a drop in ambient temperature induces a significant bradycardia and a consequent drop in cardiac output for both Pacific bluefin and yellowfin tuna hearts in situ and in vivo (5, 6, 39). However, these studies also revealed that Pacific bluefin hearts are less sensitive to temperature changes than yellowfin hearts. Remarkably, Pacific bluefin hearts sustain rhythmic beating in situ at temperatures as low as  $2^{\circ}\text{C}$ , the coldest temperature examined (5). The cellular specializations permitting bluefin tuna myocytes to maintain function at these cold temperatures remain largely unexplored.

The rates of ventricular myocyte contraction and relaxation are regulated by the cycling of  $\text{Ca}^{2+}$  into and out of the myoplasm. In mammalian and avian cardiac myocytes, sarcoplasmic reticulum (SR) functions as an intracellular  $\text{Ca}^{2+}$  store, decreasing the diffusional distances for  $\text{Ca}^{2+}$  movement during cardiac excitation-contraction (E-C) coupling (4). The significance of the SR in the contraction-relaxation cycle of cardiac muscle can vary greatly among vertebrate classes, between species within the same phylogenetic group, and during the development of an individual (29, 32, 53).

Knowledge of the processes of E-C coupling in fish hearts lags behind that of mammalian hearts. For many fish species, extracellular  $\text{Ca}^{2+}$  rather than intracellular  $\text{Ca}^{2+}$  is thought to be the most important source of activator  $\text{Ca}^{2+}$  for cardiac contraction (53). L-type calcium currents have been characterized and shown to deliver a large proportion of the  $\text{Ca}^{2+}$  in several species (34, 52, 57). The  $\text{Na}^{+}/\text{Ca}^{2+}$  exchanger may also play an important role in some fish species (33, 56). However, as first proposed by Farrell (23), the unusually high heart rates observed in tunas compared with other fish could rely on increased involvement of myocardial SR  $\text{Ca}^{2+}$  cycling (15, 23, 50, 58). Using ryanodine to block SR  $\text{Ca}^{2+}$  release through the ryanodine receptor  $\text{Ca}^{2+}$  channel in cardiac muscle strips results in a decrease of force production in trout, mackerel, and tuna (38, 48, 49). Based on such experiments, the SR appears to play a particularly important role in the atrium and ventricle of yellowfin tuna (25, 50). In fish, cold tolerance has been suggested to involve an increased capacity for SR  $\text{Ca}^{2+}$  cycling in some species. The heart of the cold stenothermal burbot displays a particularly large effect of ryanodine (54). However, interpretation of these experiments is complicated by the interaction of temperature and pacing frequency, both of which affect the ability of ryanodine to inhibit force production in isolated muscle strips (49).

In contrast to the efforts to quantitatively assess and characterize transsarcolemmal  $\text{Ca}^{2+}$  fluxes and ryanodine sensitivity in fish cardiac muscle, few studies have directly measured sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA2) activity and  $\text{Ca}^{2+}$  uptake. Aho and Vornanen (1) first revealed thapsigargin-sensitive  $\text{Ca}^{2+}$ -ATPase activity in homogenates of trout and crucian carp hearts, indicative of a potential role for SERCA-dependent  $\text{Ca}^{2+}$  uptake in fish SR. Subsequent studies have used patch-clamp techniques to calculate changes in SR  $\text{Ca}^{2+}$  content in response to stimulation of trout cardiac myocytes (33, 56). However, direct measurements of the activity and levels of expression of fish cardiac SERCA2 protein are lacking. In this study, the activity, temperature sensitivity, and content of the cardiac SERCA2 isoform were measured in SR microsomes prepared from the ventricles of three species of tuna (yellowfin, Pacific bluefin, and albacore) and a relative, the Pacific mackerel. This study addresses the hypotheses that

tuna hearts exhibit greater capacity for SR  $\text{Ca}^{2+}$  cycling than hearts of closely related ectothermic fish and that tuna species that inhabit cold waters have an increased SR  $\text{Ca}^{2+}$  uptake capacity compared with tropical tunas.

## MATERIAL AND METHODS

**Fish.** Four Pacific bluefin tuna (*Thunnus orientalis*),  $13.7 \pm 1.4$  kg, four albacore tuna (*Thunnus alalunga*),  $8.4 \pm 1.1$  kg, and three yellowfin tuna (*Thunnus albacares*),  $16.8 \pm 4.5$  kg, were caught on hook-and-line off the coast of California. The mass was calculated from measured lengths by length-weight regression equations for each species (11, 44). The fish were euthanized, and the hearts were immediately removed. Ventricles were sliced into small pieces, freeze-clamped, and stored in liquid nitrogen. Tissues were stored for periods of 1–4 mo at  $-80^{\circ}\text{C}$  before experiments. Twenty Pacific mackerel (*Scomber japonicus*),  $0.33 \pm 0.03$  kg, were caught on hook-and-line, transported to the Tuna Research and Conservation Center (TRCC), and held in a tank. Mackerel were euthanized, their hearts were excised, and the ventricles were immediately freeze-clamped in liquid nitrogen.

**Ventricular SR vesicle isolation.** SR-enriched microsomes were prepared by a modified protocol of Harigaya and Schwartz (30). Briefly, in a glass 50-ml tissue homogenizer with a Teflon pestle, between 5 and 10 g of frozen ventricular tissue was homogenized in 10 vol of buffer containing 20 mM MOPS-Tris, pH 7.0, 100 mM KCl, 100 mM NaCl, 1 mM  $\text{MgCl}_2$  and a cocktail of protease inhibitors [0.1 mM phenylmethylsulfonylfluoride (PMSF), 1  $\mu\text{M}$  pepstatin A, 1 mM iodoacetamide, 1  $\mu\text{M}$  leupeptin, 1 mM benzamide, 0.1  $\mu\text{M}$  aprotinin, and 6 mg/ml trypsin inhibitor]. The homogenate was centrifuged twice at 10,000 g for 20 min at  $4^{\circ}\text{C}$  in a Sorval SS34 rotor (Newtown, CT). The supernatants were filtered through two layers of cheesecloth and then centrifuged at 37,000 g for 30 min at  $4^{\circ}\text{C}$  in a Beckman Ti50.2 rotor (Palo Alto, CA). The pellet was resuspended in a high-ionic-strength medium containing 20 mM MOPS-Tris, pH 7.0, and 0.6 M KCl using a Teflon pestle and glass 30-ml homogenizer and then centrifuged at 100,000 g for 30 min at  $4^{\circ}\text{C}$ . 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, divided into aliquots and stored in liquid nitrogen until use. The protein concentration was determined according to Bradford (13).

**$\text{Ca}^{2+}$  uptake.** For measurement of  $\text{Ca}^{2+}$  uptake, 0.6 mg/ml (tunas) or 1 mg/ml (mackerel) of microsomes was added to a temperature-controlled cuvette containing 50 mM MOPS-Tris, pH 7.0, 100 mM KCl, 1 mM  $\text{MgCl}_2$ , 10 mM sodium azide, 10 mM potassium oxalate, 5 mM creatine phosphate, 10  $\mu\text{g}/\text{ml}$  creatine kinase (as an ATP-regenerating system), and 1.5  $\mu\text{M}$  of the  $\text{Ca}^{2+}$ -sensitive fluorescent dye fura 2. The cuvette was placed in a Shimadzu RF5301 spectrofluorophotometer (Kyoto, Japan), and the vesicles and media were allowed to equilibrate to temperature for 2 min.  $\text{Ca}^{2+}$  uptake was stimulated by the addition of 1.5 mM MgATP. After the steady state was reached,  $\text{CaCl}_2$  was added to a final concentration of 10  $\mu\text{M}$ , and the reaction was allowed to reach steady state again. At the end of each experiment, 1  $\mu\text{l}$  Triton X-100 or 1.5  $\mu\text{M}$  of the  $\text{Ca}^{2+}$  ionophore A23187 was added to collapse the SR  $\text{Ca}^{2+}$  gradient. The initial rate of  $\text{Ca}^{2+}$  uptake was calculated from the  $t_{1/2}$  (the time for one-half of the  $\text{Ca}^{2+}$  to be taken up into the vesicles).

In control experiments, neither a 30-min preincubation of vesicles with 350  $\mu\text{M}$  ryanodine nor addition of ryanodine after steady state was reached significantly affected the rate of  $\text{Ca}^{2+}$  uptake catalyzed by the SERCA2 pump (data not shown). Also, the addition of 2  $\mu\text{M}$  ruthenium red before ATP addition had no effect on  $\text{Ca}^{2+}$  uptake rates (data not shown).

**ATPase activity.** ATP hydrolysis was measured using the colorimetric method of Fiske and Subarow (24).  $\text{Mg}^{2+}$ -dependent activity was measured in the presence of 2 mM EGTA.  $\text{Ca}^{2+}$ -ATPase activity was determined by subtracting the  $\text{Mg}^{2+}$ -dependent activity from the

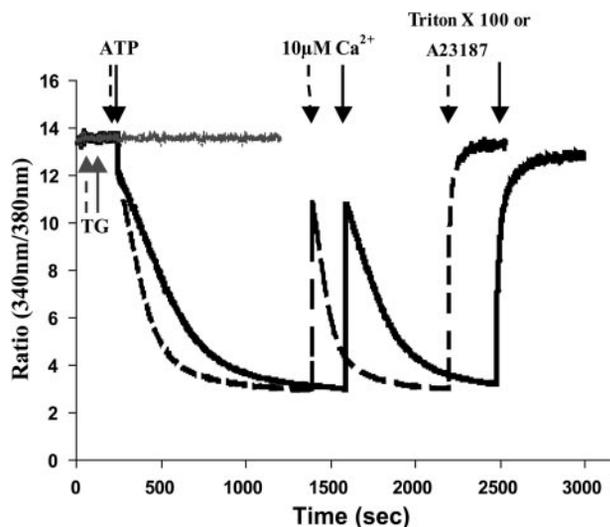


Fig. 1. Representative experiment showing time-dependence of  $\text{Ca}^{2+}$  uptake by ventricular microsomes from Pacific bluefin (dashed line) and albacore (straight line) tunas. Arrows indicate additions during course of reaction. TG indicates addition of  $1 \mu\text{M}$  thapsigargin to the reaction medium of both fish, before addition of ATP. Experimental conditions are as described in MATERIAL AND METHODS, and temperature of assay medium was  $25^\circ\text{C}$ .

total activity measured in the presence of both  $\text{Mg}^{2+}$  and  $10 \mu\text{M}$   $\text{Ca}^{2+}$ . The reaction medium was composed of 50 mM MOPS-Tris, pH 7.0, 100 mM KCl, 1 mM  $\text{MgCl}_2$ , 10 mM sodium azide, 1.5 mM  $\text{MgATP}$ , and  $10 \mu\text{M}$   $\text{Ca}^{2+}$  or 2 mM EGTA. At the desired temperature, the reaction was started by the addition of 0.1 mg/ml SR microsomes and stopped by the addition of 10% TCA. Aliquots from the reaction medium were taken at times of 3, 5, 10, and 30 min. The rate of  $\text{P}_i$  released was linear over this period, and the rate of ATP hydrolysis per minute was calculated.

**SDS-PAGE and Western blot analyses.** Microsomal preparations from bluefin, yellowfin, albacore, and mackerel ventricles were separated by electrophoresis on a 7.5% polyacrylamide gel according to the method of Laemmli (40). Proteins were visualized with silver stain (46). For Western blot analysis, proteins were transferred to PVDF membranes and probed with a polyclonal antibody specific to cardiac SERCA2 (47) at a 1:750 dilution. Blots were developed using an alkaline phosphatase goat anti-rabbit secondary antibody kit (Bio-Rad amplified alkaline phosphatase immunoblot assay kit). SERCA2 content was assessed densitometrically using NIH Image software.

## RESULTS

**$\text{Ca}^{2+}$  uptake.** Microsomal vesicles derived from the ventricles of three species of tunas, Pacific bluefin (*Thunnus orientalis*), albacore (*Thunnus alalunga*), and yellowfin (*Thunnus albacares*), and one species of mackerel (*Scomber japonicus*) retained a functional  $\text{Ca}^{2+}$ -ATPase that was capable of pumping  $\text{Ca}^{2+}$  at the expense of ATP (Fig. 1). The addition of  $1 \mu\text{M}$  thapsigargin, a specific inhibitor of the SERCA pump, which has little to no effect on the plasma membrane  $\text{Ca}^{2+}$ -ATPase, completely inhibited  $\text{Ca}^{2+}$  transport in bluefin, albacore (Fig. 1), and yellowfin tuna ventricular preparations (data not shown). After the steady state was reached, the SR-specific  $\text{Ca}^{2+}$  ionophore A23187 or Triton X-100 could be added to the cuvette to release the stored  $\text{Ca}^{2+}$  (Fig. 1).

Ventricular SR vesicles isolated from Pacific bluefin tuna had the highest  $\text{Ca}^{2+}$  uptake rate among the fish studied. SR  $\text{Ca}^{2+}$  uptake activity in bluefin tuna ventricle was two to three times higher than albacore tuna and four to five times higher

than yellowfin tuna (Fig. 1, Table 1).  $\text{Ca}^{2+}$  uptake in ventricular vesicles from mackerel was extremely slow and difficult to measure, even at a higher protein concentration (1 mg/ml). Using the fura 2  $\text{Ca}^{2+}$  fluorescence methodology, the rate of  $\text{Ca}^{2+}$  uptake in the mackerel preparations could be accurately measured only at the highest temperature of  $25^\circ\text{C}$  and was 20-fold slower than the bluefin  $\text{Ca}^{2+}$  uptake rate at this temperature (Table 1).

Measurements of  $\text{Ca}^{2+}$  uptake over a range of temperatures ( $5$ – $30^\circ\text{C}$ ) indicate that SERCA2 activity is highly temperature dependent in all species tested. The average  $Q_{10}$  values for  $\text{Ca}^{2+}$  uptake found in the three tuna species were  $3.7 \pm 0.76$  between 15 and  $20^\circ\text{C}$  and  $6.9 \pm 0.26$  between 10 and  $15^\circ\text{C}$ . Pacific bluefin tuna ventricular microsomes showed the highest rate of  $\text{Ca}^{2+}$  uptake at all temperatures tested (Fig. 2A). Albacore ventricular microsomes had the second fastest  $\text{Ca}^{2+}$  uptake rate, and yellowfin displayed the slowest uptake rate over this temperature range (Fig. 2A). The temperature sensitivity of preparations from all three tuna species was similar (Fig. 2B).

**Activation energy.** To investigate the kinetics of the tuna ventricular SERCA2 isoforms, Arrhenius plots of the data from Fig. 2 were constructed (data not shown) and used to calculate energies of activation ( $E_a$ ) over  $5^\circ\text{C}$  intervals by linear regression analysis (Table 2). The  $E_a$  values were similar among the three tuna species at each temperature interval. These data indicate that the ventricular SERCA2 isoforms are kinetically similar between the three species of tunas studied and that any intrinsic differences that may exist between the enzymes cannot explain the differences in the rates of  $\text{Ca}^{2+}$  uptake described above.

**ATPase activity.** The rate of  $\text{Ca}^{2+}$ -dependent ATP hydrolysis catalyzed by SERCA2 from bluefin and albacore tuna ventricles also showed a high dependence on temperature. Similar to the rate of  $\text{Ca}^{2+}$  uptake, bluefin tuna displayed a twofold higher ATPase hydrolysis rate than albacore at all temperatures tested (Fig. 3).  $\text{Ca}^{2+}$ -dependent ATP hydrolysis could not be accurately measured in yellowfin tuna or mackerel ventricular vesicles due to the lower activity of the SERCA pump in these tissues.

**SDS-PAGE and Western blot analyses.** A polyclonal antibody raised against a conserved cardiac SERCA2 protein sequence was used to identify and quantify the amount of  $\text{Ca}^{2+}$ -ATPase from the different microsomal preparations (47). The immunoblots revealed a single SERCA2 band corresponding to a protein of  $\sim 110$  kDa in each of the ventricular

Table 1. Comparative rate of  $\text{Ca}^{2+}$  uptake catalyzed by the ventricular SERCA2 from different Scombrid fish ventricular microsomes

Fish	Rate of $\text{Ca}^{2+}$ Uptake, $\text{nmol Ca}^{2+} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$
Bluefin tuna	$10.424 \pm 2.033$ ( $n=4$ )
Albacore tuna	$5.422 \pm 0.444$ ( $n=4$ )
Yellowfin tuna	$1.793 \pm 0.205$ ( $n=3$ )
Mackerel	$0.573 \pm 0.042$ ( $n=3$ )

Values are means  $\pm$  S/E  $n$  = no. of experiments made with preparations from at least 3 individual fish. The assay medium and experimental conditions were as described in MATERIAL AND METHODS. The temperature of the assay medium was  $25^\circ\text{C}$ . SERCA, sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase.

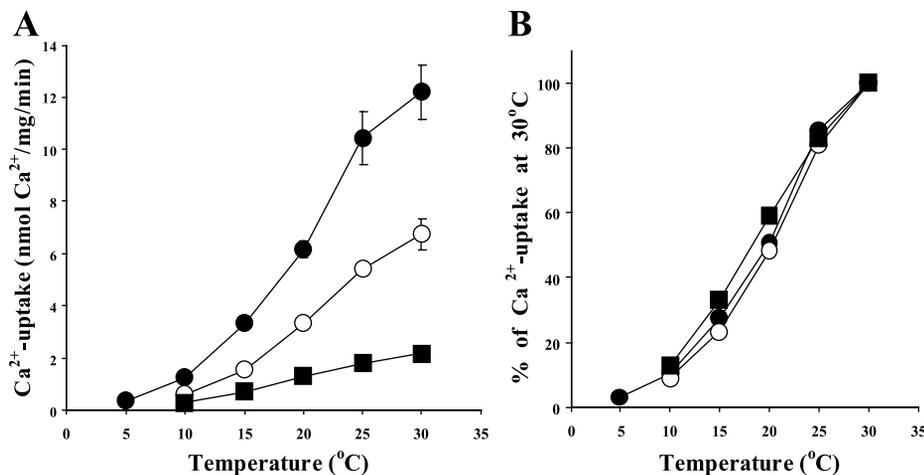


Fig. 2. Temperature dependence of  $\text{Ca}^{2+}$  uptake catalyzed by sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA2) in ventricular sarcoplasmic reticulum (SR) microsomes from different tuna species. A: experimental conditions are described in MATERIAL AND METHODS. ●, Pacific bluefin; ○, albacore; ■, yellowfin. Values represent mean  $\pm$  SE of 3 or 4 experiments performed with preparations from at least 3 individual fish. Absence of error bars indicates that the error bars are smaller than symbol. B: temperature sensitivity of  $\text{Ca}^{2+}$  uptake in 3 species of tunas. Experimental data from A were normalized to a value of 100% at 30°C for each species.

preparations. SERCA2 levels in bluefin tuna were three times higher than albacore and four times greater than yellowfin. Bluefin express the SERCA2 protein at a level  $\sim$ 20 times higher than that of mackerel (Fig. 4). Similar results were obtained from silver stained SDS-PAGE (data not shown).

#### DISCUSSION

In this study, the role of  $\text{Ca}^{2+}$  cycling by SR in the hearts of scombrid fishes was investigated by comparing the properties of the  $\text{Ca}^{2+}$ -ATPase (SERCA2) in ventricular microsomal preparations. Three species of tunas with different thermal environmental preferences and different capacities for heat conservation were compared with an ectothermic mackerel. Measurements of oxalate-supported  $\text{Ca}^{2+}$  uptake in SR-enriched ventricular vesicles indicate that the endothermic tunas (e.g. Pacific bluefin) were capable of sustaining a rate of  $\text{Ca}^{2+}$  uptake that was significantly higher than in the mackerel (Fig. 1 and Table 1).

In all of the tuna ventricular vesicle preparations, the rates of  $\text{Ca}^{2+}$  uptake and ATP hydrolysis declined as temperature dropped (Figs. 2 and 3). Low SERCA2 activity at low temperatures is likely to slow relaxation rates in the ventricle and may place a limit on the heart rate at which the ventricle can operate effectively. Thus temperature-related changes in SERCA2 activity are consistent with data from in situ experiments in yellowfin and Pacific bluefin tuna, indicating cold-induced bradycardia (5).

SERCA2 activities measured in microsomes of all three tuna species displayed similar temperature sensitivities and  $E_a$  (Fig. 2B and Table 2). This suggests similar rate-limiting steps in the catalytic cycle of the enzyme and is an indication that SERCA2

isoforms expressed in the different species are likely to be kinetically similar.

Analysis of the density of SERCA2 pump found in the ventricular SR vesicles using Western blots with an antibody that specifically recognizes cardiac SERCA2 protein revealed that the SERCA2 content varies between scombrid species and follows the pattern of the enzyme activity (Fig. 4). Taken together, the enzymatic data and Western blot data indicate that the differences in  $\text{Ca}^{2+}$  uptake and ATPase activity in scombrid fishes are related to the SERCA2 protein content in these vesicles.

*Variation in SERCA expression among scombrid fishes.* The SERCA2 pump activity varies between all three *Thunnus* species examined. Importantly, all tunas express a higher level and activity of SERCA2 than the mackerel. These results suggest a fundamental difference in the way cardiac myocytes initiate contraction in tunas and mackerel. The increased amounts of SERCA2 in the microsomes derived from the ventricles of tunas indicate increased reliance on internal  $\text{Ca}^{2+}$  stores for contraction and relaxation. In the mammalian myocardium,  $\text{Ca}^{2+}$  influx is initiated through sarcolemmal voltage-gated channels, which serve to trigger a larger  $\text{Ca}^{2+}$  release from the SR in a mechanism known as  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (22). Because SR stores are intracellular, diffusion distances to the myofilaments are relatively short, and the time course of  $\text{Ca}^{2+}$  release and resequestration by the SR can be rapid. Data from this study and previous work (25, 37, 48, 50) indicate that tuna hearts have evolved a more advanced mode of E-C coupling and are less reliant on extracellular  $\text{Ca}^{2+}$  than mackerel.

Table 2.  $E_a$  of SR ventricular  $\text{Ca}^{2+}$ -ATPase in different tuna species

Tuna	Temperature Interval			
	5–10°C	10–15°C	15–20°C	20–25°C
	$E_a$ , kcal/mol			
Bluefin	39.61 $\pm$ 2.95 (n=3)	31.61 $\pm$ 3.57 (n=3)	22.56 $\pm$ 1.36 (n=3)	16.75 $\pm$ 1.19 (n=3)
Albacore	ND	30.70 $\pm$ 2.4 (n=3)	21.50 $\pm$ 2.50 (n=3)	15.60 $\pm$ 2.50 (n=3)
Yellowfin	ND	30.50 $\pm$ 1.41 (n=3)	17.70 $\pm$ 2.30 (n=3)	12.76 $\pm$ 5.13 (n=3)

Values represent means  $\pm$  SE of 3 experiments performed with preparations from at least 3 individual fish.  $E_a$ , activation energy =  $(-\text{slope} \times R)$ . SR, sarcoplasmic reticulum; ND, not determined.

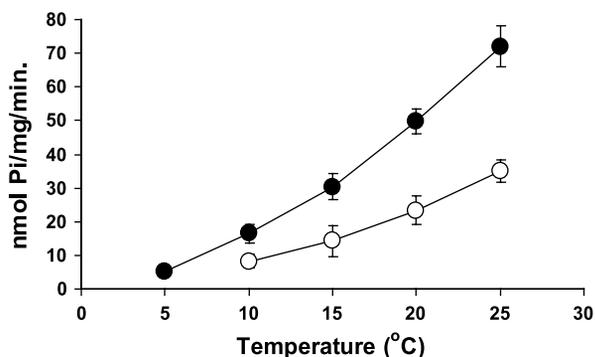


Fig. 3. Temperature dependence of the rate of ATP hydrolysis catalyzed by the SERCA2 enzyme from ventricular SR microsomes from Pacific bluefin (●) and albacore (○). Values represent mean  $\pm$  SE of 3 or 4 experiments performed with preparations from at least 3 individual fish. The absence of error bars indicates that the error bars are smaller than the symbol.

Several recent studies have applied ryanodine to cardiac muscle strips to block SR  $\text{Ca}^{2+}$  release channels to quantify the involvement of SR in fish cardiac muscle contraction. Ventricular strips from Atlantic cod and sea raven were found to be ryanodine insensitive (21). Multiple studies of rainbow trout ventricular strips indicate that ryanodine decreases force production only at subphysiological pacing frequencies or warmer temperatures (31, 38, 49, 51). Similar results were found in ventricular trabeculae from Pacific mackerel, in which ryanodine reduced isometric forces by 20–30% when tested at 20°C but not at all at 15°C (48). Thus, for trout and mackerel, operating at physiological pacing frequencies and at their preferred environmental temperatures, the contribution of SR  $\text{Ca}^{2+}$  in ventricular contraction is most likely minor compared with that of extracellular  $\text{Ca}^{2+}$ .

Work by Keen et al. (37), Freund (25), and Shiels et al. (50) on tropical tuna species indicate that the SR plays a significant role in E-C coupling in the hearts of these fish. Ryanodine decreased force by 30% in skipjack atrial strips without affecting force-frequency relationships (37). Yellowfin atrial strips showed a similar but larger effect, exhibiting a 50% drop in force after ryanodine treatment (50). In addition, yellowfin tuna ventricular strips exhibit strong postrest potentiation across a range of frequencies, indicating a reliance on SR  $\text{Ca}^{2+}$  for contraction (25). Pacific mackerel ventricular strips exhibit only a small degree of postrest potentiation (25). The SERCA2 data presented here further corroborate these earlier studies and provide more evidence for a significant role for the SR in the hearts of tunas.

One hypothesis for the increased expression of SERCA2 and the increased reliance on SR in tuna hearts is that this may reflect a key step in the evolution of high metabolic rates in the tuna lineage. Routine metabolic rates of yellowfin and albacore tuna are at least twofold higher than those of Pacific mackerel (19, 20, 25, 27). A high metabolic rate requires an increased cardiac output. High heart rates and high cardiac outputs have been demonstrated by *in vivo* and *in situ* studies on tunas (5, 6, 15, 36). Evolution of a ventricular myocyte capable of delivering a high cardiac output via increased heart rate may be a cellular requirement for high metabolic rates at an organismal level. Indeed, key steps in the evolution of the high metabolic rates observed in tunas likely include increased expression of E-C coupling proteins in the heart, increased perfusion of the

heart, and increased capillarization of peripheral tissues (2, 45, 55). Ongoing work focusing on the expression of ryanodine receptors in scombrid hearts indicates that the  $\text{Ca}^{2+}$  release channels appear to follow an expression pattern similar to the SERCA2 proteins (J. M. Morrisette and B. A. Black, unpublished data). Future efforts are needed to continue to examine the expression of SR proteins and their role in the myocardium of tunas, bonitos, and mackerels to tease apart the relationship between the evolution of internal SR  $\text{Ca}^{2+}$  stores, increased heart performance, and elevated metabolic rates.

*Variation in SERCA expression among Thunnus species.* The most striking distinction among the three *Thunnus* species is the increase in SERCA2 expression in the bluefin tuna hearts. Results from *in situ* experiments indicate that at cold temperatures Pacific bluefin achieve higher heart rates than Pacific yellowfin tuna (5). The increased SERCA2 expression observed in the bluefin tuna in this study may underlie this capacity for higher heart rates. In addition, the higher cardiac SERCA2 expression in bluefin may be directly related to the cold tolerance of this species. Acoustic and electronic tagging data indicate that all bluefin species have a significantly colder thermal niche than the yellowfin and albacore (7–9, 11, 14, 28, 41, 44). Atlantic and Pacific bluefin tuna encounter waters as cold as 2–4°C and the Pacific bluefin has the highest density of SERCA2 pumps among the species examined. The yellowfin tuna has the lowest SERCA2 activity and protein expression, while albacore, which occupy cooler waters (8–20°C), have intermediate levels (Fig. 4). Results from the heart of a single big-eye tuna (*Thunnus obesus*), a species with the ability to tolerate cold temperatures (15), demonstrated a cardiac SR  $\text{Ca}^{2+}$  uptake rate intermediate in value between yellowfin and albacore tuna ( $2.52 \pm 0.27 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$  at 20°C,  $n = 3$  experiments). The higher SERCA2 expression among the cold-tolerant tunas may permit the rhythmic beating of the heart to continue under cold conditions that may limit  $\text{Ca}^{2+}$  diffusion.

Several studies in fish support the idea that increases in SR expression are involved in cold acclimation and improve cardiac contractility at cold temperatures. In rainbow trout, the rate of  $\text{Ca}^{2+}$  uptake into SR measured in crude ventricular homogenates was enhanced almost twofold by cold acclimation (1), thereby increasing the relative importance of the SR during E-C coupling. A study on burbot, a cold-active fish, found that ryanodine caused a 44% inhibition of maximal ventricular force, indicating a well-developed SR (54). Studies of mammalian hibernators also support the linkage between cardiac SR expression and cold tolerance. Hibernators, whose

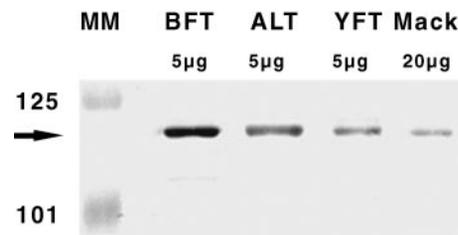


Fig. 4. Western blot analysis of microsomes from different scombrid fish ventricles. MM, molecular mass markers (kDa); BFT, Pacific bluefin tuna; ALT, albacore tuna; YFT, yellowfin tuna; Mack, Pacific mackerel. Nos. above each lane indicate  $\mu\text{g}$  protein used. Arrow indicates the SERCA2 pump with an estimated molecular mass of 110 kDa, calculated based on the relative mobility of the protein.

hearts must continue to function at low temperatures, have a greater capacity for SR  $\text{Ca}^{2+}$  uptake than nonhibernators (43). The increased expression of SR in hibernator myocardium is seasonally regulated, as is the ability of the heart to tolerate cold temperatures (3).

This study has revealed distinct cellular differences in the expression of a key protein associated with the beat-to-beat contraction of scombrid hearts. It suggests that there are fundamental differences in the way tunas and mackerels initiate myocyte contraction and provides additional evidence for the role of the SR  $\text{Ca}^{2+}$ -ATPase in cardiac function of tunas. Further comparative studies of tuna, bonito, and mackerel hearts are required to delineate the roles of other proteins involved in E-C coupling, such as the sarcolemmal  $\text{Ca}^{2+}$  channels and the ryanodine receptor. Speciation of the bluefin tuna group into cooler niches among the tunas may be directly related to cardiac cellular function. Examining cardiac performance at the cellular level in a wider range of *Thunnus* species over a larger range of body masses should shed light on cardiac function and its relationship to thermal niche expansion in these species.

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