

# Characterization of the Sarcoplasmic Reticulum Proteins in the Thermogenic Muscles of Fish

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**Abstract.** Marlins, sailfish, spearfishes, and swordfish have extraocular muscles that are modified into thermogenic organs beneath the brain. The modified muscle cells, called heater cells, lack organized myofibrils and are densely packed with sarcoplasmic reticulum (SR), transverse (T) tubules, and mitochondria. Thermogenesis in the modified extraocular muscle fibers is hypothesized to be associated with increased energy turnover due to  $\text{Ca}^{2+}$  cycling at the SR. In this study, the proteins associated with sequestering and releasing  $\text{Ca}^{2+}$  from the SR (ryanodine receptor,  $\text{Ca}^{2+}$  ATPase, calsequestrin) of striated muscle cells were characterized in the heater SR using immunoblot and immunofluorescent techniques. Immunoblot analysis with a monoclonal antibody that recognizes both isoforms of nonmammalian RYRs indicates that the fish heater cells express only the  $\alpha$  RYR isoform. The calcium dependency of [ $^3\text{H}$ ]ryanodine binding to the RYR iso-

form expressed in heater indicates functional identity with the non-mammalian  $\alpha$  RYR isoform. Fluorescent labeling demonstrates that the RYR is localized in an anastomosing network throughout the heater cell cytoplasm. Measurements of oxalate supported  $^{45}\text{Ca}^{2+}$  uptake,  $\text{Ca}^{2+}$  ATPase activity, and [ $^{32}\text{P}$ ]phosphoenzyme formation demonstrate that the SR contains a high capacity for  $\text{Ca}^{2+}$  uptake via an ATP dependent enzyme. Immunoblot analysis of calsequestrin revealed a significant amount of the  $\text{Ca}^{2+}$  binding protein in the heater cell SR. The present study provides the first direct evidence that the heater SR system contains the proteins necessary for  $\text{Ca}^{2+}$  release, re-uptake and sequestration, thus supporting the hypothesis that thermogenesis in the modified muscle cells is achieved via an ATP-dependent cycling of  $\text{Ca}^{2+}$  between the SR and cytosolic compartments.

**S**KELETAL muscles are most often examined at the cellular level in relationship to their primary role in force generation. Throughout the animal kingdom, the skeletal muscle cell has a secondary role in thermogenesis. Shivering and non-shivering thermogenesis provide excess warmth and increase whole body metabolism in birds, mammals, reptiles, fishes, and insects (5). The first response to a lowering of body temperature in many endotherms is shivering thermogenesis where heat production is increased due to muscle contractions that produce no useful work. Thermogenesis is a result of stimulation of muscle cell respiration and substrate oxidation due to cross-bridge cycling and  $\text{Ca}^{2+}$  cycling, ATP consuming processes. A role for skeletal muscle in heat generation without contraction or non-shivering thermogenesis has been debated for many years (5). Long-term cold exposure is known to stimulate whole body metabolism in endotherms (2) and the exact mechanisms involved in increasing tissue level oxygen consumption are as yet unknown.

Recent investigations indicate similar cellular mechanisms

may be associated with skeletal muscle non-shivering thermogenesis in fish thermogenic organs, mammalian malignant hyperthermia (MH),<sup>1</sup> and avian non-shivering thermogenesis (5, 13, 33). The common theme emerging is that skeletal muscle based non-shivering thermogenesis is linked with the energy turnover associated with the release and re-uptake of  $\text{Ca}^{2+}$  ions by the intracellular membrane system of muscle, the sarcoplasmic reticulum (SR). The SR of striated muscle cells is a complex membranous network that serves as an intracellular storage site for  $\text{Ca}^{2+}$  (35). The SR regulates the contraction-relaxation cycle of muscle by rapidly releasing and reaccumulating  $\text{Ca}^{2+}$  ions. The longitudinal SR mediates  $\text{Ca}^{2+}$  uptake by a membrane-bound  $\text{Mg}^{2+}$ -dependent  $\text{Ca}^{2+}$  ATPase that accounts for up to 90% of the total membrane protein of this portion of the SR (35).  $\text{Ca}^{2+}$  release from the SR occurs at morphologically distinct sites called the terminal cisternae, via the SR  $\text{Ca}^{2+}$  release channel or ryanodine receptor (RYR; 17,39). The RYR is a tetramer of a 565-kD polypeptide that forms both the  $\text{Ca}^{2+}$  conducting channel and a large cytoplasmic foot structure that bridges the 10-nm gap between the junctional SR and

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1. *Abbreviations used in this paper:* MH, malignant hyperthermia; RYR, ryanodine receptor; SR, sarcoplasmic reticulum; T, transverse.

transverse (T) tubular membranes in skeletal muscle (18). The release of  $\text{Ca}^{2+}$  into the sarcoplasm results in stimulation of the  $\text{Ca}^{2+}$ -dependent ATPase and enhances mitochondrial oxidative metabolism which promotes the synthesis of ATP (36).

Excessive  $\text{Ca}^{2+}$  release from the SR of skeletal muscle fibers has a measurable thermogenic effect which is most clearly manifested in mammalian MH. MH is an inherited genetic disorder of mammals that is initiated by anesthetics in humans and by stress in domestic animals (22). The syndrome is characterized by skeletal muscle hyperthermia, accelerated muscle metabolism, metabolic acidosis, and if un-stopped, death (33). In domestic swine and susceptible humans the condition is due to abnormalities in the regulation of  $\text{Ca}^{2+}$  release via the skeletal muscle ryanodine receptor (RYR). Biochemical studies indicate that the MH RYR channel is more prone to channel opening by ligands that activate the channel such as  $\text{Ca}^{2+}$  and adenine nucleotides (16). Molecular genetic studies in swine indicate this increased sensitivity to channel opening is associated with a point mutation, resulting in substitution of Cys for Arg<sup>615</sup> in the pig skeletal muscle RYR1 gene (20), and in humans Cys for Arg<sup>614</sup> (21).

In fish, a specialized thermogenic organ, modified from extraocular muscle, provides a unique model system for examining the structural and functional attributes of skeletal muscle that are evolutionarily selected for heat generation (3–5). Thermogenic organs have been identified in oceanic fishes from three families: Xiphiidae, Istiophoridae, and Scombridae (5, 12). In billfishes (Xiphiidae and Istiophoridae) the heat generating portion of the superior rectus muscle is composed of muscle fibers called heater cells that lack organized myofibrils and are, instead, packed with mitochondria, SR and T tubules (6). Ultrastructural studies reveal the heater SR system has a dense packing of the  $\text{Ca}^{2+}$  ATPase (6). Golgi labeling of the T tubule system and intermediate voltage electron microscopy have demonstrated a complex anastomosing T tubular network situated between the mitochondria. Junctional regions between SR and T membranes systems comparable to those in skeletal muscle have been difficult to identify via conventional structural techniques. In vitro metabolic studies indicate that heater cell mitochondria have high capacities for oxidative metabolism and oxidize both fatty acids and carbohydrate substrates (52). Block (4, 5) has proposed that the hypertrophy of the cellular machinery for aerobic metabolism as well as the SR and T tubule volume in the modified muscle cells is associated with a pathway for thermogenesis involving  $\text{Ca}^{2+}$  cycling across the SR membrane. In this study we provide key biochemical evidence in support of this hypothesis by identifying the necessary protein components for  $\text{Ca}^{2+}$  cycling in the heater cell SR. Deciphering the molecular pathway associated with thermogenesis in the modified fish muscle cells should help to elucidate the cellular components of the skeletal muscle fiber that are important for non-shivering thermogenesis in all vertebrates.

## Materials and Methods

### Tissue Collection

Two species of billfishes, the blue marlin (*Makaira nigricans*) and the

swordfish (*Xiphias gladius*) were used in these studies. The fish were caught by commercial and sport fishermen and the elapsed time between capture of the fish and preparation of the microsomal fractions or fixation of material ranged from 30 min to 1.5 h. The eye muscles with the heater tissue attached were removed from the fish and placed in ice cold 0.3 M sucrose, or in 0.1 M NaCl and 25 mM K-Pipes (pH 7.0) and transferred in the buffer on ice, to the laboratory. For immunofluorescent preparations, tissues were isolated and either fixed or freeze clamped within 30 min of capture of a fish. Trout tissues (*Salmo gairdneri*) were obtained from a local supplier and maintained at 20°C prior to use.

### Preparation of SR Membrane Fractions

Heater tissue and extraocular muscle microsomal fractions were isolated from 2 to 10 g of tissue. Heater tissue was minced, added to 10 vol of ice-cold homogenizing solution (0.1 M NaCl, 20 mM Na-Pipes, pH 7.3, 5 mM EGTA, 0.5 mM DIFP, 1 mM benzamide, 1  $\mu\text{M}$  leupeptin, 1  $\mu\text{g}/\text{ml}$  aprotinin, 2  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor) and homogenized with a Potter-Elvehjem teflon glass homogenizer. Extraocular muscle and control muscle preparations were prepared in the same buffer and homogenized in three 20-s bursts with a Tekmar homogenizer at half speed. Small aliquots of the heater and muscle homogenates were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for later use. Muscle and heater centrifugation steps were carried out simultaneously on the homogenates prepared from the skeletal muscle and heater tissue removed from an individual fish. Homogenates were centrifuged for 20 min at 1,900 g and the supernatants filtered through three layers of cheesecloth. An additional spin at 9,750 g for 20 min was included to reduce contamination by mitochondrial membranes in heater tissue preparations. SR vesicles in the 9,750 g supernatant were subsequently pelleted by a 2 h, 20,000 g spin in a Sorvall SS34 rotor. The pellets were resuspended at 10 to 20 mg protein per ml in 0.3 M sucrose, 5 mM K-Pipes, pH 7.0, 0.5 mM DIFP, and frozen in liquid nitrogen in small aliquots.

### Gel Electrophoresis and Immunoblotting

The protein profile of SR vesicle fractions were routinely analyzed by one-dimensional SDS-PAGE using the protocol of Laemmli (29) on 7 or 10% gels for calsequestrin, or 3–12 and 3–15% gradient gels for ryanodine receptor analyses. SR vesicles were solubilized in an SDS sample buffer containing 0.1 M Tris-Cl, pH 6.8, 0.5 mM EDTA, 2% SDS, and 10% glycerol, and loaded for Coomassie blue staining (20 to 60  $\mu\text{g}/\text{lane}$ ). Stains-all staining of  $\text{Ca}^{2+}$  binding proteins was carried out as described in King and Morrison (28) and Campbell et al. (11) in 0.75-mm thick, 7.5% acrylamide gels. Protein was estimated either by the method of Lowry et al. (32) using bovine serum albumin as a standard or via the BCA assay (49).

Ryanodine receptor isoforms were identified by immunoblotting on 5–12% SDS-PAGE gradient gels according to (40). For calsequestrin immunoblots, 30–50  $\mu\text{g}$  of protein was electrophoresed by SDS-PAGE (0.75 mm, 7.5% acrylamide gels) and transferred to PVDF membranes (Millipore Corporation, Bedford, MA) in a Novablot apparatus for 2 h at 0.8 mA/cm<sup>2</sup>. Alkaline phosphatase-linked secondary antibodies (BioRad Laboratories, Hercules, CA) were used to detect calsequestrin as described above and the bands were visualized by reaction with bromochloroindolyl phosphate and nitro blue tetrazolium. Calsequestrins from dog ventricle and rabbit skeletal muscles were isolated from heavy SR vesicles using ammonium sulfate precipitation, followed by DEAE-Sephadex column chromatography according to (48). Fractions from the DEAE-Sephadex column containing calsequestrin were pooled and further purified by phenyl-Sepharose column chromatography (10). Molecular weights of the transferred proteins were calibrated with prestained molecular weight markers (Sigma Chemical Co., St. Louis, MO). The prestained markers were initially calibrated to unstained markers in Coomassie blue-stained gels by scanning densitometry. The markers and their apparent  $M_r$ 's are  $\alpha$ -2 macroglobulin (186 kD),  $\beta$ -galactosidase (118 kD), fructose-6-phosphate kinase (87 kD), pyruvate kinase (68 kD), and fumarate (57 kD).

### Isolation of the Ryanodine Receptor

The 30 S ryanodine receptor complex was isolated by gradient density centrifugation according to Lai et al. (31). [<sup>3</sup>H]Ryanodine binding was assayed according to reference 55 in a medium consisting of 0.2 M KCl, 20 mM K-Pipes, pH 7.1, 1 mM EGTA, 2 mM NTA, 1.91 mM  $\text{CaCl}_2$  (100  $\mu\text{M}$  free calcium), 1 mM AMP-PCP, 0.1  $\mu\text{g}/\text{ml}$  BSA, 1  $\mu\text{g}/\text{ml}$  leupeptin, 1  $\mu\text{g}/\text{ml}$  aprotinin, 0.6 mM DIFP, and 1–100 nM [<sup>3</sup>H]ryanodine. Data for each assay represent means for two filtered aliquots. At least two replicate assays

were performed per preparation. The calcium dependency of [<sup>3</sup>H]ryanodine binding was assayed in the medium described above but in addition, contained 0.5 mg/ml BSA, 6.4 nM [<sup>3</sup>H]ryanodine, and CaCl<sub>2</sub> to set the free calcium concentration from 1 nM to 3.3 mM. Free calcium concentrations were calculated with a computer program using stability constants for EGTA and NTA from Fabiato (15).

### Immunofluorescent Labeling

Small pieces of heater tissue were fixed in 2% paraformaldehyde in 0.1 M sodium cacodylate, cryoprotected by immersion in 0.5 M sucrose in PBS for 30 min, followed by 1 h in 1 M sucrose. Tissues were fast-frozen between polished copper plates pre-cooled in liquid nitrogen. Samples were stored in liquid nitrogen during the length of each cruise at sea (swordfish, up to one month) and subsequently stored at -80°C. Muscle fibers from the swimbladder of the toadfish (*Opsanus tau*) were fixed by perfusion through the major artery supplying this muscle according to Block et al. (7). Fixed tissues were cryoprotected by immersion in 0.5 M sucrose in PBS for 30 min followed by immersion in 1.0 M sucrose in PBS for 1 h and frozen in liquid nitrogen. For comparison with fixed sections, the swimbladder was dissected out and unfixed thin bands of muscle were quickly frozen in liquid nitrogen. For immunofluorescent staining, 8-10- $\mu$ m-thick cryostat sections of fixed and unfixed heater tissue or toadfish swimbladder muscle were mounted on subbed slides (0.5% gelatin-0.05% KCr<sub>2</sub>SO<sub>4</sub>) and air-dried 30 min. The sections were rinsed briefly with 0.1 M PBS, pH 7.5. Heater sections have a high autofluorescent background and were soaked for 30 min in a 0.1 M PBS solution that contained 0.5 mM ascorbic acid and 0.05 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Nonspecific binding was blocked by incubating the sections for 30 min in 10% normal goat serum. Sections were incubated with either a RYR monoclonal antibody CO10 (1:200) for 1-2 h at 22°C, a myosin antibody (1:100) for 1 h at 22°C or a calsequestrin polyclonal antibody (1:200 provided by Dr. Larry Jones, Krannert Institute of Cardiology, Indiana University School of Medicine). For the calsequestrin labeling the step with the primary antibody included 0.1% Triton-X. Sections treated with monoclonal antibodies were incubated with DTAF-conjugated affinity-purified goat anti-mouse IgG (1:40) for 30 min at room temperature. The sections were washed four times 10 min each in PBS. Sections treated with primary antibodies to calsequestrin were incubated for 30 min with a 1:40 dilution of DTAF-conjugated, affinity-purified goat anti-rabbit IgG. The sections were washed four times 10 min in PBS and mounted in 4% *n*-propyl gallate, 80% glycerol in 20 mM Tris, pH 9.0. Heater and muscle were examined in a Zeiss Confocal microscope. Samples for electron microscopy were removed from a fish 5 min after capture and fixed, embedded, sectioned, and photographed as described in Block and Franzini-Armstrong (6).

### Ca<sup>2+</sup> Efflux, Loading, and ATPase Activity

<sup>45</sup>Ca<sup>2+</sup> efflux from SR vesicles passively loaded with <sup>45</sup>Ca<sup>2+</sup> was determined by Millipore filtration as previously described (38). Vesicles (1-5 mg of protein/ml) were incubated at 23°C in a medium containing 20 mM K-Pipes, pH 7.0, 0.1 M KCl, 0.1 mM EGTA, 5 mM <sup>45</sup>Ca<sup>2+</sup>, and 1 mM DIFP. Ca<sup>2+</sup> release behavior of the vesicles was assayed after 90 min by diluting vesicles into an isosmolar unlabeled release medium that either inhibited (10 mM Mg<sup>2+</sup>, 10  $\mu$ M ruthenium red, 1 mM EGTA) or activated (1 mM EGTA, 0.85 mM Ca<sup>2+</sup>, and 1 mM AMP-PCP) the SR Ca<sup>2+</sup> release channel. Untrapped as well as released <sup>45</sup>Ca<sup>2+</sup> was removed by placing the vesicles on 0.45  $\mu$  Millipore filters followed by rapid rinsing to remove extravesicular <sup>45</sup>Ca<sup>2+</sup>. <sup>45</sup>Ca<sup>2+</sup> radioactivity retained by the vesicles on the filters was determined by liquid scintillation counting. Ca<sup>2+</sup> loading by SR vesicles was measured in the presence of the Ca<sup>2+</sup> precipitating agent oxalate. Unless otherwise indicated Ca<sup>2+</sup> loading rates in the crude membrane fractions were assayed at 23°C in a solution containing 0.15 M KCl, 6.5 mM MgCl<sub>2</sub>, 5 mM ATP, 5 mM potassium oxalate, 200  $\mu$ M EGTA, 200  $\mu$ M Ca<sup>2+</sup> (10  $\mu$ M free Ca<sup>2+</sup>), and 30 mM K-Pipes, pH 6.9. Ca<sup>2+</sup> loading in homogenates was measured in a similar medium with 140  $\mu$ M EGTA and 100  $\mu$ M Ca<sup>2+</sup> (1.5  $\mu$ M free Ca<sup>2+</sup>). To inhibit mitochondrial uptake of calcium the following inclusions were made: freshly prepared 2.5  $\mu$ M CCCP along with either 5 mM sodium azide or oligomycin (2  $\mu$ g/ml). Ca<sup>2+</sup> loading was measured by adding 3.0 ml of the above reaction mixture to an aliquot (0.15 mg protein) of heater or muscle crude fractions or homogenates. At timed intervals, loading of the vesicles was terminated by filtering 0.5 ml aliquots of the reaction mixture through a Millipore filter (0.45  $\mu$ m pore size). The <sup>45</sup>Ca<sup>2+</sup> taken up in the vesicles was retained on the filters and determined by scintillation counting. Ca<sup>2+</sup>-dependent ATPase activity in heater tissue and muscle fractions was determined at 25°C according to

Meissner (38). Unless otherwise noted, in the ATPase activity assays, the mitochondrial ATPase inhibitor, oligomycin (2  $\mu$ g/ml) was present under all conditions.

The formation of Ca<sup>2+</sup>-dependent [<sup>32</sup>P] labeled phosphoenzyme intermediate was determined according to Meissner (37).

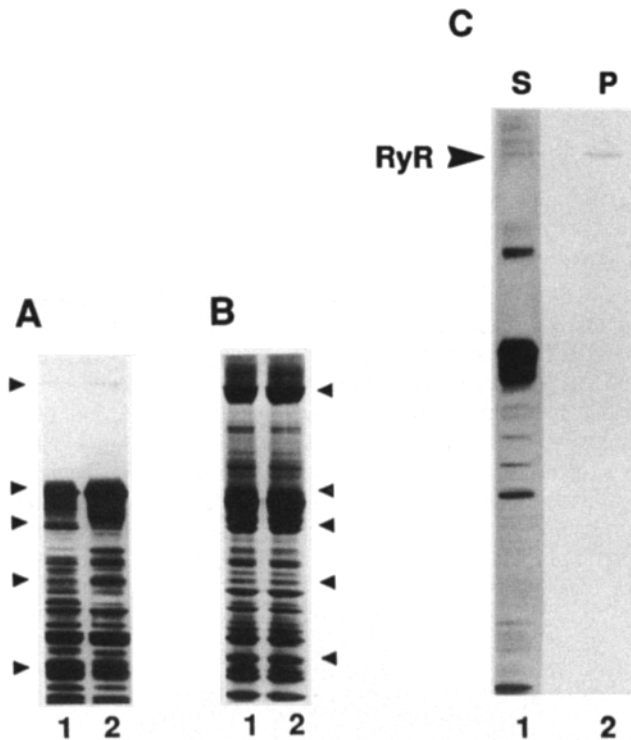
## Results

### Ultrastructure of Heater Cells

Electron micrographs of heater cells reveal the tight packing of membranes and mitochondria characteristic of this modified muscle fiber (Fig. 1). The cellular space in between the mitochondria is occupied with sarcoplasmic reticulum and T tubular membranes (6). Many of the membrane cisternae have an electron-dense content (Fig. 1). Terminal cisternae of skeletal muscle have a similar electron-dense content that has been identified as calsequestrin (19). Cisternae without an electron-dense content are also apparent and regions of stacked SR have been shown by freeze fracture to contain the Ca<sup>2+</sup> ATPase (6).



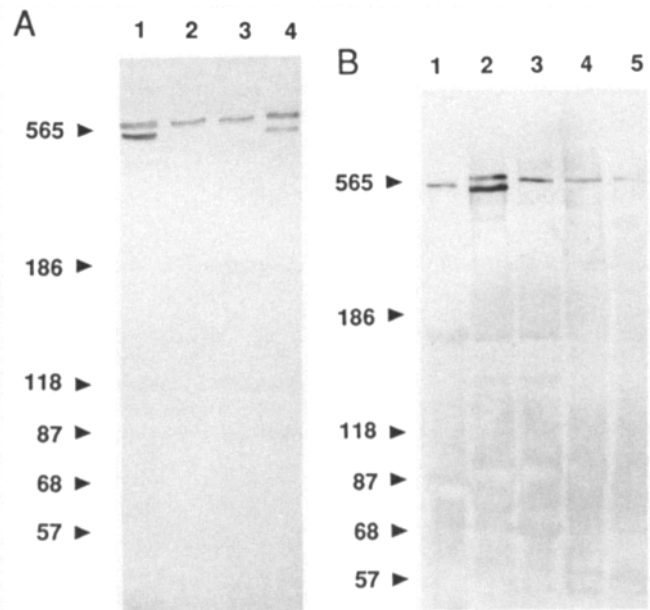
Figure 1. Electron micrograph of heater cells from a blue marlin demonstrating the extensive smooth membrane system situated between the mitochondria in heater cells and electron dense content presumed to be calsequestrin (arrows) of the SR. Bar, 0.5  $\mu$ m.



**Figure 2.** SDS-PAGE characterization of heater microsomal fractions. (A) Marlin heater homogenate (lane 1) and unfractionated SR microsomes (lane 2) resolved by SDS-PAGE reveal a predominance of the 100-kD band presumed to be the  $\text{Ca}^{2+}$  ATPase and a relatively small amount of myosin (apparent molecular weight of 200 kD). The location of molecular mass standards are marked by arrowheads and their values are myosin (205 kD),  $\beta$ -galactosidase (116 kD), phosphorylase b (97.4 kD), serum albumin (66 kD), and ovalbumin (45 kD). A Hoefer GS 300 scanning densitometer was used for the calibrations. (B) For comparison, SDS-PAGE of unfractionated swordfish skeletal muscle. Lane 1 is prepared from fresh tissue at sea as described in text. Lane 2 is an unfractionated SR preparation from flash frozen tissue prepared with a higher speed (100,000 g) final spin. (C) Heavy SR fractions from heater (lane 1) have one high molecular weight protein presumed to be the RyR. Purification of heater heavy SR fractions reveals a single high molecular weight polypeptide (lane 2). Molecular weights of polypeptides in the gels were estimated using as molecular weight markers: mammalian skeletal RYR (565 kD), myosin (205 kD),  $\beta$ -galactosidase (116 kD), phosphorylase b (97.4 kD), serum albumin (66 kD), and ovalbumin (45 kD).

### SDS-PAGE and Immunoblot Analysis of SR Fractions

Isolation and characterization of the SR in heater cells was performed using standard techniques for mammalian skeletal muscle (37, 38). The most prominent protein in the SR profile of heater unfractionated microsomes is a major band with an apparent molecular weight of 100,000 D (Fig. 2). This band co-migrated with a similar band from the white muscle preparations of unfractionated SR from swordfish skeletal muscle (Fig. 2 B). Since the SR  $\text{Ca}^{2+}$ ATPase migrates on SDS gels with an  $M_r$  100,000 this band likely represents this protein. A distinct difference between crude microsomal fractions of heater and muscle is the virtual absence of higher molecular weight proteins in the former preparation. In contrast heavy SR of heater contains proteins



**Figure 3.** Immunoblots with antibody RYR C010 demonstrate the presence of both isoforms of the non-mammalian RYRs. In blue marlin and toadfish swimming muscles (lanes 1 and 4) two isoforms of the RYR are apparent, but only the  $\alpha$  or upper band is present in blue marlin heater and extraocular muscle (lanes 2 and 3). (B) Immunoblot as in A, with mouse skeletal muscle (lane 1), striped bass swimming muscle (lane 2), blue marlin eye muscle (lane 3,  $\alpha$  only), blue marlin heater tissue (lane 4,  $\alpha$  only), and swordfish heater ( $\alpha$  only). Molecular weight calibration is based upon the mammalian skeletal muscle RYR as a standard known to be 565 kD, and prestained molecular weight markers calibrated to unstained markers by scanning densitometry. The prestained markers and their apparent  $M_r$ 's are  $\alpha$ 2-macroglobulin (in kD) (186),  $\beta$ -galactosidase (118), fructose-6-phosphate kinase (87), pyruvate kinase (68), and fumarase (57).

with  $M_r > 100,000$  including a  $\sim 200$ -kD band co-migrating with the myosin standard and several bands with a relative mobility comparable to  $M_r$  565,000 (Fig. 2 C, lane 1). The identity of one of the high molecular weight polypeptides was determined by sedimenting CHAPS solubilized heavy heater SR labeled with  $^3\text{H}$  through a linear sucrose gradient according to Lai (31). A single high molecular weight polypeptide specifically co-migrates in the linear sucrose gradient (Fig. 2 C, lane 2) with the  $^3\text{H}$ ryanodine binding peak (not shown), indicative of the RYR protein.

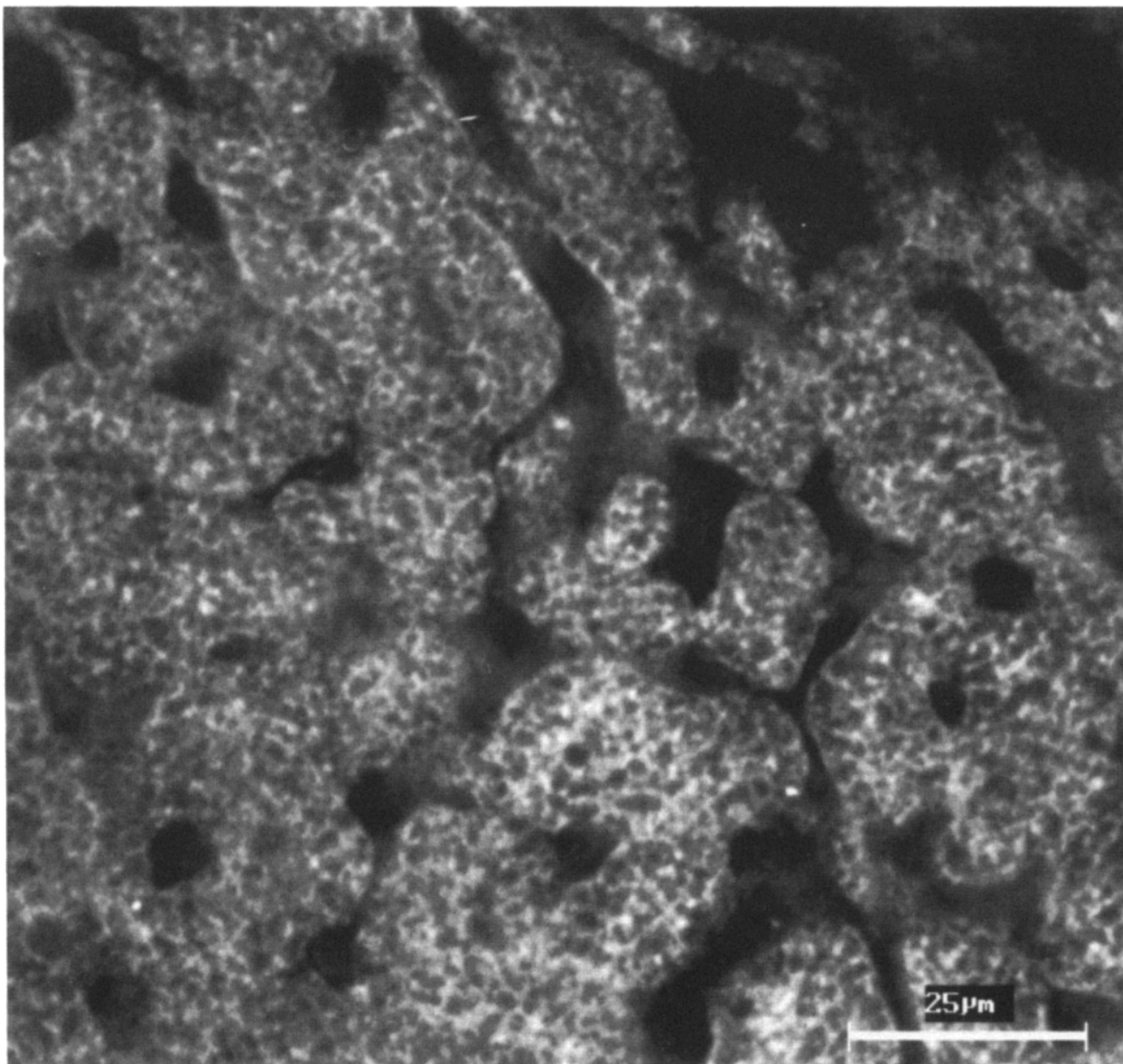
Immunoblot analysis using a monoclonal antibody prepared against mammalian cardiac RYR (mAb C010) confirmed the identity of the  $M_r$  565,000 protein band. The monoclonal antibody cross-reacts with  $\alpha$  and  $\beta$  isoforms of the RYR in skeletal muscle of fish (40) and reveals that two RYR isoforms are present in marlin and toadfish epaxial and hypaxial (swimming) muscles (Fig. 3 A, lanes 1 and 4). In contrast, only one isoform is detected in marlin heater (lane 2) and superior rectus muscles (lane 3). Fig. 3 B demonstrates that the mobility of the RYR isoform expressed in marlin heater and superior rectus muscle (lanes 3 and 4) is similar to that of mouse skeletal muscle RYR (lane 1). SR fractions from the swordfish heater organ also reveal only the

$\alpha$  isoform of the RYR (lane 5). A rat skeletal muscle RYR antiserum that recognizes only the skeletal form of the mammalian RYRs cross-reacts only with the upper bands in fish RYRs, including the single RYR present in heater organs of marlin and swordfish (not shown).

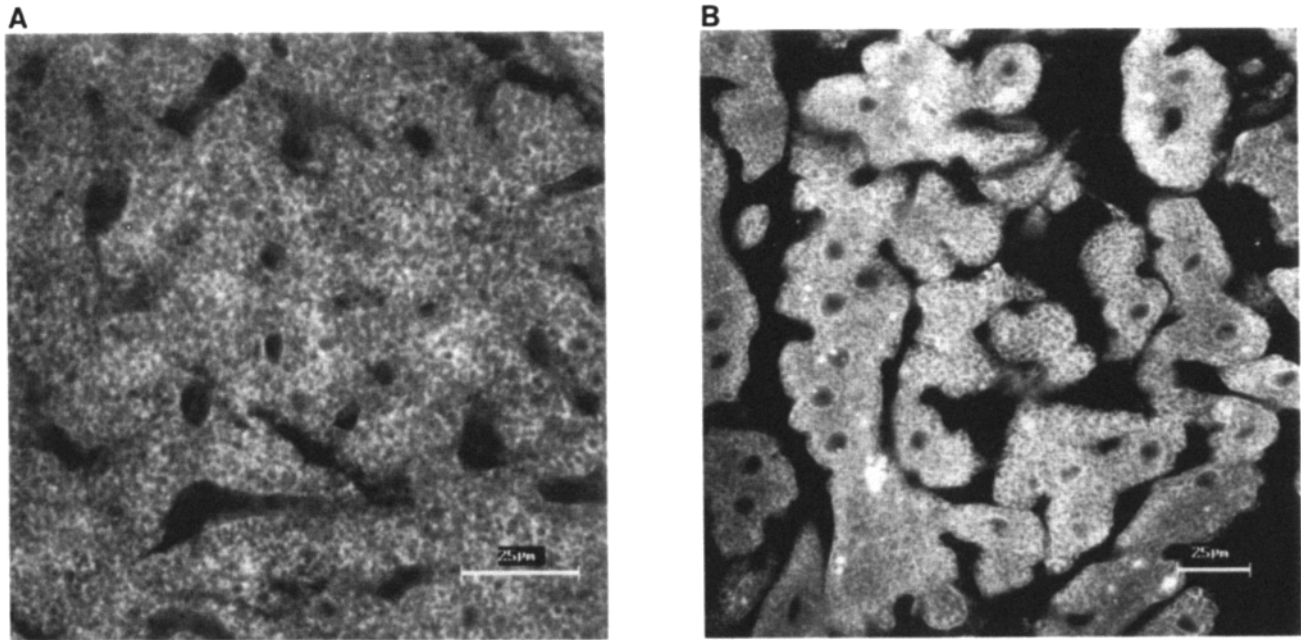
#### ***Immunolocalization of RYR in Heater***

The monoclonal antibody used for immunoblotting was also an excellent probe for examining the location of the RYR in the heater SR membranes using immunofluorescent techniques. For all experiments, marlin skeletal muscle, extraocular muscle or toadfish swimbladder muscle were used as controls on the same slides with sections of heater tissue. The toadfish muscle, specialized for high frequency contraction for sound production, is enriched in the junctional SR and T tubule membranes and is a structural tool for studying junctional proteins (7).

Figs. 4, 5, and 7 a show confocal images of localization of the RYR in heater tissue. Immunofluorescence labeling of the RYR in heater cells reveals a fluorescent network of signal corresponding to the disposition of the SR (Fig. 1) in this cell type. Higher magnification images provide some evidence for an occasional punctate localization (Fig. 4). The immunostaining reveals a continuous network of the RYR label throughout the SR system and outlines the regions of both the mitochondria and nucleus which remain unlabeled. Vasculature in between the heater cells also remains unlabeled. The staining pattern with the monoclonal antibody is highly specific as evidenced by the lack of any signal around the smooth muscle of arteries which have the appearance of the negative controls (Fig. 6). For comparison, longitudinal sections of the toadfish swimbladder muscle reveal highly organized rows of punctate fluorescence (27) with a characteristic 1- $\mu$ m spacing (in toadfish swimbladder muscle that has



**Figure 4.** Confocal laser scanning immunofluorescent image of the blue marlin heater tissue labeled with C010. The label corresponds to the SR network which surrounds the unlabeled mitochondria (see Fig. 1). The stain is occasionally punctate but primarily labels in a homogenous fashion. Nuclei are centrally located and remain unstained and the rich vasculature is also not labeled.



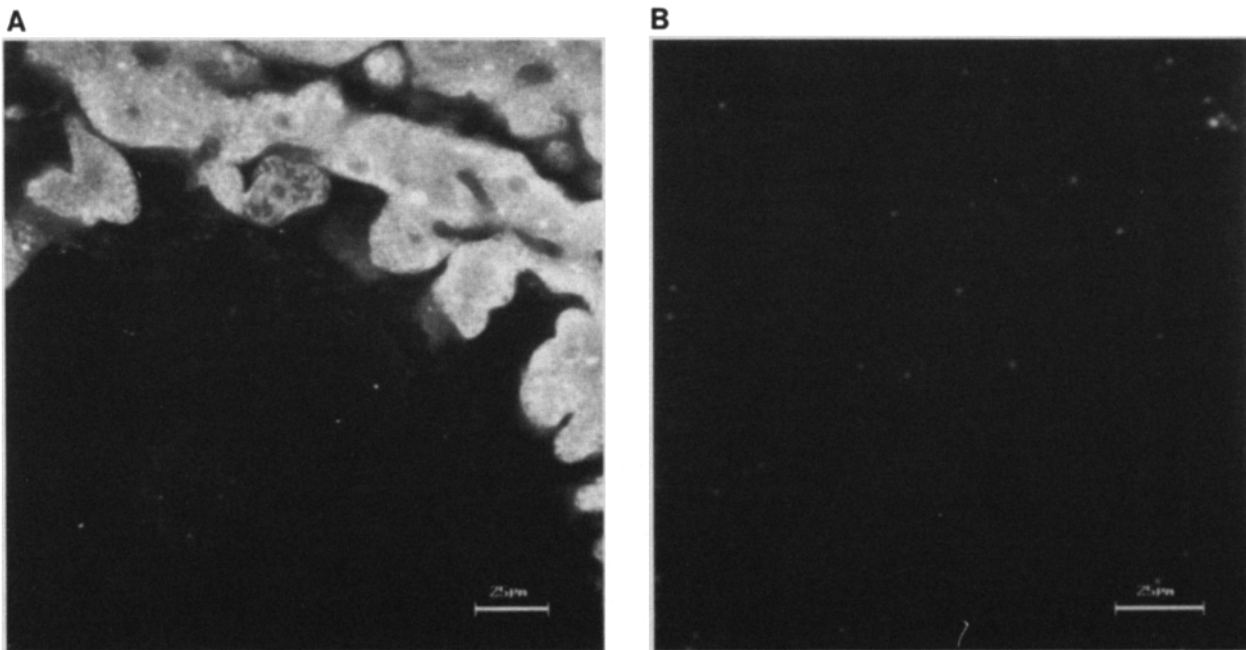
**Figure 5.** Confocal micrograph of heater tissue labeled with RYR C010 in two species of billfishes with heater organs, the swordfish (*A*) and blue marlin (*B*) showing the same distribution of the RYR label as seen in the blue marlin heater cell.

not contracted), running transversely to the long axis of the muscle fiber (Fig. 7 *b*).

#### **Functional Properties of Heater Ryanodine Receptor**

[<sup>3</sup>H]Ryanodine binding to heavy SR fractions has indicated that the plant alkaloid ryanodine specifically binds at nanomolar concentrations to the Ca<sup>2+</sup> release channel of rabbit skeletal and canine heart SR (44). The binding of

[<sup>3</sup>H]ryanodine to terminal cisternae of skeletal muscle fibers has been shown to be affected by ligands that modulate calcium release in an isoform specific manner (e.g., Ca<sup>2+</sup>, Mg<sup>2+</sup>, caffeine, and adenine nucleotides). The ryanodine receptor in the unfractionated heater SR vesicles was characterized by determining its affinity for [<sup>3</sup>H]ryanodine and the calcium dependency of binding. [<sup>3</sup>H]Ryanodine binding characteristics of the unfractionated heater microsomes are



**Figure 6.** Confocal micrograph reveals rich label of RYR in heater cells in comparison to regions of smooth muscles (artery, *A*) and negative control (*B*). The label is exceptionally clean and stops as soon as vascular tissue is encountered. Negative control has autofluorescence (*small punctate dots*) due to numerous mitochondria, which is characteristic of the heater cells.

**Table I. Ryanodine Binding Properties of Heater Tissue and Extraocular Muscle**

Sample	$K_d$	$B_{max}$
(n)	(nM)	(pmoles/mg)
Heater organ (3)	$8.7 \pm 5.4$	$2.8 \pm 0.9$
Superior rectus (3)	$9.8 \pm 2.8$	$2.2 \pm 0.8$

summarized in Table I. The affinity for ryanodine of the heater RYR is not significantly different from that of the extraocular muscle RYR. [ $^3\text{H}$ ]Ryanodine binding in heater SR was activated at a threshold of  $\sim 0.1 \mu\text{M}$ , was optimal at  $10 \mu\text{M}$  free  $\text{Ca}^{2+}$ , and was completely inhibited by  $1 \text{ mM}$  free  $\text{Ca}^{2+}$ , resulting in a bell-shaped  $\text{Ca}^{2+}$ -activation response (Fig. 8). This bell-shaped  $\text{Ca}^{2+}$  dependency closely corresponds to that observed in blue marlin superior rectus muscle SR, which expresses only the  $\alpha$  RYR isoform, the toadfish swimbladder muscle SR ( $\alpha$  only) and rabbit skeletal muscle RYR (40). Ryanodine binding to heater SR was stimulated 20-fold by addition of  $2.5 \text{ mM}$  AMP-PCP with a shift in the optimum  $\text{Ca}^{2+}$  level to  $100 \mu\text{M}$ . Only 30% inhibition of [ $^3\text{H}$ ]ryanodine binding was observed at  $3.3 \text{ mM}$  calcium under these conditions (not shown).

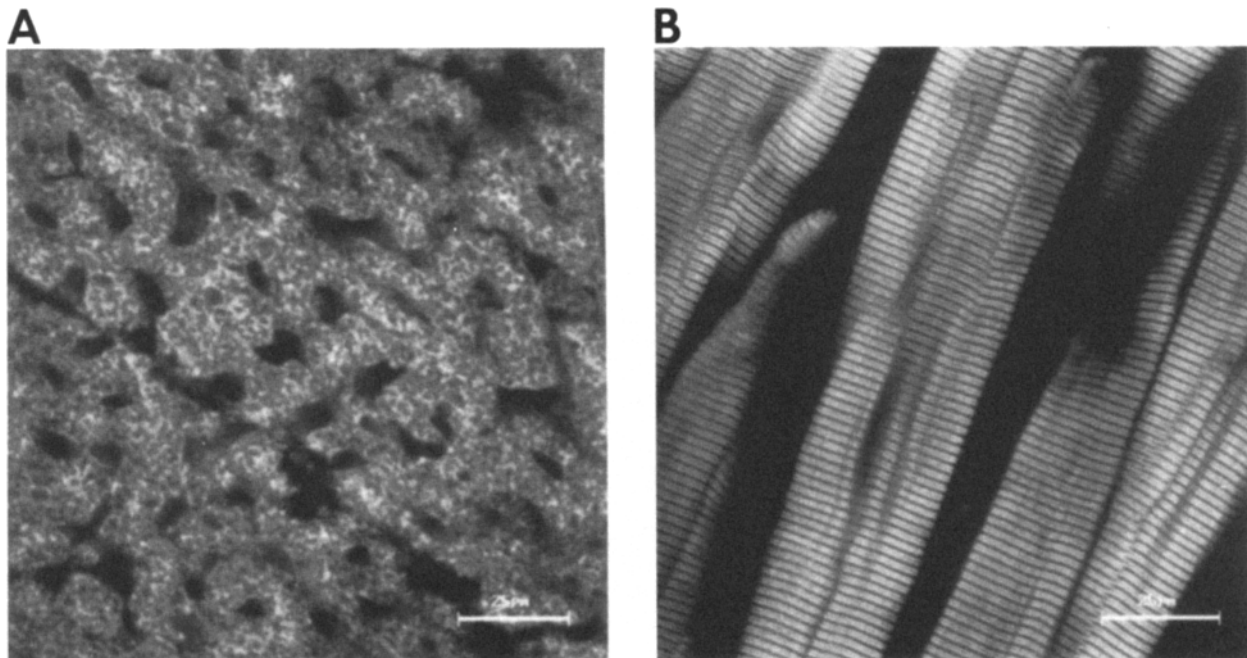
The presence of a functional  $\text{Ca}^{2+}$  release channel pathway in heater cells was shown by determining the ability of heater SR to retain  $^{45}\text{Ca}^{2+}$  in the presence of RYR channel modulators. The vesicle fractions were passively loaded with  $5 \text{ mM}$   $^{45}\text{Ca}^{2+}$  and then diluted into a medium which has been shown to inhibit or activate the  $\text{Ca}^{2+}$  release channel of rabbit skeletal and canine heart SR (38, 39).  $^{45}\text{Ca}^{2+}$  efflux in heater SR vesicles and trout unfractionated SR vesicles was slow when the vesicles were placed in a  $\text{Mg}^{2+}$  and ruthenium red containing medium which inhibited the SR

$\text{Ca}^{2+}$  release channel. Extrapolation of the efflux curve back to the time of vesicle dilution indicated that the heater and trout muscle microsomal fractions trapped  $\sim 45 \pm 18 \text{ nmol}$  and  $50 \pm 10 \text{ nmol } ^{45}\text{Ca}^{2+}/\text{mg}$  protein, respectively. When vesicles were diluted into the  $\text{Ca}^{2+}$  release channel activating medium which contained  $10 \mu\text{M}$  free  $\text{Ca}^{2+}$  and  $1.0 \text{ mM}$  AMP-PCP, reduced amounts of  $^{45}\text{Ca}^{2+}$  were retained by the heater tissue vesicles. 25% of the trapped  $^{45}\text{Ca}^{2+}$  was released from the heater vesicles in 30 s. The control trout white muscle fraction displayed a somewhat greater extent of release, releasing 55% of the trapped  $^{45}\text{Ca}^{2+}$  within 30 s. The  $^{45}\text{Ca}^{2+}$  remaining with the vesicles 30 s after dilution was released with rates comparable to those in the channel-inhibiting medium. A biphasic release behavior consisting of a rapid and slow release component has been shown to indicate the presence of two subpopulations of vesicles, a permeable fraction containing a  $\text{Ca}^{2+}$  and adenine nucleotide activatable  $\text{Ca}^{2+}$  release channel and an impermeable fraction lacking the channel (38).

### Calcium ATPase

Unfractionated vesicles from heater tissue have a protein profile enriched in the 100,000 D band presumed to be the  $\text{Ca}^{2+}$  ATPase (Fig. 2 A). The presence of a high amount of the pump in the SR profile is consistent with freeze-fracture images of the cytoplasmic surface of the heater SR (6). Immunofluorescent localization of the  $\text{Ca}^{2+}$  ATPase was not possible because monoclonal and polyclonal antibodies raised against mammalian SERCA 1 and SERCA 2 isoforms of the  $\text{Ca}^{2+}$  ATPase fail to show cross-reactivity with the isoform of the  $\text{Ca}^{2+}$  ATPase expressed in scombroid fish swimming muscles, extraocular muscle or heater tissue.

When skeletal muscle is homogenized, SR forms vesicles that retain the ability to rapidly accumulate  $\text{Ca}^{2+}$  against a



**Figure 7.** Confocal micrograph of RYR C010 label in blue marlin heater tissue (A) and control swimbladder muscle of toadfish (B). In toadfish, the RYR label has a junctional pattern of labeling for this muscle (A/I band), is punctate at higher magnification and colocalizes with the dihydropyridine receptor (not shown).

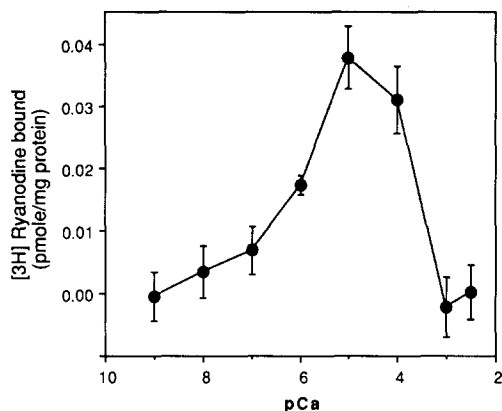


Figure 8.  $[^3\text{H}]$ Ryanodine binding in relationship to pCa in heater tissue. Marlin heater tissue (an  $\alpha$  only RYR tissue) has a  $\text{Ca}^{2+}$  activation and inactivation profile similar to other fish tissues that contain only the  $\alpha$  isoform of the ryanodine receptor (toadfish swimbladder muscle, marlin extraocular muscle).

concentration gradient when ATP,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$  are present (24, 34).  $\text{Ca}^{2+}$  loading (uptake in the presence of oxalate) was investigated in homogenates and in unfractionated SR vesicles from both heater and extraocular muscle in the presence of mitochondrial  $\text{Ca}^{2+}$  uptake inhibitors. As shown in Table II, homogenates from heater tissue accumulate  $\text{Ca}^{2+}$  with an appreciably higher rate than homogenates prepared from the superior rectus muscle. Heater homogenates accumulate about twice as much  $\text{Ca}^{2+}$  per gram tissue as the muscle homogenates. Homogenates from marlin liver tissue showed little  $\text{Ca}^{2+}$  accumulation (<5 nmoles per mg protein after 20 min) under these same conditions.

When the unfractionated SR vesicles were incubated in the standard reaction medium for  $\text{Ca}^{2+}$  loading experiments, both heater and muscle preparations showed significant  $\text{Ca}^{2+}$  uptake. The mean initial  $\text{Ca}^{2+}$  loading rate and the total  $\text{Ca}^{2+}$  taken up after 20 min (loading capacity) were not significantly different (paired *t*-test) between the muscle and heater tissue membrane fractions expressed as specific activities.  $\text{Ca}^{2+}$  uptake in both the heater and muscle SR fractions was dependent on the presence of ATP. The removal of oxalate from the medium decreased the initial rate of  $\text{Ca}^{2+}$  loading to background levels (no ATP present) in both types of vesicles.

Mitochondrial contamination was present in the SR fractions of heater and muscle.  $\text{Ca}^{2+}$  uptake in mitochondria is a carrier-mediated process and does not occur via a specific  $\text{Ca}^{2+}$  pump as in SR (36).  $\text{Ca}^{2+}$  accumulation in mitochondria is not enhanced by oxalate and is inhibited 90 to 100% by uncouplers such as CCCP or by inclusion of electron transport or mitochondrial ATPase inhibitors (8).  $\text{Ca}^{2+}$  loading experiments were performed in the presence of either CCCP and sodium azide, or CCCP and oligomycin. In the absence of the mitochondrial inhibitors,  $\text{Ca}^{2+}$  loading in the heater SR fractions was only slightly increased (by 1–10%), however a significant increase (1–4%) was also observed for the extraocular muscle SR vesicle fractions.

$\text{Ca}^{2+}$ -stimulated ATPase activities of the heater and muscle unfractionated SR vesicles were measured in the presence of the mitochondrial ATPase inhibitor oligomycin (Fig. 9). Similar "basic" ATPase activities (measured in a low free

Table II.  $\text{Ca}^{2+}$ -loading Properties of Homogenates and Unfractionated SR Fractions Prepared from Heater Tissue and Extraocular Muscle in Fish

Assay	Heater	Muscle
Homogenate $\text{Ca}^{2+}$ loading capacity $\mu\text{mol}/\text{gram tissue}/20 \text{ min}$ ( $n = 3$ )	$23.6 \pm 0.5$	$10.1 \pm 1.2$
Unfractionated vesicles $\text{Ca}^{2+}$ loading rate $\mu\text{mol}/\text{min}^{-1}/\text{mg protein}^{-1}$ ( $n = 9$ )	$1.0 \pm 0.40$	$1.0 \pm 0.2$
$\text{Ca}^{2+}$ loading capacity $\mu\text{mol}/\text{mg protein}/20 \text{ min}$ ( $n = 9$ )	$2.5 \pm 0.67$	$2.7 \pm 0.22$
$[^{32}\text{P}]$ Phosphoenzyme formation $\text{nmol } ^{32}\text{P}/\text{mg protein}$ ( $n = 4$ )	$1.1 \pm 0.29$	$1.3 \pm 0.4$

Enzymatic activities are determined as described under the Methods section.  $\text{Ca}^{2+}$ -stimulated ATPase activities were determined in the presence of the ionophore A23187. Number of experiments is given in parentheses.

$\text{Ca}^{2+}$  solution) and  $\text{Ca}^{2+}$ -stimulated ATPase activities were observed for the heater and muscle vesicle fractions.  $\text{Ca}^{2+}$ -stimulated ATPase activities of both heater and muscle fractions were stimulated when rendering the vesicles permeable to  $\text{Ca}^{2+}$  by the addition of the  $\text{Ca}^{2+}$  ionophore A23187. In the absence of the ionophore, in muscle and heater vesicles, the  $\text{Ca}^{2+}$ -stimulated ATPase is relatively low due to inhibition of the pump by the accumulated  $\text{Ca}^{2+}$ .

The molar ratio of  $\text{Ca}^{2+}$  loading to  $\text{Ca}^{2+}$ -stimulated ATPase activity is a measure of how much  $\text{Ca}^{2+}$  is translocated into the membrane vesicles per ATP hydrolyzed. The efficiency of  $\text{Ca}^{2+}$  transport was determined at pH 6.9 in the presence of 5 mM oxalate using the same medium for both assays. Studies with rabbit skeletal muscle SR have indicated that maximally two  $\text{Ca}^{2+}$  are transported per ATP hydrolyzed

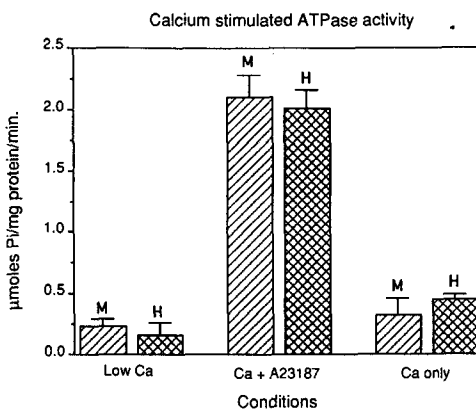


Figure 9. Calcium stimulated ATPase activities in both muscle and heater fractions in the presence of mitochondrial inhibitor oligomycin. The low calcium condition is background ATPase activity and is always low in the presence of oligomycin. The  $\text{Ca}^{2+}$  and A23187 condition is the maximally stimulated  $\text{Ca}^{2+}$  ATPase activity. The  $\text{Ca}^{2+}$  only condition is used to determine the leakiness of the membrane vesicle preparation. The large difference between the presence and absence of A23187 is indicative of intact "tight" vesicles. The enzyme stops translocating  $\text{Ca}^{2+}$  as the vesicle fills with  $\text{Ca}^{2+}$ . Data are means  $\pm$  S.D. from seven individual preparations.



(53). For three heater and billfish muscle homogenate preparations, the mean  $\text{Ca}^{2+}/\text{ATP}$  coupling ratio was  $0.962 \pm 0.213$  and  $0.968 \pm 0.15$ , respectively.

### Formation of the [ $^{32}\text{P}$ ]Phosphoenzyme Intermediate

ATP-dependent  $\text{Ca}^{2+}$  transport by the  $\text{Ca}^{2+}$  ATPase occurs through the transient formation of a covalently bound, acyl phosphate intermediate (50). The formation of the intermediate, referred to here as the phosphoenzyme, is correlated with the number of active  $\text{Ca}^{2+}$  pump protein sites of the SR and can therefore be used to estimate the SR  $\text{Ca}^{2+}$  ATPase content of the membrane preparation. As indicated in Table II, unfractionated SR vesicles from heater and muscle produced a similar amount of the [ $^{32}\text{P}$ ]phosphoenzyme intermediate in the presence of  $\text{Ca}^{2+}$ . In the presence of 1 mM EGTA there was a 75% decrease in heater and a 85% decrease in muscle [ $^{32}\text{P}$ ]phosphoenzyme formation.

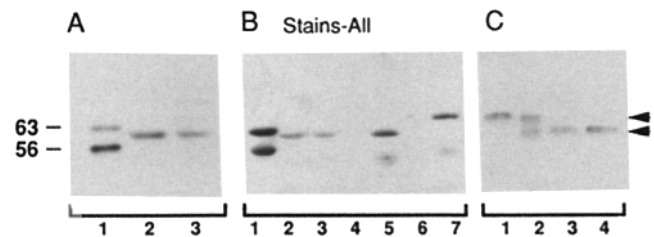
### Heater SR Contains Calsequestrin

Calsequestrin is a prominent  $\text{Ca}^{2+}$  binding protein of skeletal and cardiac muscle SR (34). Electron micrographs of heater tissue (Fig. 1) demonstrate that the lumen of the SR in heater cells often has an electron dense content similar to the electron dense content in skeletal muscle terminal cisternae (19, 25, 26). To confirm the presence of calsequestrin we analyzed the protein composition of unfractionated SR vesicles by SDS PAGE using the cationic carbocyanine dye "stains all" and by immunoblotting techniques. Calsequestrin was then localized using immunofluorescent techniques.

Mammalian striated muscles express two distinct isoforms of calsequestrin that can be distinguished on the basis of their different mobilities in SDS-polyacrylamide gels (34).  $\text{Ca}^{2+}$  binding proteins in fish heater SR, extraocular muscle SR, and fast-twitch skeletal muscle were analyzed on SDS-PAGE gels, with calsequestrin purified from mammalian SR fractions (dog heart and rabbit skeletal muscle) as standards. Using the stains-all procedure unfractionated heater vesicles showed one deep blue staining band with an apparent mobility of 60,000 D (Fig. 10 B, lane 2). Extraocular muscle of marlin had a blue staining band that was identical in mobility to the heater band (Fig. 10 B, lane 3). The heater and eye muscle blue staining bands ran faster than the mammalian skeletal calsequestrin but slower than mammalian cardiac calsequestrin controls (Fig. 10 B, lane 1). The mobility of the extraocular and heater blue staining bands were faster than a blue staining protein of fish fast-twitch skeletal muscle of apparent  $M_r$  66,000 (Fig. 10 B, lane 7).

Immunoblot techniques were used to confirm the identification of the proteins identified in the stains-all gels (Fig. 10, A and C). A canine anti-cardiac calsequestrin polyclonal antiserum (a gift from Dr. Larry Jones) cross-reacted with the fish SR preparations. In all of the fish SR vesicles the calsequestrin antibody cross-reacts with the same protein bands identified as a  $\text{Ca}^{2+}$  binding protein band in the stains-all gels. The immunoblots confirm that the heater and extraocular muscle isoform of calsequestrin runs between the two mammalian isoforms. Occasionally fractions of the extraocular muscle revealed the presence of both isoforms of fish calsequestrin (Fig. 10 C, lane 2) perhaps indicative of the mixed fiber population of these muscles.

Localization of calsequestrin in heater tissue was con-



**Figure 10.** Immunoblot and "stains-all" analysis of calsequestrin in heater and extraocular muscle tissues. (A) Immunoblot of unfractionated SR vesicles from heater tissue (lane 2) and superior rectus eye muscle (lane 3), with purified mammalian skeletal and cardiac calsequestrin shown in lane 1. The blot was performed with an affinity purified polyclonal antibody raised against canine cardiac calsequestrin. Heater and extraocular muscles have a similar band that has a mobility in between the two mammalian isoforms. (B) Stains-all corroborates the immunoblot results. Purified skeletal and cardiac muscle calsequestrins are used for controls (lane 1), heater tissue (lane 2), eye muscle (lane 3), red muscle (lane 5), and white, fast-twitch swimming muscles are in lane 7. The blue staining bands in heater and extraocular muscle run in between the mammalian bands. (C) Immunoblot staining of SR vesicles from blue marlin white muscle (lane 1), eye muscle (lane 2), heater tissue (lane 3), and cardiac muscle (lane 4).

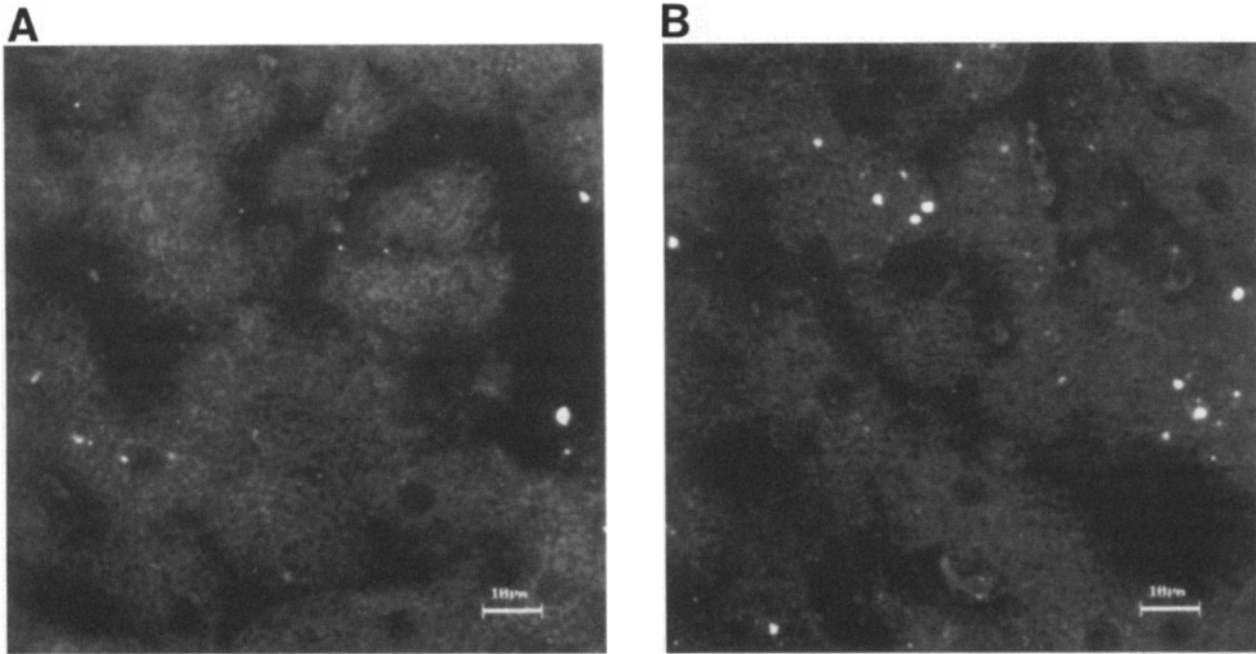
siderably more difficult than the localization of the RYR due to a weaker signal with the polyclonal calsequestrin antibody in fish muscle tissues. Although the fluorescent signal was not as strong in fish heater tissue, it did produce a characteristic terminal cisternae binding pattern in both marlin and toadfish skeletal muscle (not shown). Calsequestrin localization had a diffuse distribution throughout the heater cell SR, significantly above background, and was comparable to the RYR stain (Fig. 11). The label outlined the position of the mitochondria which remained unstained. This localization is consistent with the EM images of SR with an electron dense luminal content throughout the heater cytoplasm.

### Discussion

The heater cell of billfishes is a specialized type of skeletal muscle fiber that has been evolutionarily modified to generate significant amounts of heat (up to  $250 \text{ watts} \times \text{kg}^{-1}$ ) rather than force (5, 52). Thermogenesis in the heater cells is hypothesized to be associated with a thermogenic process involving  $\text{Ca}^{2+}$  cycling between the SR and cytosol, and subsequent stimulation of substrate oxidation and mitochondrial respiration (4, 5). In this study, three SR proteins associated with  $\text{Ca}^{2+}$  release and reuptake in normal muscle have been identified and characterized. In support of a putative role for the heater cell SR in  $\text{Ca}^{2+}$  cycling and thermogenesis, the heater cell SR was shown to contain a rich distribution of the  $\alpha$  RYR isoform, a  $\text{Ca}^{2+}$  ATPase that has a high capacity to translocate  $\text{Ca}^{2+}$ , and the  $\text{Ca}^{2+}$  binding protein calsequestrin.

### Heater Cells Express Only the $\alpha$ Isoform of the RYR

In skeletal and cardiac muscle, the SR calcium release channel (RYR) is the major site for  $\text{Ca}^{2+}$  release from the SR. The RYR has been most extensively studied in mammalian striated muscles where the protein family was first functionally and structurally characterized from the SR of skeletal



**Figure 11.** Indirect immunofluorescent localization of calsequestrin in blue marlin heater tissue demonstrates that this protein is distributed in a pattern that is similar to that observed for the RYR. This is consistent with the distribution of the electron dense content within the lumen of the SR that is observed ultrastructurally (Fig. 1).

and cardiac muscles (17, 39, 42, 51, 54). Three isoforms of the RYR have been identified in mammals, a skeletal muscle isoform (RYR1) expressed primarily in skeletal muscle (51), a cardiac isoform (RYR2) present in heart and brain tissue (42), and a brain isoform (RYR3) expressed predominantly in brain and smooth muscle (23). All three isoforms exist as homotetrameric proteins composed of a monomer of approximately  $M_r$  565,000. While mammalian skeletal muscles express only the single skeletal muscle RYR isoform (RYR1), avian, amphibian, certain reptilian and fish skeletal muscles coexpress two isoforms termed  $\alpha$  and  $\beta$  (1, 31, 40). Immunological, biochemical, and physiological data indicate the two RYR isoforms,  $\alpha$  and  $\beta$ , are functionally distinct (1, 40, 41). Physiological and molecular data indicate that the non-mammalian  $\alpha$  is homologous to the mammalian skeletal RYR while the  $\beta$  isoform is homologous with the mammalian type 3 receptor (41, 43). Molecular results of cloning and sequencing of the marlin extraocular  $\alpha$  isoform indicate homology with the mammalian skeletal RYR (Franck, J., personal observation).

In this study, the presence of the RYR in heater cells has been demonstrated using several independent techniques. Solubilization of heater SR and sucrose gradient purification of the RYR yields a single high molecular weight polypeptide with an apparent molecular weight equivalent to that of the  $\alpha$  isoform from fish skeletal muscle and to the rabbit skeletal muscle type 1 RYR. The fast contracting extraocular muscles which give rise to the heater phenotype also express only a single high molecular weight polypeptide identifiable as the  $\alpha$  isoform of non-mammalian skeletal muscle (40).

The neutral plant alkaloid ryanodine binds with nanomolar affinity to all isoforms of the RYR and also provided a sensitive means for identifying the presence of a  $Ca^{2+}$  release channel in heater tissue. The unfractionated SR

vesicles from heater tissue showed specific, high-affinity [ $^3H$ ]ryanodine binding, demonstrating the presence of the RYR. The  $B_{max}$  and  $K_d$  are similar to values reported for toadfish swimbladder muscles (both  $\alpha$  isoform only), as well as mammalian skeletal muscle RYRs (41). The bell-shaped  $Ca^{2+}$  dependency of the ryanodine binding curve has properties identical to the calcium dependency of binding in the other fish muscles that express only the  $\alpha$  isoform (41) and similar to the  $Ca^{2+}$  dependency of binding in rabbit skeletal muscle. Calcium-dependent inactivation of [ $^3H$ ]ryanodine binding to the heater RYR indicates that high calcium concentrations likely shut down SR calcium release. SDS-PAGE and immunoblot results also indicate that the RYR expressed in the heater organ is the  $\alpha$  isoform, which is functionally equivalent to the skeletal (type 1) RYR of mammals. The expression of a single isoform in the heater and extraocular muscle is a specialized condition, presumably linked with the superfast contraction capabilities of extraocular muscle. The expression pattern for the heater and eye muscle is in sharp contrast to the slow and fast-twitch muscles of marlin and swordfish which have been shown here via immunoblot analysis and SDS-PAGE to have the ancestral non-mammalian condition of two RYR isoforms.

Immunofluorescent localization of the RYR in heater SR provides a striking result. The RYR was situated throughout the SR. Specific foci indicating a concentration of the stain or the receptor were not seen and thus junctional regions of SR, homologous with the terminal cisternae of normal skeletal muscle, were not readily identified. In the previous ultrastructural study of the heater cell it was shown that triads, similar to those of skeletal muscle with a high density of feet are present in the heater tissue. The presence of triads would indicate a discontinuous distribution of high density foci of RYRs on the SR. However, the immunofluorescent results

suggest a more continuous distribution of the RYR. The network appearance of the RYR immunofluorescent label is complementary to the appearance of the T tubule with Golgi labeling and the disposition of the immunofluorescent signals when staining for the  $\alpha_1$  subunit of the DHPR (Block, B. A., and S. Y. Kim, unpublished results). The network disposition of labeling is similar to that found in smooth muscle and neuronal cells and suggests a more homogeneous localization of the  $\alpha$  RYR isoform throughout the SR of the specialized heater organ (14). Visualization of the foot portion of the RYR using a variety of electron microscopy techniques produced images of extensive or long terminal cisternae which may result in the continuity between regions of localization (6).

Studies of calcium release properties of the unfractionated vesicles should be considered preliminary in this report and should be viewed as further indication of the presence of the  $\text{Ca}^{2+}$  release channel. The heater SR calcium release channel was opened and closed by the modulators of the channel.

### *Ca<sup>2+</sup> ATPase*

Ultrastructural studies indicated previously that the extensive SR present in the heater cell had structural properties equivalent to those of skeletal muscle SR (6). Freeze-fracture studies revealed the presence of intramembranous particles with dimensions and densities that are appropriate for the  $\text{Ca}^{2+}$  pump protein, and vanadate treated vesicles readily formed crystalline arrays of the pump. Comparison of the protein profile of the heater tissue SR fraction with purified SR from muscle suggests that the major protein band with an apparent molecular weight between 100,000 to 105,000 D seen in all of the heater tissue preparations is the  $\text{Ca}^{2+}$  ATPase. The functional studies reported here demonstrate that homogenates and microsomal fractions prepared from heater tissue are able to accumulate  $\text{Ca}^{2+}$  in the presence of ATP and  $\text{Mg}^{2+}$ . This indicates an ATP dependent  $\text{Ca}^{2+}$  translocating protein is present in the heater cells.  $\text{Ca}^{2+}$  loading,  $\text{Ca}^{2+}$ -stimulated ATPase activities and phosphoenzyme assays confirm that the heater SR vesicles sequester  $\text{Ca}^{2+}$  through an ATP-dependent  $\text{Ca}^{2+}$  pump.

Comparison of heater tissue  $\text{Ca}^{2+}$  loading rates and ATPase activities with other studies on more conventional muscles is difficult due to the differences in the protocols used. Briggs et al. (9) found that the  $V_{\text{max}}$  values of  $\text{Ca}^{2+}$  loading for homogenates from fast-twitch and slow-twitch rat muscles were 50 and 10  $\mu\text{mol Ca}^{2+}$  per g wet tissue per min, respectively. For the extensor digitorum longus (predominantly fast twitch) and soleus (slow twitch) of the cat the  $V_{\text{max}}$  values were 34 and 5.6  $\mu\text{mol Ca}^{2+}$  per g per min, respectively. By comparison,  $\text{Ca}^{2+}$  loading rates observed in this study for heater tissue homogenates were about 1  $\mu\text{mol Ca}^{2+}$  per g wet tissue per min. For unfractionated heater vesicles,  $\text{Ca}^{2+}$  loading rates of 0.6–1.8  $\mu\text{mol Ca}^{2+}$  per  $\mu\text{g}$  protein per min were measured. These rates compare favorably with  $\text{Ca}^{2+}$  loading rates (1–6  $\mu\text{mol Ca}^{2+}$  per  $\mu\text{g}$  protein per min) obtained with purified rabbit skeletal muscle SR vesicle preparations (37).

In heater tissue, as in extraocular muscle,  $\text{Ca}^{2+}$  ATPase activity was stimulated several fold in the presence of the  $\text{Ca}^{2+}$  ionophore A23187. The presence of the ionophore dissipates the  $\text{Ca}^{2+}$  gradient across the membrane of the SR vesicles which results in stimulation of the enzyme activity

due to release of intravesicular inhibitory  $\text{Ca}^{2+}$ . The increase of the ATPase activity in the presence of the ionophore is indicative of intact vesicle preparations in both heater and muscle fractions and also indicates that there is no endogenous leak (or enhanced  $\text{Ca}^{2+}$  permeability) in a majority of the heater vesicles.

Most of the studies on the heater ATPase were done with the extraocular muscle ATPase as a control. Recent studies by A. Tullis and B. A. Block (unpublished results) have indicated that the heater and muscle cells express the same isoform of the protein. By amplifying from cDNA functionally important conserved domains, their study has revealed that heater cells express the same form of the pump as fast-twitch muscles in fish. By convention with the mammalian paradigm of isoform classification this indicates that the heater cells express the SERCA 1 isoform of the pump. This coincides well with the RYR expression pattern which would indicate selection for rapid *in vivo* release kinetics by virtue of the presence of the  $\alpha$  isoform (40).

### *Calsequestrin*

Calsequestrin is a major protein component of the junctional SR and is localized intralumenally in mammalian cardiac and skeletal muscles (26, 34). In mammalian muscles, cardiac, and skeletal isoforms of calsequestrin have been purified, cloned, and sequenced. Both isoforms serve as high capacity, moderate affinity  $\text{Ca}^{2+}$  binding proteins. The cardiac form is expressed in vertebrate cardiac and slow-twitch skeletal muscle but not in fast-twitch skeletal muscle (47). The skeletal form is only expressed in fast-twitch muscles. In addition to sequestering  $\text{Ca}^{2+}$ , the protein has been postulated to play a role in facilitating  $\text{Ca}^{2+}$  release from the skeletal muscle ryanodine receptor although the details of the interaction or facilitation of release have not been determined. The mammalian isoforms are 65% identical at the amino acid level and differ primarily in the carboxyl terminus where the cardiac isoform has an extension of 31-amino acid residues (47). In this study we have identified the presence of calsequestrin in the heater cells by electron microscopy, staining with the stains-all procedure, immunoblot analysis and light microscopy level immunolocalization. The polyclonal antibody used in the present study has cross-reactivity with both calsequestrin isoforms in fishes (skeletal and cardiac). Previous studies have established that mammalian skeletal and cardiac calsequestrins differ in their mobilities in alkaline SDS-PAGE gels (47, 48). The heater and extraocular muscle isoforms of calsequestrin run between the two mammalian forms under alkaline conditions on SDS gels. Calsequestrin from fish fast-twitch muscle (swimming musculature) has a lower mobility than the mammalian fast-twitch form and does not have a similar mobility to the predominant calsequestrin isoform expressed in heater and extraocular muscle. Interestingly, fish cardiac calsequestrin has a mobility that is identical to that of heater (Block, B. A., unpublished results).

### *Excitation-Thermogenic Coupling*

In normal skeletal muscles, intracellular  $\text{Ca}^{2+}$  concentrations are controlled by the T tubule and sarcoplasmic reticulum membranes (46). Release of  $\text{Ca}^{2+}$  from the SR is triggered by an action potential at the neuromuscular junction. The transverse tubule system relays the signal to the SR via

the coupling between the T tubule DHPR (voltage sensor) and the skeletal ryanodine receptor, resulting in  $\text{Ca}^{2+}$  efflux into the sarcoplasm (18, 45).  $\text{Ca}^{2+}$  release is thought to occur only at the sites where junctional T tubules and SR are morphologically associated with one another (at the triads). Activation by the DHPR of  $\text{Ca}^{2+}$  release by the skeletal ryanodine receptor may be due either to a direct interaction between the two proteins or to activation via a third intermediate protein (18). Muscle contracts when the sarcoplasmic  $\text{Ca}^{2+}$  concentration reaches  $10^{-6}$  M, and relaxes when the  $\text{Ca}^{2+}$  concentration is reduced below  $10^{-7}$  M as  $\text{Ca}^{2+}$  is pumped back into the membrane system by the  $\text{Ca}^{2+}$  ATPase located primarily in the longitudinal regions of the SR (35). A similar cycle of calcium release and uptake, excitation-thermogenic coupling, may also operate in the heater cells.

The present study provides the first direct evidence for the presence of the SR proteins generally deemed to be necessary for SR  $\text{Ca}^{2+}$  uptake, storage and release thus providing the biochemical support for the SR  $\text{Ca}^{2+}$  cycling hypothesis of Block (5). In addition to the SR, an abundance of mitochondria and an elaborate transverse (T) tubule network have been demonstrated in the heater cells (6). One possibility is that upon depolarization,  $\text{Ca}^{2+}$  release from the SR leads to an increase in cytoplasmic  $\text{Ca}^{2+}$  which would stimulate the  $\text{Ca}^{2+}$  ATPase resulting in increased ATP utilization. The increased  $\text{Ca}^{2+}$  would also stimulate respiration-dependent cycling of  $\text{Ca}^{2+}$  at the mitochondria. Both of these processes would result in an increase of heat production and are similar to the mechanisms associated with heat production in malignant hyperthermia (22, 33).

If heat production in the fish thermogenic muscle tissue is related to a  $\text{Ca}^{2+}$  cycle that involves  $\text{Ca}^{2+}$  release and sequestration from the SR one question that needs to be addressed is: how would such a system be regulated? Motor innervation from the oculomotor nerve is still apparent in the intact portion of the superior rectus and innervation is also found in the modified region of the muscle. To determine if the heat producing cycle in these modified muscle cells is mediated by the same depolarization induced pathway as in skeletal muscle excitation-contraction coupling it will be necessary to establish the precise structural and functional links between the SR and T-system in heater tissue.

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