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Characterization of ryanodine receptor and Ca^{2+}-ATPase isoforms in the thermogenic heater organ of blue marlin (*Makaira nigricans*)

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Summary

A thermogenic organ is found beneath the brain of billfishes (Istiophoridae), swordfish (Xiphiidae) and the butterfly mackerel (Scombridae). The heater organ has been shown to warm the brain and eyes up to 14°C above ambient water temperature. Heater cells are derived from extraocular muscle fibers and express a modified muscle phenotype with an extensive transverse-tubule (T-tubule) network and sarcoplasmic reticulum (SR) enriched in Ca^{2+}-ATPase (SERCA) pumps and ryanodine receptors (RyRs). Heater cells have a high mitochondria content but have lost most of the contractile myofilaments. Thermogenesis has been hypothesized to be associated with release and reuptake of Ca^{2+}. In this study, Ca^{2+} fluxes in heater SR vesicles derived from blue marlin (*Makaira nigricans*) were measured using fura-2 fluorescence. Upon the addition of MgATP, heater SR vesicles rapidly sequestered Ca^{2+}. Uptake of Ca^{2+} was thapsigargin sensitive, and maximum loading ranged between 0.8 μmol Ca^{2+} mg^{-1} protein and 1.0 μmol Ca^{2+} mg^{-1} protein. Upon the addition of 10 mmol l^{-1} caffeine or 350 μmol l^{-1} ryanodine, heater SR vesicles released only a small fraction of the loaded Ca^{2+}. However, ryanodine could elicit a much larger Ca^{2+} release event when the activity of the SERCA pumps was reduced. RNase protection assays revealed that heater tissue expresses an RyR isoform that is also expressed in fish slow-twitch skeletal muscle but is distinct from the RyR expressed in fish fast-twitch skeletal muscle. The heater and slow-twitch muscle RyR isoform has unique physiological properties. In the presence of adenine nucleotides, this RyR remains open even though cytoplasmic Ca^{2+} is elevated, a condition that normally closes RyRs. The fast Ca^{2+} sequestration by the heater SR, coupled with a physiologically unique RyR, is hypothesized to promote Ca^{2+} cycling, ATP turnover and heat generation. A branch of the oculomotor nerve innervates heater organs, and, in this paper, we demonstrate that heater cells contain large 'endplate-like' clusters of acetylcholine receptors that appear to provide a mechanism for nervous control of thermogenesis.

Key words: ryanodine receptor, RyR, Ca^{2+}-ATPase, SERCA, thermogenesis, heater organ, marlin, *Makaira nigricans*.

Introduction

Most fish are obligate ectotherms. However, several large pelagic fishes of the suborder Scombroidei have independently evolved a unique form of cranial endothermy (Carey, 1982; Block, 1994). The billfishes (family Istiophoridae), swordfish (family Xiphiidae) and the butterfly mackerel (family Scombridae) warm the brain and eyes using a specialized thermogenic organ that has evolved from skeletal muscle (Block et al., 1993; Block and Franzini-Armstrong, 1988; Carey, 1982). The thermogenic 'heater' organs are bilaterally situated beneath the brain and serve to warm the brain and eyes up to 14°C above ambient water temperature (Carey, 1982).

Heater organs are derived from modified extraocular muscle cells that have been optimized for heat production rather than contractility. Heater cells have lost organized myofilaments (Block and Franzini-Armstrong, 1988; Tullis and Block, 1997) but have retained an extensive transverse-tubule (T-tubule) network and sarcoplasmic reticulum (SR) complete with triads (Block et al., 1988a,b, 1994). Electron microscopy studies revealed that as much as 60% of the heater cell in billfishes is composed of tightly packed mitochondria (Tullis et al., 1991). Biochemical studies have shown that heater cells have an exceptional aerobic capacity (Ballantyne et al., 1992; Tullis et al., 1991) and a tight coupling of oxidative phosphorylation and electron transport in the blue marlin (O'Brien and Block, 1996). However, Ballantyne et al. (1992) demonstrated that high millimolar [Ca^{2+}] uncoupled swordfish mitochondria (Ballantyne et al., 1992). Uncoupling proteins have not been found in heater tissue (Block, 1986). The presence of an extensive T-tubule and SR network in close proximity to coupled mitochondria indicates that a Ca^{2+}-dependent process

may underlie thermogenesis in heater cells (Block and Franzini-Armstrong, 1988). However, due to the challenges of conducting *in vivo* work on large pelagic fishes, this hypothesis has never been fully tested. The present study provides an *in vitro* assay that indicates that blue marlin heater tissue has exceptional Ca^{2+} uptake activity with rates comparable with the superfast-contracting toadfish swimbladder muscle. Furthermore, molecular and physiological studies indicate that the extraocular muscle fibers and the heater cells express physiologically unique isoforms of the SR ryanodine receptor (RyR1-slow) and Ca^{2+}-ATPase (SERCA 1B), which may predispose these cells for Ca^{2+} cycling and thermogenesis. In addition, innervation of heater tissue by a branch of the oculomotor neuron and the presence of extensive clusters of acetylcholine receptors (AchR) on the heater plasma membrane indicate that thermogenesis may be under neural control.

Materials and methods

Heater organ microsomal vesicle preparation

Heater organs and extraocular muscles were collected from freshly caught blue marlin (*Makaira nigricans*) in Hawaiian waters according to Block et al. (1994). Tissues were collected from fish captured in a hook-and-line sport fishery and freeze-clamped in liquid nitrogen within 1–2 h of removal. Tissues were stored in liquid nitrogen or at –80°C until used for biochemical preparations. Between 5 g and 10 g of frozen heater tissue was homogenized in 10 vol. of buffer containing 0.3 mol l^{-1} sucrose and 20 mmol l^{-1} K-Pipes ($C_6H_{16}N_2O_6S_2K_2$), pH 7.3 using a Teflon pestle and glass 50 ml homogenizer. The homogenate was centrifuged at 2600 ***g*** for 20 min in a Sorvall SS34 rotor (Kendro, Newtown, CA, USA). The supernatant was filtered through two layers of cheesecloth, and the microsomes were pelleted by centrifugation at 100 000 ***g*** in a Beckman Ti50.2 rotor (Beckman Coulter, Fullerton, CA, USA). The pelleted microsomes were resuspended in 0.3 mol l^{-1} sucrose, 5 mmol l^{-1} K-Pipes, pH 7.3, snap frozen in liquid nitrogen and stored at –80°C for later use.

Ca^{2+}-ATPase antibody production and purification

Two peptides, one corresponding to amino acids 328–342 of blue marlin SERCA 1 (Londraville et al., 2000) and one corresponding to amino acids 192–205 of rabbit SERCA 2 (Brandl et al., 1986), were synthesized by Research Genetics Inc. (Huntsville, AL, USA) and used to immunize four rabbits for polyclonal antibody production. Serum immunoglobulin G (IgG) purification was performed using ImmunoPure Affinity Pak™ protein A columns following the instructions provided by the manufacturer (Pierce, Rockford, IL, USA). Two affinity columns were made by immobilization of 5.0 mg of the synthesized peptides provided by Research Genetics Inc. using an EDC [1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide]/Diaminodipropylamine Immobilization Kit (Pierce, Rockford, IL, USA). 1 ml of the IgG fractions from the protein A columns followed by 2.0 ml of PBS (phosphate-buffered saline) were applied to the appropriate affinity column and incubated for 1 h at room temperature. The columns were washed with an additional 14 ml of PBS, after which the SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) antibodies were eluted with 0.1 mol l^{-1} glycine, pH 3.0. 1 ml fractions were collected and their absorbance at 280 nm was monitored to determine the presence of antibody. Fractions with the highest absorbance were pooled, adjusted to neutral pH and stored at –80°C for later use. This protocol resulted in the production and purification of two SERCA antibodies, one able to recognize SERCA 1 and the other able to recognize SERCA 2.

Immunohistochemistry and α-bungarotoxin labeling

Immunohistochemical analysis of sectioned heater tissue was performed as previously described (Block et al., 1994; Tullis and Block, 1996). Briefly, 10 μm thick slices of frozen heater tissue were mounted on slides and incubated in PBS containing 0.5 mmol l^{-1} ascorbic acid and 0.05 mmol l^{-1} *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, pH 7.5 for 40 min to reduce auto-fluorescence. Sections were then incubated in PBS supplemented with 10% goat serum and 0.3% Triton-X for 30 min followed by an overnight incubation in AchR antibody diluted 1:200 in PBS or 10 μg ml^{-1} of rhodamine-conjugated α-bungarotoxin in PBS at 4°C. Sections were washed for 3×10 min in PBS. Antibody-labeled sections were incubated for 30 min at room temperature in a fluorescein-conjugated secondary antibody at 1:50 dilution in PBS and then washed for 3×10 min in PBS. α-bungarotoxin-labeled sections were washed for 3×10 min in PBS. Sections were mounted in 4% n-propyl gallate, 80% glycerol, 20 mmol l^{-1} Tris, pH 9.0 and examined using a Zeiss Axioplot microscope (Carl Zeiss Inc., Thornwood, NY, USA) equipped with an epifluorescence attachment. Control sections were incubated with secondary antibody only or with 100 μg ml^{-1} unlabeled α-bungarotoxin prior to rhodamine-conjugated toxin.

Ca^{2+} fluorimetry

Sarcoplasmic reticulum Ca^{2+} uptake and release was measured using the Ca^{2+}-sensitive dye fura-2 and a Shimadzu RF 5301 spectrofluorophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). SR microsomes were added at a final concentration of 0.5 mg ml^{-1} to a cuvette containing 2.0 ml of transport buffer [95.0 mmol l^{-1} KCl, 20.0 mmol l^{-1} H-Mops, 7.5 mmol l^{-1} $Na_4P_2O_7$ (sodium pyrophosphate), 5.0 mmol l^{-1} creatine phosphate, 0.01 mg ml^{-1} creatine phosphokinase and 1.5 μmol l^{-1} fura-2 (potassium salt), pH 7.0]. Extravesicular Ca^{2+} was monitored as the ratio of fura-2 fluorescence emission intensity at excitation wavelengths of 340 nm and 380 nm. Ca^{2+} uptake was initiated with the addition of 2.5 mmol l^{-1} MgATP and allowed to proceed to steady state. Sequential 75 nmol additions of $CaCl_2$ were made to the cuvette to load the vesicles to a filling capacity of 0.8–1.0 μmol Ca^{2+} mg^{-1} protein. 10 mmol l^{-1} caffeine and

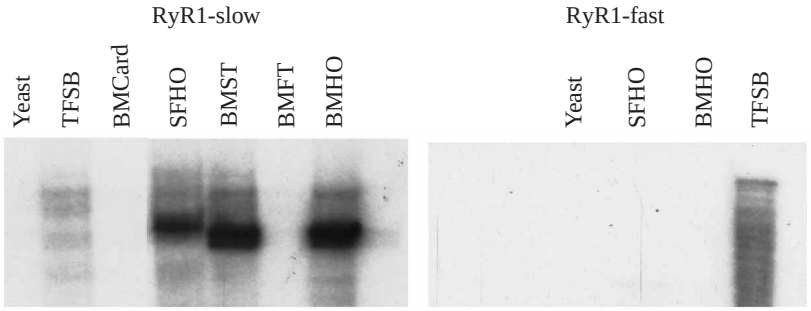

Fig. 1. RNase protection assay reveals the expression of RyR1-slow in heater tissue. A ^{32}P-labeled RNA probe, synthesized from a blue marlin extraocular muscle clone, hybridized to slow-twitch skeletal muscle and heater organ but not to fast-twitch skeletal muscle. This isoform was named RyR1-slow to distinguish it from RyR1-fast, which is expressed in fish fast-twitch skeletal muscle. The probe was hybridized to 20 μg total RNA from each tissue. Yeast, control RNA; TFSB, toadfish fast-twitch swimbladder muscle; BMCard, blue marlin cardiac muscle; SFHO, swordfish heater organ; BMST, blue marlin slow-twitch skeletal muscle; BMFT, blue marlin fast-twitch skeletal muscle; BMHO, blue marlin heater organ.

350 μmol l^{-1} ryanodine were added to stimulate Ca^{2+} release. In some experiments, the activity of the SERCA pumps was reduced by the omission of creatine phosphate and creatine phosphokinase from the transport buffer.

Gel electrophoresis and western blot analysis

Heater tissue SR microsomes were run on 7.5% SDS (sodium dodecyl sulfate)–polyacrylamide gels according to the method of Laemmli (1970). Gels were blotted onto PVDF (polyvinylidine fluoride) membranes that were subsequently blocked in 5% non-fat milk, 0.2% tween in PBS for >1.5 h. Blots were incubated overnight at 4°C in primary antibody diluted 1:500 in PBS. Blots were washed three times in PBS and subsequently incubated with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody diluted 1:1000 in PBS for 1 h. Blots were developed with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium).

Ribonuclease protection assays (RPAs)

RNase protection assays were performed as previously described (Franck et al., 1998). Briefly, two ryanodine receptor antisense probes were constructed: one that hybridized strictly to transcripts of RyR1-slow, an RyR specific to slow-twitch muscle, and one that hybridized strictly to transcripts of RyR1-fast, an RyR specific to fast-twitch muscle. The RyR1-slow probe was synthesized from a subcloned region amplified from a blue marlin eye muscle RyR clone [nucleotides 4075–4315 in the blue marlin RyR1-slow open reading frame (ORF)], while the RyR1-fast probe was synthesized from a subcloned region amplified from a toadfish swimbladder muscle RyR clone (nucleotides 13 737–14 130 in the blue marlin RyR1-slow ORF). Both antisense probes were synthesized from *Eco*RI linearized plasmids with T7 RNA polymerase and [^{32}P]dUTP according to the Ambion Maxiscript T7/T3 *in vitro* transcription kit protocol (Ambion Inc., Austin, TX, USA). Total RNA used for the RPAs (20 μg $assay^{-1}$) was prepared using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) with the RPA performed using the Ambion Direct Protect RPA kit (Ambion Inc.). All hybridizations were performed at 37°C. Samples were separated on a 6% sequencing gel that was dried and exposed to x-ray film for 24–72 h at –80°C.

Results

Identification and characterization of heater cell ryanodine receptors

The complete 16.3 kb ryanodine receptor has been cloned and sequenced from blue marlin extraocular muscle (Franck et al., 1998). The RyR cDNA sequence encodes a protein of 5081 amino acids with a molecular mass of 576 302 Da. The deduced amino acid sequence was aligned to the multiple published RyR sequences from chicken, frog, rabbit and human and determined to belong to the RyR1 (skeletal muscle) family (Franck et al., 1998). A ^{32}P-labeled RNA probe was synthesized from the blue marlin extraocular muscle RyR sequence, and RNase protection assays were performed to determine the distribution of this RyR1 isoform in heater and extraocular muscle tissues. Fig. 1 shows that this RNA probe hybridized to total RNA from marlin and swordfish heater organ and marlin slow-twitch skeletal muscle but not to marlin or toadfish swimbladder fast-twitch skeletal muscle or to marlin cardiac muscle. Thus, heater tissue expresses the skeletal type RyR1 isoform that we have shown previously to be expressed in a fiber-type-specific manner. The isoform that is expressed in slow-twitch muscle and heater tissue was designated RyR1-slow to indicate that it is part of the skeletal muscle RyR1 gene family but is distinct from RyR1-fast, the skeletal RyR isoform expressed in fish fast-twitch skeletal muscle.

Identification and characterization of heater cell SERCA

Western blotting with antibodies constructed to specifically label SERCA 1 or SERCA 2 epitopes indicates that heater cells express the SERCA 1 isoform of the Ca^{2+}-ATPase (Fig. 2). The SERCA 1-specific antibody was raised to amino acids 328–342 of blue marlin SERCA 1 (Londraville et al., 2000) and labeled SR vesicles from blue marlin and bluefin tuna fast-twitch swimming muscle, blue marlin extraocular muscle and heater organ but did not detect the SERCA 2 isoforms in bluefin heart or slow-twitch muscle. Conversely, the SERCA 2-specific antibody, raised to amino acids 192–205 of rabbit SERCA 2, did not label heater organ SR vesicles but positively labeled heart and slow-twitch muscle. The complete cDNA sequence for the SR Ca^{2+}-ATPase (SERCA) has been cloned from blue marlin extraocular muscle (Londraville et al., 2000). The sequence

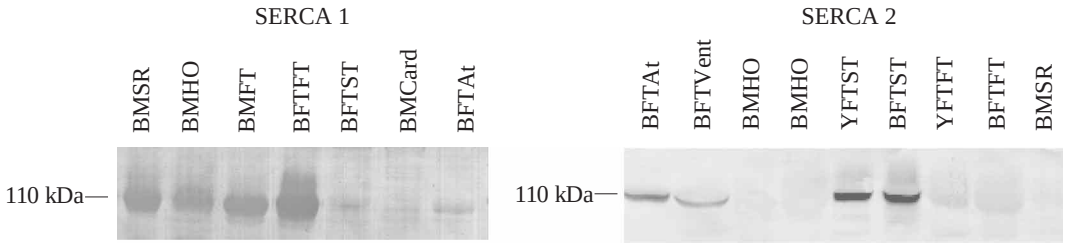

Fig. 2. Western blot analysis with SERCA 1- and SERCA 2-specific antibodies reveals the expression of SERCA 1 in heater cells. Two peptides, one corresponding to amino acids 328–342 of blue marlin SERCA 1B and one corresponding to amino acids 192–205 of rabbit SERCA 2, were used to synthesize isoform-specific Ca^{2+}-ATPase antibodies. The SERCA 1 antibody labeled an approximately 110 kDa band in superior rectus extraocular muscle, heater organ and fast-twitch muscle, while the SERCA 2 antibody labeled atrium, ventricle and slow-twitch muscle. BMSR, blue marlin superior rectus muscle; BMHO, blue marlin heater organ; BMFT, blue marlin fast-twitch skeletal muscle; BMCard, blue marlin cardiac muscle; BFTFT, bluefin tuna fast-twitch skeletal muscle; BFTST, bluefin tuna slow-twitch skeletal muscle; BFTAt, bluefin tuna atrial muscle; BFTVent, bluefin tuna ventricular muscle; YFTFT, yellowfin tuna fast-twitch skeletal muscle; YFTST, yellowfin tuna slow-twitch skeletal muscle.

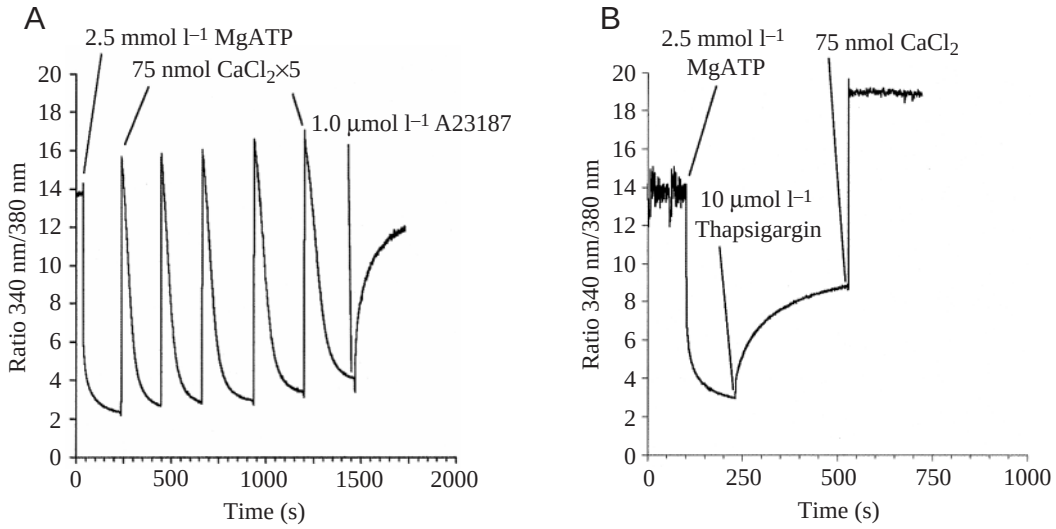

Fig. 3. Heater organ sarcoplasmic reticulum (SR) vesicles sequester Ca^{2+} in an ATP-dependent manner. 0.5 mg ml^{-1} of heater tissue SR vesicles were added to a cuvette containing 1.5 ml transport buffer, 10 µmol l^{-1} carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) and 10 µmol l^{-1} oligomycin. The extravesicular Ca^{2+} concentration was monitored using the fura-2 fluorescence ratio (340 nm/380 nm). Ca^{2+} uptake was initiated with the addition of 2.5 mmol l^{-1} MgATP, and sequential additions of 75 nmol $CaCl_2$ were used to load the vesicles. (A) The Ca^{2+} ionophore A23187 releases the SR-loaded Ca^{2+}. (B) The uptake of Ca^{2+} is blocked by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin.

codes for 996 amino acids and corresponds to a neonatal isoform of Ca^{2+}-ATPase found in mammals (SERCA 1B). The marlin SERCA 1B sequence is identical to marlin SERCA 1A expressed in marlin fast-twitch muscle except for an additional five amino acids at the carboxyl terminus. RNase protection assays using probes constructed to hybridize to SERCA 1B reveal that heater tissue expresses SERCA 1B (Londraville et al., 2000). The western blot results show that heater cells express the SERCA 1 protein and corroborate the molecular work of Londraville et al. (2000).

Characterization of Ca^{2+} uptake and release by heater cell SR

The Ca^{2+}-sensitive dye fura-2 was used to monitor the uptake and release of Ca^{2+} by blue marlin heater SR vesicles. The addition of 2.5 mmol l^{-1} MgATP stimulated Ca^{2+} uptake by heater SR vesicles, evident as a decrease in the ratio of the fura-2 fluorescence (Fig. 3A). Sequential additions of 75 nmol of $CaCl_2$ actively loaded the vesicles to an apparent steady-state filling capacity of approximately 0.8–1.0 µmol Ca^{2+} mg^{-1} protein. Once Ca^{2+} has been loaded into the SR lumen, it can be rapidly released by the addition of the Ca^{2+} ionophore A23187. The sequestration of Ca^{2+} by heater SR is completely blocked by the addition of 10 µmol l^{-1} thapsigargin, indicating the active nature of the loading phase (Fig. 3B). Ca^{2+} uptake rates for blue marlin SR vesicles were 1.52±0.36 nmol Ca^{2+} mg^{-1} s^{-1} for uptake of the first-added Ca^{2+} bolus and declined to 0.83±0.19 nmol Ca^{2+} mg^{-1} s^{-1} for uptake of the fifth Ca^{2+} addition. A slowing of the uptake rate can be expected as the vesicles become progressively filled. These uptake rates are comparable with rates of initial Ca^{2+} uptake displayed by toadfish swimbladder muscle SR vesicles

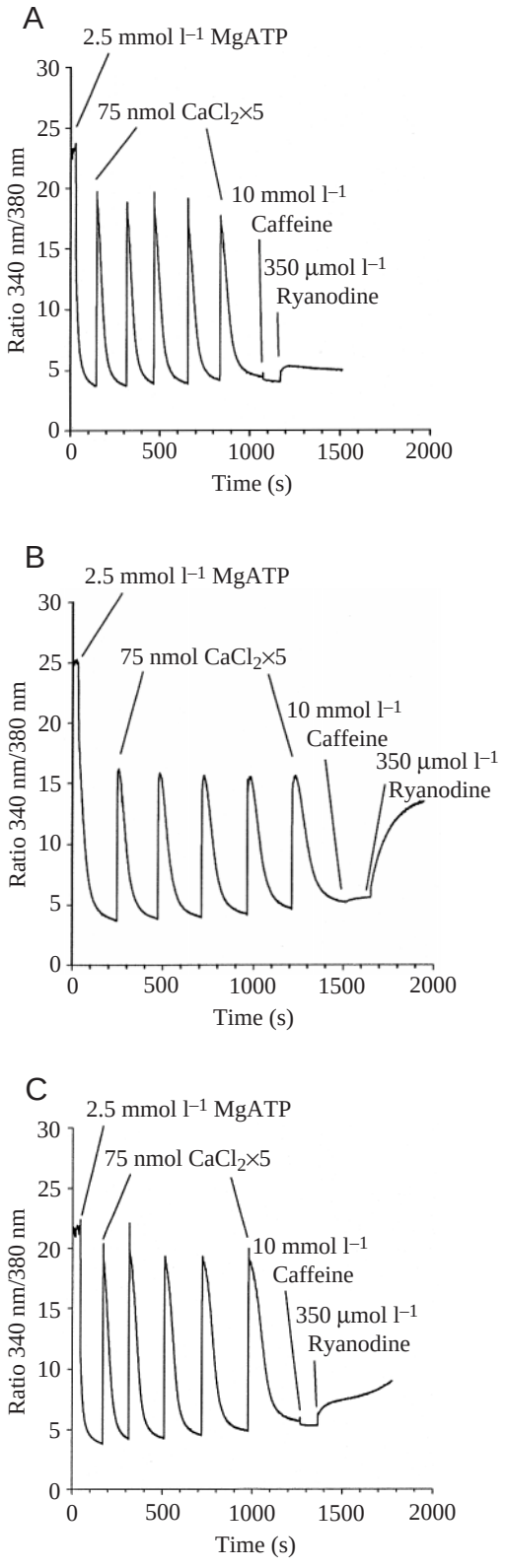

Fig. 4. Heater organ sarcoplasmic reticulum (SR) vesicles release Ca^{2+} through ryanodine receptor (RyR) channels. (A) After the heater SR vesicles have been loaded with Ca^{2+}, a very small Ca^{2+} release event can be evoked by the addition of 10 mmol l^{-1} caffeine and 350 µmol l^{-1} ryanodine. (B) In similarly loaded SR vesicles isolated from toadfish swimbladder fast-twitch muscle, the addition of 10 mmol l^{-1} caffeine and 350 µmol l^{-1} ryanodine resulted in a large Ca^{2+} release event. (C) In the absence of an ATP-regenerating system (creatine phosphate and creatine phosphokinase), a much larger release event is evoked in heater tissue by the addition of 10 mmol l^{-1} caffeine and 350 µmol l^{-1} ryanodine, indicating that the strong presence of the pump in heater tissue limits SR Ca^{2+} release in this assay.

(1.41±0.31 nmol Ca^{2+} mg^{-1} s^{-1}; Fig. 4B) but are considerably faster than the 0.69 nmol Ca^{2+} mg^{-1} s^{-1} uptake rate observed in fast-twitch skeletal muscle (data not shown).

Caffeine and ryanodine are two agonists of RyRs commonly used to release Ca^{2+} from SR stores. Surprisingly, the addition of caffeine and ryanodine to Ca^{2+}-loaded blue marlin heater SR vesicles caused little release (Fig. 4A). An identical experiment using fast-twitch toadfish swimbladder muscle SR vesicles, which sequester Ca^{2+} at a similar rate and to a similar degree as heater tissue SR, resulted in a large proportion of SR Ca^{2+} released upon stimulation by caffeine and ryanodine (Fig. 4B). One explanation for the apparent lack of significant Ca^{2+} release in heater SR vesicles is that the release is masked by the high activity of the SERCA pump. This hypothesis is supported by the fact that omission of the creatine phosphate/creatine phosphokinase ATP regenerating system, thus limiting the pump's access to ATP, causes a much larger Ca^{2+} release event in response to RyR agonists (Fig. 4C). However, even with the SERCA pump activity constrained, the Ca^{2+} released from heater tissue SR is still less than that from swimbladder muscle SR (Fig. 4C). This suggests that other factors, such as the distinct RyR isoforms in heater and swimbladder muscle (RyR1-slow *vs* RyR1-fast), may contribute to the differences in caffeine- and ryanodine-evoked Ca^{2+} release in the two tissues.

Heater organ 'endplates'

Heater organs are located beneath the brain, bilaterally situated between the two superior rectus extraocular muscles. As seen during dissections, a branch of the oculomotor nerve courses through the heater organ and is suggestive of a neuronal control of heater organ thermogenesis. To examine this further, we used immunohistochemistry to identify whether acetylcholine receptors (AchR) were present along the surface of the heater cells. Using two methodologies (antibodies with epitopes specific to the AchR or rhodamine-conjugated α-bungarotoxin), we have identified large clusters and extensive labeling of AchR on the surface of heater cells (Fig. 5). The presence of these 'endplate-like' labels is indicative of significant junctional membranes, which may function to couple heater cells and oculomotor nerve endings electrically in a fashion analogous to the motor endplates of skeletal muscle, making excitation–thermogenic coupling possible.

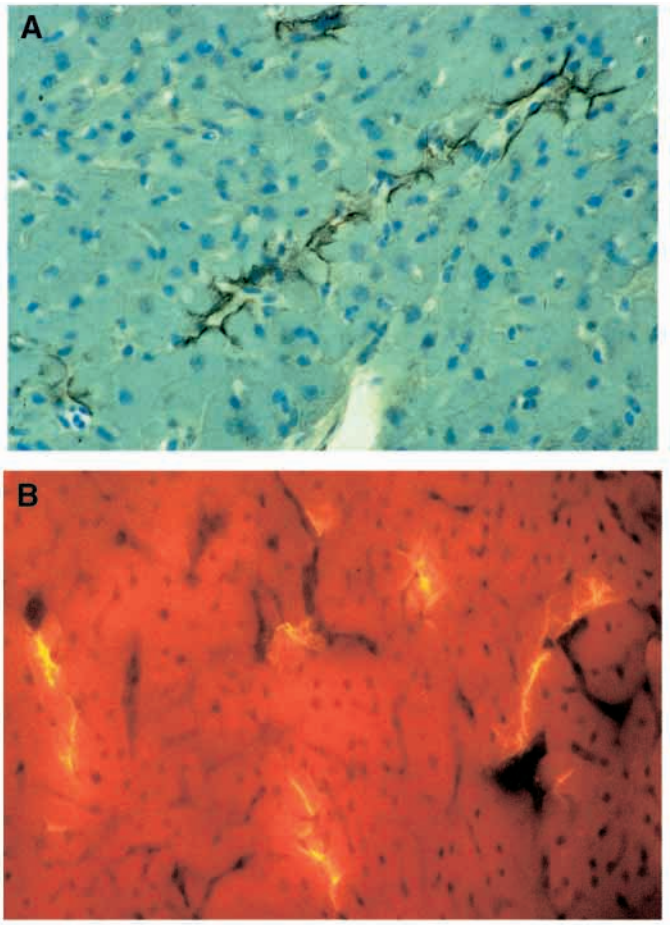

Fig. 5. Light micrographs of heater tissue labeled with (A) an acetylcholine receptor antibody or (B) rhodamine-conjugated α-bungarotoxin. Extensive junctional endplates are present on the surface membrane of heater cells, suggestive of a nervous control of thermogenesis.

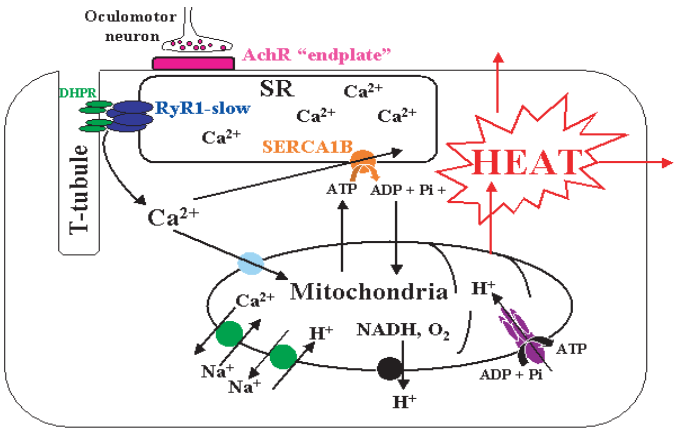

Fig. 6. A model of excitation–thermogenic coupling in heater cells. Thermogenesis in heater cells is proposed to occur *via* depolarization-induced Ca^{2+} release pathways. Nervous stimulation mediated by acetylcholine receptors (AchR) results in heater cell depolarization and DHPR–RyR1-mediated Ca^{2+} release. Increased cytoplasmic Ca^{2+} stimulates Ca^{2+} transport and ATP turnover by SERCA 1B and mitochondrial influx and efflux pathways. The physiological properties of the RyR1-slow isoform expressed in heater cells may facilitate prolonged channel openings under these conditions (high Ca^{2+} and the presence of adenine nucleotides) and promote further release of Ca^{2+} in a 'futile' cycle that results in thermogenesis. Abbreviations: T-tubule, transverse-tubule; SR, sarcoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; RyR, ryanodine receptor; DHPR, dihydropyridine receptor.

Discussion

Studies on the heater tissue have employed structural, biochemical and molecular techniques (Block et al., 1988a,b, 1994; Block and Franzini-Armstrong, 1988; O'Brien and Block, 1996; Londraville et al., 2000; Tullis and Block, 1996, 1997; Tullis et al., 1991). These studies have indicated that the heater cell is a muscle cell derivative enriched in T-tubule and SR membrane systems that function to sequester and release calcium. The cells have been shown to express the protein components of the SR that are involved in excitation–contraction coupling but, to date, the isoforms of the SR calcium release channel and the calcium ATPase have not been identified. From ultrastructural, histochemical and biochemical data, Block (1986, 1994) has hypothesized that the thermogenic process is associated with calcium cycling involving excitation–thermogenic coupling between the T-tubule and SR membranes. The present study provides direct evidence from *in vitro* work that supports this hypothesis and provides a strong indication for neural control of heater cell thermogenesis.

Thermogenesis in fish heater organs occurs in modified extraocular muscle cells that have lost much of their organized contractile apparatus but express a high content of mitochondria and SR. Heater cells are innervated by a branch of the oculomotor nerve (Block, 1986), and, in this study, we have demonstrated the presence of large clusters of AchR on the plasma membrane, indicative of an endplate-like structure. The presence of AchR is consistent with a thermogenic mechanism involving the nervous depolarization of heater cells. The first step in an excitation–thermogenic coupling mechanism probably involves the transmission of action potentials down the oculomotor neuron, resulting in release of Ach and depolarization of the heater endplate (see Fig. 6). Once stimulated, heater cell depolarization would lead to Ca^{2+} release from the SR in much the same manner as it occurs in skeletal muscle.

Previous studies have established an extensive T-tubule system in heater cells using intermediate voltage electron microscopy and Golgi labeling (Block and Franzini-Armstrong, 1988). Junctional complexes between the T-tubule and SR membrane systems have also been identified (Block et al., 1988b). Depolarization of the T-tubule membrane would lead to a conformational change in the voltage-sensitive dihydropyridine receptors (DHPR) or L-type Ca^{2+} channels. To date, no antibodies have been identified that crossreact with the fish DHPR isoform, and identification and localization of the DHPR expressed in heater cells has yet to be resolved.

Depolarization of the T-tubule membrane would induce the opening of the RyR, possibly through a physical mechanical coupling between DHPR and RyR. A direct mechanical connection between DHPRs and RyR1s is the prevailing hypothesis by which these two proteins interact in skeletal muscle. However, a mechanism involving Ca^{2+}-induced Ca^{2+} release in which RyRs are opened by the inward Ca^{2+} current through the DHPR cannot be ruled out.

In the present study, we have shown that heater tissue expresses RyR1-slow, an RyR isoform that is also expressed in fish slow-twitch muscle and displays several physiological properties that may promote thermogenesis. RyR1-slow has been isolated and characterized from tuna slow-twitch muscle and compared with RyR1-fast from tuna fast-twitch and toadfish swimbladder muscle (Morrissette et al., 2000). The most important finding of this comparative analysis is that adenine nucleotides attenuate the Ca^{2+}-dependent inhibition of RyR1-slow but have little effect on the Ca^{2+}-dependent inhibition of RyR1-fast. When cytoplasmic [Ca^{2+}] rises, most skeletal muscle RyR channels close; however, in the presence of adenine nucleotides, RyR1-slow remains open. This property may contribute to a prolonged release of Ca^{2+} from the SR in heater cells. Prolonged release of Ca^{2+} would promote thermogenesis. Remarkably, the physiological characteristics of the naturally expressed heater RyR resembles in many aspects the physiological properties exhibited by the mutated RyR responsible for the lethal muscular disease malignant hyperthermia (MH). MH is induced by clinical doses of general anesthetics that stimulate prolonged Ca^{2+} release due to a mutated RyR (Loke and MacLennan, 1998; Mickelson and Louis, 1996). MH patients develop a condition characterized by increased muscle metabolism coupled with muscle rigidity and massive hyperthermia. The physiological properties of RyR1-slow would promote prolonged channel opening and Ca^{2+} release in heater tissue during thermogenic episodes. The increase in cytoplasmic Ca^{2+} would stimulate Ca^{2+} transport and ATP turnover by the Ca^{2+}-ATPase pump and mitochondrial influx and efflux pathways that would consume oxidative energy and promote thermogenesis. The high activity of the Ca^{2+}-ATPase would attempt to restore cytoplasmic Ca^{2+} to resting values; however, the prolonged open state of RyR1-slow could create a continuous Ca^{2+} 'leak pathway' from the SR and increase the rate of ATP hydrolysis and heat production.

In the present study, the presence of a SERCA 1 protein in heater tissue is established by western blots. This is consistent with Londraville et al. (2000), who used RNase protection assays to show that heater tissue expresses SERCA 1B, the neonatal isoform of the Ca^{2+} pump. However, it is still uncertain whether heater cells may also express the SERCA 1A isoform. Tullis and Block (1996) amplified a PCR product that indicated that a SERCA 1A isoform was present in heater cells; however, it was unclear whether the amplified product was from the heater or muscle cellular component of the tissue (Tullis and Block, 1996). Electron microscopy of heater tissue has shown that muscle fibers expressing a normal muscle cell architecture are often in close association with the heater cell phenotype. *In situ* hybridization studies are needed to definitively resolve the expression pattern of SERCA 1 isoforms in heater cells. Regardless of the expression pattern of SERCA 1 isoforms, the data in the present study allow the characterization of the physiological properties of the heater SERCA isoform(s). In identical microsomal preparations, heater vesicles sequester Ca^{2+} at a rate comparable with toadfish swimbladder muscle, one of the fastest contracting vertebrate muscles (Rome, 1999), and more than 2-fold faster than fast-twitch swimming muscle. This high rate of pump activity would clearly be advantageous in an excitation–thermogenic coupling process, enhancing ATP turnover and oxidative phosphorylation. The extent to which the high rate of Ca^{2+} uptake in heater tissue is due to the individual properties of the isoforms expressed, or to increased expression of pump proteins, remains to be seen. Currently, experiments are underway to further characterize the rate of ATP hydrolysis and the expression patterns of heater SERCA in a variety of billfish heater cell phenotypes.

In summary, the data indicate that Istiophorid billfish, represented by the blue marlin, have the membrane components to initiate nervous depolarization of heater cells. The resulting depolarization would result in heat production *via* RyR1-slow mediated Ca^{2+} release and SERCA 1-mediated Ca^{2+} reuptake by the SR. The cycling of Ca^{2+} between the SR and cytoplasm would increase substrate oxidation to fuel the process as well as generate additional heat. The RyR and SERCA isoforms expressed in heater tissue are unique and their physiological properties probably contribute to the heat production process. In these fish species, the presence of slow-twitch fibers close to the brain and eyes probably provides the raw material for the evolution of the heater phenotype.

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