The Structure of the Membrane Systems in a Novel Muscle Cell Modified for Heat Production

Barbara A. Block and Clara Franzini-Armstrong

Departments of Biology and Anatomy, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract. A thermogenic organ, modified from an eye muscle, warms the brain and eyes of several oceanic fish. The extraocular muscles associated with thermogenesis are composed of modified muscle cells that are structurally distinct from all other types of muscle previously described. In "heater" cells, contractile filaments are virtually absent and the cell volume is packed with mitochondria and smooth membranes. Freeze-fracture studies and negative staining of microsomal fractions treated with vanadate indicate that most of the membrane system of heater cells has a high Ca^{2+}-ATPase density and is equivalent to skeletal muscle sarcoplasmic reticulum (SR). High voltage electron micrographs of heater cells infiltrated with the Golgi stain demonstrate that the cells also have an extensive transverse tubule system with a complicated three-dimensional structure. Junctional regions between transverse tubules and SR occur in the heater cell and contain feet protein. Activation of thermogenesis in heater cells may occur through the same protein components involved in excitation–contraction coupling and appears to be associated with the ATP-dependent cycling of calcium at the SR.

SEVERAL groups of large oceanic fish, commonly known as billfish, have extraocular muscles that function as a heat-generating organ located beneath the brain and close to the eyes (1–3, 5). The presence of this heater organ allows these ectothermic fish to maintain elevated brain and eye temperatures, an unusual condition in animals that have large heat losses associated with the gill and body surfaces. A striking alteration in the blood supply to the head accompanies the presence of the heat-generating muscle tissue. The entire carotid circulation to the brain and eye passes through the modified portion of the muscle thus sending a convective supply of warm blood to the retina and central nervous system. In the case of the swordfish, *Xiphias gladius*, measurements on free-swimming fish indicate that temperature in the cranial cavity remains ~29°C while the body temperature of the fish fluctuated with the ambient water temperatures (≤15°C) for several hours during deep dives (5).

The eye muscles associated with thermogenesis have an unusual structure that appears to optimize the muscle cell for heat production rather than force production. In the billfish (marlins, sailfish, and spearfish), the superior rectus muscles are primarily composed of modified muscle cells (heater cells) which have few, if any, myofibrils and contractile filaments. The cells associated with heat production are tightly packed with mitochondria and have an extensive membrane system comprising tubules, cisternae, and membranous stacks located throughout the cytoplasm in between the mitochondria (1–3). The mitochondria of the heater cell occupy between 55 and 70% of the cell volume and studies on isolated mitochondria indicate they generate ATP and lack the 32-kD uncoupling protein associated with thermogenesis in mammalian brown adipose tissue (1–3). A large portion of the heater cell membrane network has been identified as sarcoplasmic reticulum (SR)[1] based on its high calcium-activated ATPase activity (2, 3; Block, B. A., and G. Meissner, manuscript submitted for publication). The heater cell SR also has calcium-release activity, as shown by ion flux studies, and binds the calcium-release channel probe ryanodine.

Contraction and relaxation in normal skeletal muscle are regulated by the cycling of Ca^{2+} ions which is under the control of the SR and transverse (T) tubule membrane systems. The SR and T membranes of normal muscle have two structurally and functionally defined regions characterized either as junctional or nonjunctional. The nonjunctional or free SR is primarily involved with the uptake of Ca^{2+} and the Ca^{2+}-ATPase transport protein comprises 90% of the membrane protein (24). A direct interaction between T and SR occurs at specialized junctional regions, called triads, which contain the proteins thought to be involved in excitation–contraction coupling. Release of Ca^{2+} from the junctional SR is coupled to T tubular membrane depolarization by a mechanism which involves voltage-sensing molecules in the junctional T tubular membrane and Ca^{2+}-release channels of the junctional SR (11, 16, 17, 28, 30, 31).

---

1. *Abbreviations used in this paper*: Na_3VO_4, sodium vanadate; SR, sarcoplasmic reticulum; T, transverse.

Thermogenesis in the eye muscles of billfish may be associated with the ATP-dependent recycling of calcium at the SR. The heater cells have the cellular machinery of muscle cells for regulating calcium movements, a Ca^{2+} pump and Ca^{2+}-release channel, and it has been proposed that the muscle membrane components are involved in a heat-producing cycle centered upon SR and mitochondria rather than a force-generating cycle involving actin and myosin (1–3). In this paper we provide a thorough description at the ultrastructural level of this modified muscle cell, using a combination of four EM techniques (thin section, freeze-fracture, freeze-drying, and selective staining) to identify two distinct membrane systems. The disposition of the SR membranes and of the Ca^{2+}-ATPase molecules is examined. In addition, we now recognize that a portion of this extensive membrane system is equivalent to the T tubule system of skeletal muscle fibers. Ultrastructural examination of heater cells indicates that the triad structure, long recognized as the morphological basis for communication between the SR and T system, is structurally conserved in the heater cell, despite the lack of the myofilament lattice. This suggests that activation of thermogenesis in heater cells may occur through the same protein components involved in excitation–contraction coupling.

Materials and Methods

The eye muscle and heater tissues of the following species were examined in this study: the blue marlin, *Makaira nigricans*; the striped marlin, *Tetrapturus audax*; the sailfish, *Istiophorus platypterus*; and the spearfish, *Tetrapturus pfluegeri*. Most of the fish were captured on hook and line aboard commercial fishing boats along the Kona coast of Hawaii during the summer months. Blue marlins, sailfish, and spearfish, used for isolation of the SR vesicles were caught in the Gulf Stream off of North Carolina in the months of June and July. In all, 20 blue marlins ranging in body size from 32 kg to 350 kg, 4 sailfish all <30 kg, 2 striped marlins ~50 kg, and 1 10-kg spearfish were examined for this study. The age and sex of the individual fish varied but all were considered to be adults. The superior rectus eye muscle and the associated heater organ were removed within 10 min of capture. The heater tissue was trimmed free of the muscle and blood vessels and was either cut into pieces and immersed into cold fixative or injected with fixative via a syringe. Samples were also taken from fish brought into the docks within 1.5 h of capture that were judged to be in excellent condition for sampling (i.e., good color in the skin and firm muscles).

Thin Sectioning

For thin sections, the tissue was fixed with 3% glutaraldehyde, 0.1 M cacodylate, pH 7.2, with or without either 0.1 or 0.05% EM-grade tannic acid. Tissues were rinsed in 0.1 M cacodylate, postfixed in 1% OsO_4 for 1 h, en bloc stained in 2% aqueous uranyl acetate for 30 min, dehydrated in alcohol, and embedded in Spurr. To enhance staining of junctional feet, tissues were en bloc stained in 2–4% uranyl acetate at 65°C. Thin sections were cut on an MT6000 microtome, stained with uranyl acetate and lead, and photographed with an electron microscope (model 410; Philips Electronic Instruments, Inc., Mahwah, NJ).

Freeze-Fracture

Heater tissue samples used for freeze-fracture were fixed as above. The tissue was infiltrated with a sequential series of glycerol (10–30% for 15 min each), frozen in freon 22 cooled by liquid nitrogen, fractured at −110°C, and shadowed with platinum at an angle of 45°. Tissues were removed from the replicas by floating them in clorox, and the clean replicas were picked up either on collodion- and carbon-coated grids or bare grids, and photographed in an electron microscope (model 410; Philips Electronic Instruments, Inc.).

Freeze-Drying and Gel Electrophoresis of SR Vesicles

Isolated "crude" membrane fractions from blue marlin were prepared from homogenates of heater tissue at 4°C following procedures developed for rabbit skeletal muscle SR (25, 26). Heater tissue was cut away from the attached eye muscle and vascular tissues and trimmed with a scissors and razor blade on all sides to remove any remaining muscle tissue. SR preparations from skeletal muscle were prepared from the portion of the superior rectus eye muscle remaining attached to the heater organ. Membrane fractions were isolated from 4 to 25 g of heater tissue, minced, and then homogenized with a Potter-Elvehjem teflon glass homogenizer in 0.1 M NaCl, 20 mM Tris/Pipes (pH 7.3), 5 mM EGTA, and 1 mM of the protease inhibitor diisopropyl fluorophosphate. Muscle homogenates were prepared from 35 g of tissue and homogenized with a tissue grinder (Tekmar Co., Cincinnati, OH). Heater and muscle crude membrane fractions were prepared similarly by centrifuging the homogenates at 4,000 rpm for 20 min at 4°C in a GSA rotor in a centrifuge (model RC-2; Sorvall Instruments Div., Newton, CT). The pellet was discarded and the supernatant was filtered through several layers of cheese cloth. The supernatant was centrifuged at 15,000 rpm for 10 min in the rotor (model SS34; Sorvall Instruments Div.). The pellet, which was primarily composed of mitochondria, was discarded and the supernatant was centrifuged at 20,000 rpm for 30 min in the rotor (model SS34; Sorvall Instruments Div.). Pellets obtained were resuspended in a small volume of buffer containing 0.3 M sucrose, 0.1 M NaCl, 0.1 mM EGTA, 5 mM K-Pipes (pH 7.0), and 1 mM diisopropyl fluorophosphate. The resulting suspension was centrifuged at 30,000 rpm in a Ti 55.2 rotor for 30 min in an ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). The final pellet was taken up a small volume of buffer containing 0.3 M sucrose, 10 mM K-Pipes (pH 7.0). Vesicle suspensions were quickly frozen in liquid nitrogen and stored at −80°C before use.

SR vesicles were freeze-dried and rotary shadowed at 25° according to Franzini-Armstrong and Ferguson (13). Ordered arrays of the Ca^{2+}-ATPase were produced in the heater SR vesicles using sodium orthovanadate (Na_3VO_4) following procedures for skeletal muscle SR (9, 13). SR vesicles (1 or 2 mg protein/ml) were suspended at pH 7.0 in 10 mM phosphate, 5 mM Na_3VO_4, 5 mM $MgCl_2$, 5 mM EGTA, and 1 mM $MnCl_2$ at 2°C for 48 h. Vesicles were prepared for EM by washing with 100 mM ammonium acetate and negatively staining with 2% uranyl acetate.

Protein profiles of the SR vesicles from heater and muscle tissue were examined by SDS-PAGE according to Laemmli (19), using a 10% running gel and a 4.0% stacking gel. Protein samples were reduced for 1 min at 100°C in 10 mM Tris (pH 8.0), 4% SDS, 10% beta-mercaptoethanol, and 20% glycerol. Aliquots (20 to 30 µl) containing 1 µg of protein were loaded on the gel. Electrophoresis was for 4.5 h at 200 V and the gel was fixed and stained according to Merril et al. (27). Protein was estimated by the method of Lowry et al. (23).

Golgi Infiltration

Heater and eye muscle tissues were infiltrated with the Golgi stain (32) as modified by Franzini-Armstrong and Peachey (15). Large pieces of tissue were fixed by immersion in 3% glutaraldehyde and 0.1 M cacodylate for 4–6 h, washed in three changes of 3% potassium dichromate, postfixed in 3% potassium dichromate, and 1% osmium tetroxide (pH 7.4) for 7 d at 4°C, followed by washing in 0.75% silver nitrate for 2 d at 4°C. If necessary a shorter cycle was repeated to increase the level of infiltration. Tissues were stored in the silver nitrate for 3 mo before embedding. For embedding, peripheral portions of well-infiltrated areas were dehydrated in alcohol and embedded in Epon. Thick sections, 0.5–2 µm, were cut with a histo-knife on a microtome (model MT600; Diatome-US Co., Fort Washington, PA) and examined either in an electron microscope (model 410; Philips Electronic Instruments, Inc.) at 100 kV or in an intermediate voltage electron microscope (model 4000; JEOL USA, Peabody, MA) at 400 kV.

Ouabain Binding

[³H]Ouabain binding in heater SR membrane fractions was determined in a medium containing 150 mM NaCl, 12.5 mM $MgCl_2$, 1.25 mM EGTA, 50 mM Tris-HCl (pH 7.4), in the presence of 100 mM Tris-ATP and 0.4% Saponin. SR membrane fractions were incubated in the assay medium for 1 h at 30°C. The [³H]ouabain (14.4 Ci/mmol) concentration in the medium ranged from 5 to 160 nM. After incubation, the sample was passed through a filter (Millipore Corp., Bedford, MA) and washed three times with 5 ml ice-cold medium that contained no ouabain. The filters were
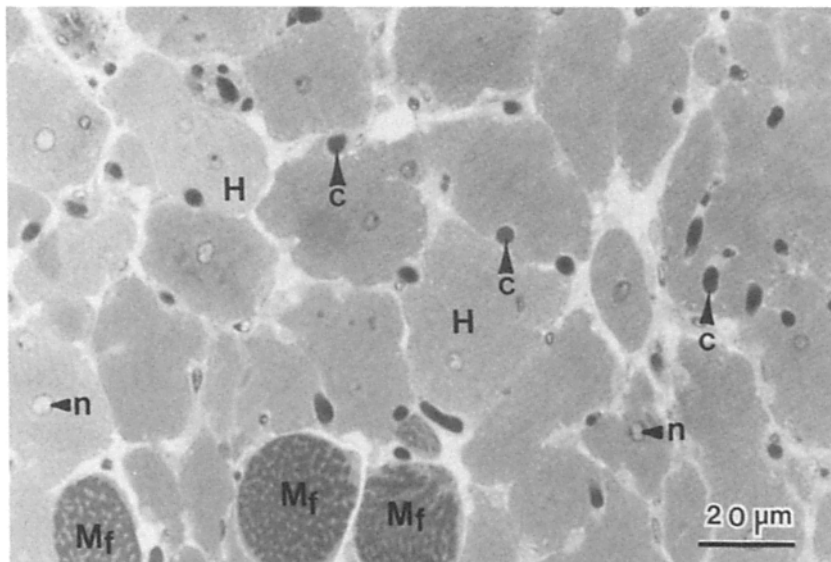
Figure 1. Light micrograph of a transverse section of the heater organ revealing both heater cells (*H*) and muscle fibers (*Mf*). Heater cells have a granular cytoplasm lacking myofibrils and have an irregular, occasionally circular shape and are similar in diameter to the mitochondria-rich muscle fibers shown here. Muscle fibers with a normal structure are often situated adjacent to heater cells in these modified eye muscles. Numerous capillaries are found around the heater cells (*c*). *n*, heater cell nuclei.

digested in a scintillation fluid and the [^{3}H]ouabain remaining with the filter was determined by scintillation counting.

Results

General

The most frequently examined fish in this study, the blue marlin, is one of the largest bony fish in the oceans weighing >900 kg. These fish have large eyes and the muscles that rotate the eye are robust (individual eye muscles weigh as much as 250 g) and have a deep red coloration. The superior rectus muscle has a normal insertion on the eye but as this muscle courses toward the brain the muscle tissue undergoes a distinct transition from normal muscle fibers into the heat-generating cell type (1–3). The heat-generating portion of the eye muscle is highly vascularized and there is a rete mirabile, which serves as a countercurrent heat exchanger, located ventrally to the heat-producing tissue.

Heater cells are easily distinguished in the light microscope from muscle fibers by their granular cytoplasm, the lack of characteristic striations associated with the myofibrils, and their round often centrally located nuclei (Fig. 1). The cells have an irregular, occasionally circular cross section and are 20–35 μm in diameter.

In thin sections of heater cells, profiles of mitochondria with densely packed cristae dominate the images (Fig. 2, *A* and *B*). An extraordinary labyrinth of smooth membranes occupies the entire cytoplasmic space in between the mitochondria. As described in detail below, the internal membranes consist of two distinct systems homologous to the SR and T tubules of muscle fibers.

Sarcoplasmic Reticulum

The SR of heater cells forms a continuous network throughout the cell and has several components: vesicles and tubules, large stacks of round flat cisternae, and dilated sacs (Fig. 2, *A* and *B*). The vesicles, tubules, and stacks are similar to the nonjunctional regions of the SR due to their dense disposition of the Ca^{2+}-ATPase in these membranes described below. The dilated sacs contain a visible content and have a structural similarity to terminal cisternae of skeletal muscle SR that contain the Ca^{2+}-binding protein calsequestrin (4, 26).

In freeze-fracture images, the cytoplasmic leaflet of all portions of the heater cell SR is densely and uniformly packed with 8–12-nm particles and the luminal leaflet has a smooth surface with few particles (Fig. 3, *A–D*). There is an even distribution of the intramembranous particles throughout the heater SR including the surface of the pancake-like membranous stacks (Fig. 3, *C* and *D*). Previous studies on skeletal muscle SR indicate the principal component of the nonjunctional regions of the SR is the Ca^{2+}-ATPase (4, 24, 26), and most, if not all, intramembranous particles in the cytoplasmic leaflet of the nonjunctional regions of the SR in skeletal muscle represent various aggregations of the pump molecules. Biochemical assays on crude membrane fractions of the heater tissue have established that the heater cells have a high Ca^{2+}-ATPase activity (2, 3). SDS gels as shown below indicate that the Ca^{2+} pump protein is the most prominent protein in the heater membrane fractions. Thus, the numerous intramembranous particles on the cytoplasmic surface of the heater SR represent the crowded disposition of the pump protein in the membrane. The fracturing properties and density of the intramembranous particles in heater cell SR are consistent with the distribution of the Ca^{2+}-ATPase in the nonjunctional regions of the SR of fast-twitch skeletal muscle.

When heater SR vesicles are exposed to a solution containing 5 mM Na$_3$VO$_4$ crystalline arrays of Ca^{2+}-ATPase form over a large portion of the surface of the SR vesicles (Fig. 4, *A–C*). The formation of extensive crystalline arrays was apparent 6 h after exposure to vanadate and continued for 48 h. Polymerization of the Ca^{2+}-ATPase in heater SR vesicles often resulted in elongation of the vesicles into the tubular shapes that are characteristic of Ca^{2+}-ATPase crystalline formations in SR of skeletal muscle (9, 10, 24).

1102
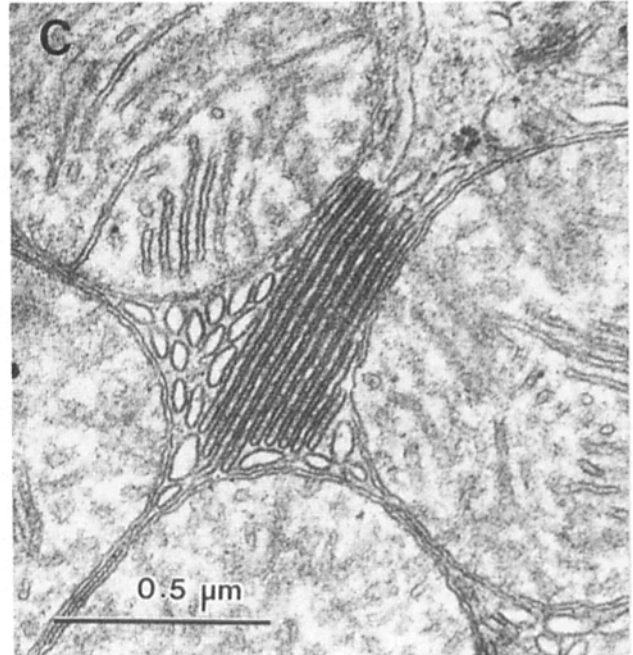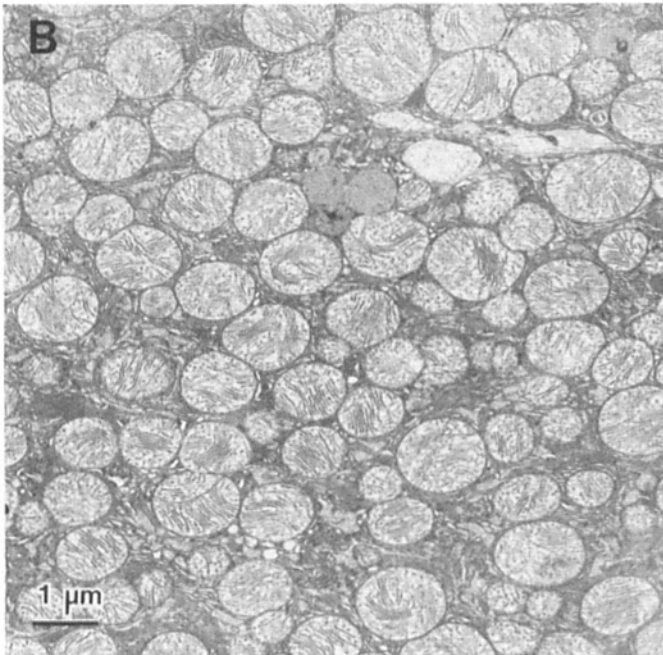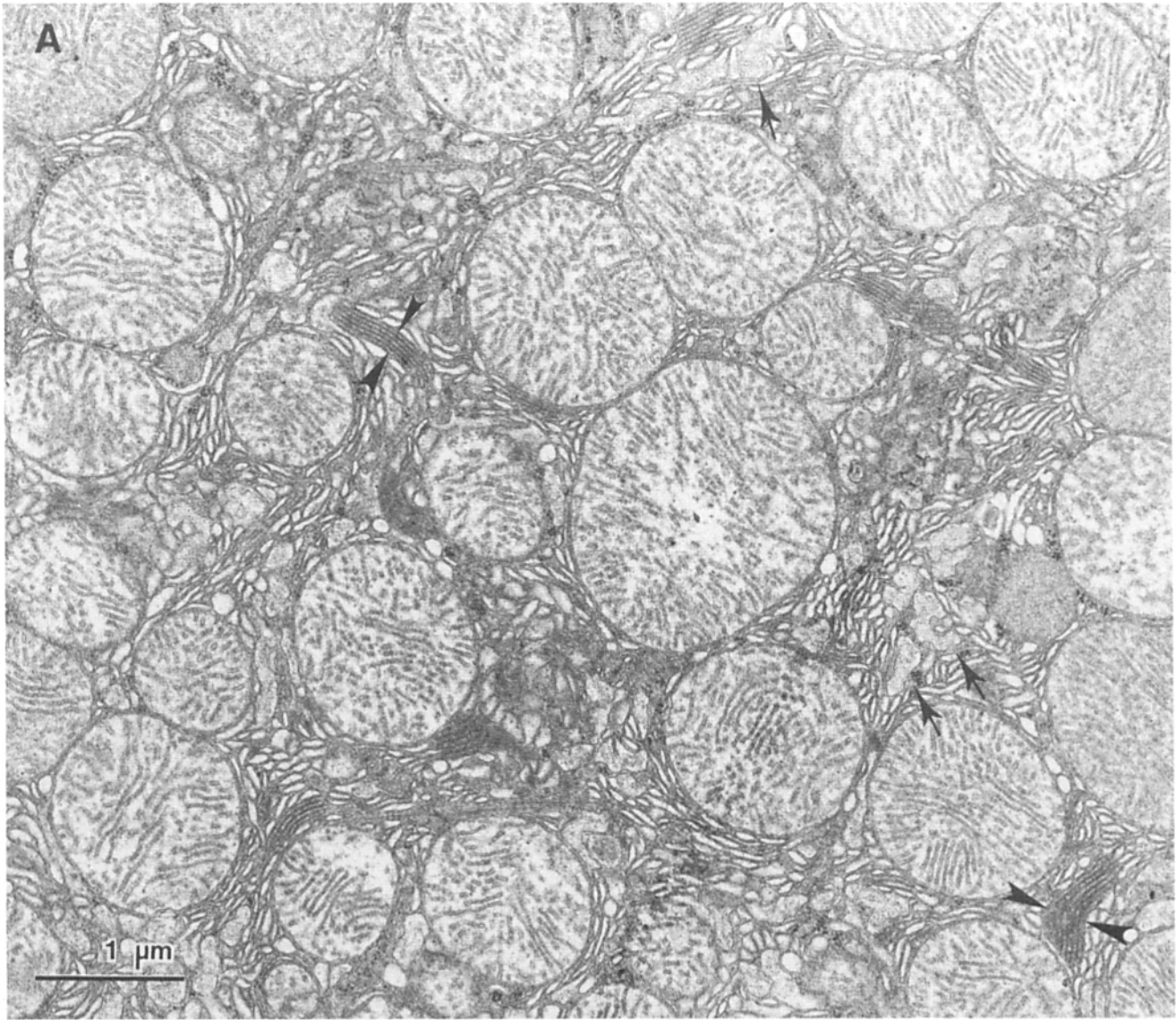
Freeze-drying and rotary shadowing of isolated SR vesicles also allow visualization of the hydrophilic tails of individual Ca^{2+}-ATPase molecules as they project into the cytoplasm. This technique can be used to further determine the surface density of the pump in the SR membranes. In Fig. 4 D, rotary shadowed SR vesicles from the blue marlin are covered by a finely grained shadow. Individual ATPase molecules are represented by the random dots which cover the surface of the vesicles (see also references 10, 13, 24). In these images, areas of membrane not occupied by the pump (lipid patches) have a more finely grained shadow and a lower profile which can best be viewed in stereo images. Such lipid patches were rare and occupy a small percentage of the heater tissue nonjunctional SR surface area. The surface topography of the shadowed heater SR vesicles is comparable to that of similarly prepared SR fractions from white muscle of rabbit hind legs (see reference 11). In the latter, the surface density of ATPase is calculated to be 31,000–34,000 molecules per μm^2 of membrane.

Further confirmation of the dense disposition of the Ca^{2+} pump in the heater SR vesicles examined by the above EM techniques is obtained from SDS–polyacrylamide gels (Fig. 5). The most prominent protein band in the heater SR vesicle preparation is the polypeptide with an apparent molecular mass of $\sim$100 kD corresponding to the Ca^{2+}-ATPase in skeletal muscle (in Fig. 5, compare heater SR in lanes 4 and 5 with muscle SR in lanes 1 and 2). The presence of a prominent protein band corresponding to the Ca^{2+}-ATPase corresponds to high ATP-dependent Ca^{2+} uptake activity measured in the heater SR vesicles (2).

SR Stacks

Extensive stacks of SR tubules are found throughout the cytoplasm of the heater cells (see Figs. 2, A and C; and 3, A, C, and D). The SR stacks are composed of 3–10 layers of closely apposed lamellae with a narrow lumen, separated by a narrow cytoplasmic cleft. Individual lamellae profiles from the stacks range in length from 0.2 to 1.8 μm. The entire stack is either flat or gently curved, and the membranes of the individual lamellae are parallel to each other at a constant distance through the stack. The most peripheral portions of each lamellae is slightly dilated and occasional continuities with the rest of the SR are visible. The average center-to-center spacing between the lamellae in the center of the stacks (from 11 stacks in 10 micrographs) is 31 nm. The separation between the luminal surfaces of the membranes of two adjacent lamellae (a distance which includes the thickness of two membranes and the width of the cytoplasmic layer between them) is $\sim$20 nm.

As described above, the surface of the cytoplasmic leaflet of the stacks is packed with intramembranous particles, thus the pancake-like arrangement of the SR combines a dense disposition of the ATPase with a tight packing of membranes. Considering that the total length of the intramem-

branous and cytoplasmic portions of the calcium ATPase molecules is 10–12 nm, the lamellae in the stacks are packed as closely as possible, with the tips of the ATPase either touching or slightly overlapping. This results in small regions of high ATPase density.

T Tubules

T tubules were first identified in heater cells using the Golgi infiltration which fills the T tubules with a precipitate that is visible in the light and electron microscope. Due to its complicated three-dimensional disposition, the network is best studied in thicker (0.5–2.0 μm) slices at higher accelerating voltages (400 kV). In these images, the filled T tubules are visible while the rest of the cell components (primarily mitochondria and SR) appear ghostlike in the background.

The T tubules form a complex network of interconnecting elements, which fills the entire cell and is excluded only from the nuclear region (Fig. 6, A and B). In contrast to the planar network of skeletal muscle, there is no particular orientation of the individual components of the T network with respect to internal structures within the cell (see reference 15 for review of T network in muscle). This is likely due to the lack of aligned contractile elements that provide a scaffolding for the T network and the loss of specific anchoring points on the myofibrillar lattice for the membrane network. The most common feature of the T network in heater cells is the circular arrangement of the tubules surrounding the mitochondria, and an anastomosing and branching pattern as tubules connect and form multifaceted rings. The individual components are curved and straight segments of connecting tubules, but at nodal points there are enlarged cisternal elements with variable shapes and some with fenestrations. The cisternae may either be elongated or have circular shapes. The latter are more often spherical but occasionally appear as flattened discs (Fig. 7 A).

Freeze-fracture was used to demonstrate homology between T tubules of heater and muscle cells (Fig. 7 B). The network nature of the T tubules is well recognizable in freeze-fracture and the shapes of the T tubules are comparable to those seen with the Golgi stain (compare Fig. 7, A and B). The T membranes can be readily distinguished from the SR membranes due to the paucity of intramembranous particles in comparison to the SR, on both the cytoplasmic and luminal leaflets of the membrane. The cytoplasmic leaflets of T membranes have small particles of fairly uniform size and low frequency that are most apparent on the expanded portions of the T tubules.

The presence of the T system in heater cells was assessed biochemically by measuring the binding of [³H]ouabain, an enzymatic marker for surface and T tubule membranes (21). The crude membrane preparations from heater homogenates were used for this assay. Initial preparation of the membrane fractions in these fish did not involve sucrose gradient centrifugation, hence it was assumed that some surface mem-

Figure 2. (A) Thin section view of a heater cell. Mitochondria and smooth membranes occupy most of the volume of the modified muscle cell. The SR has numerous stacks of parallel SR membranes (*arrowheads*) and dilated sacs that contain an electron-dense content (*arrows*). (B) Lower magnification of a heater cell showing the numerous mitochondria that pack the cytoplasm of this modified muscle cell. (C) Higher magnification of stacks of SR. Stacks occur frequently throughout the cytoplasm and occasionally have continuities with cisternal elements. Individual lamellae of a stack are parallel to each other at a constant distance throughout the stack.
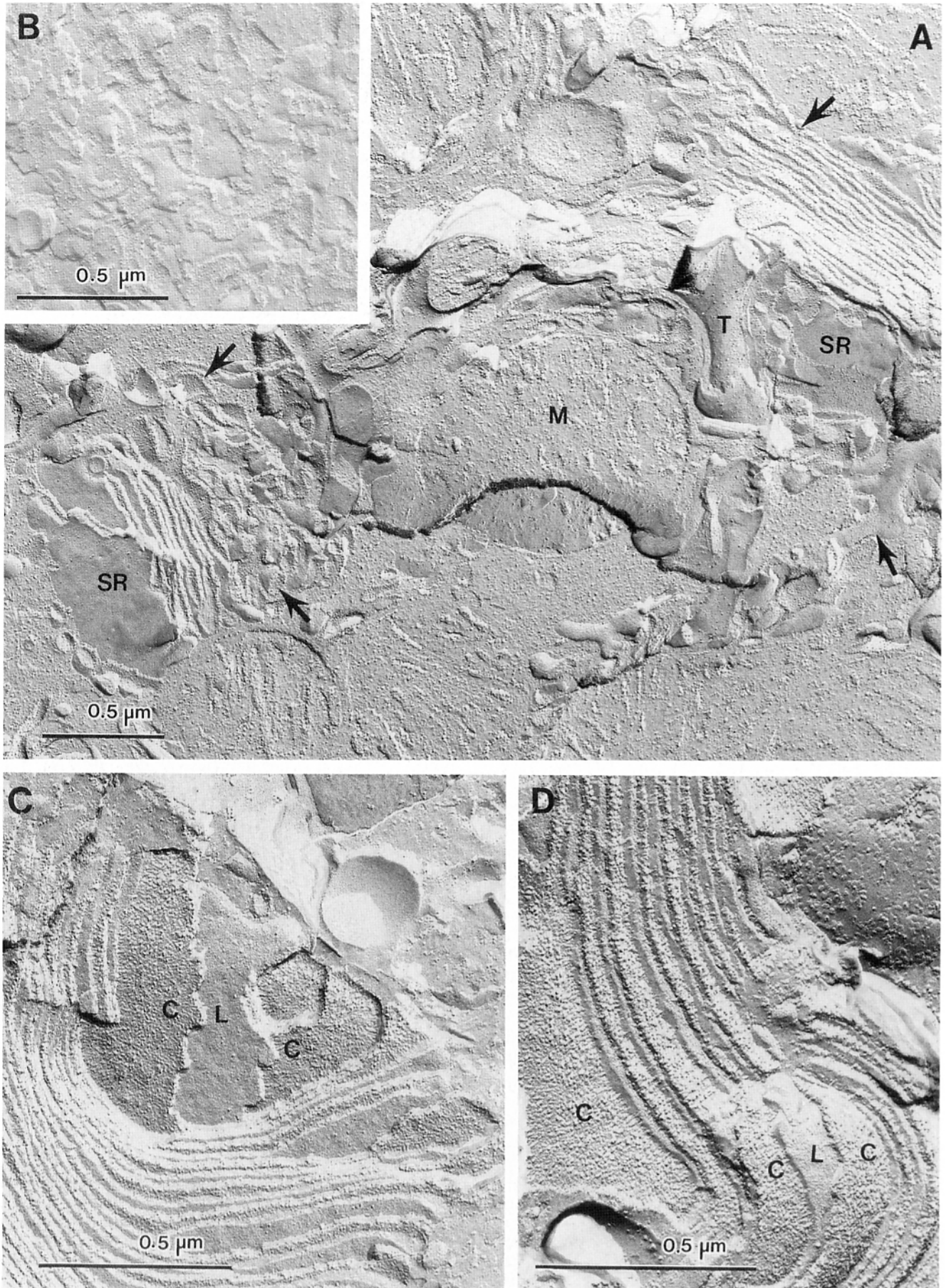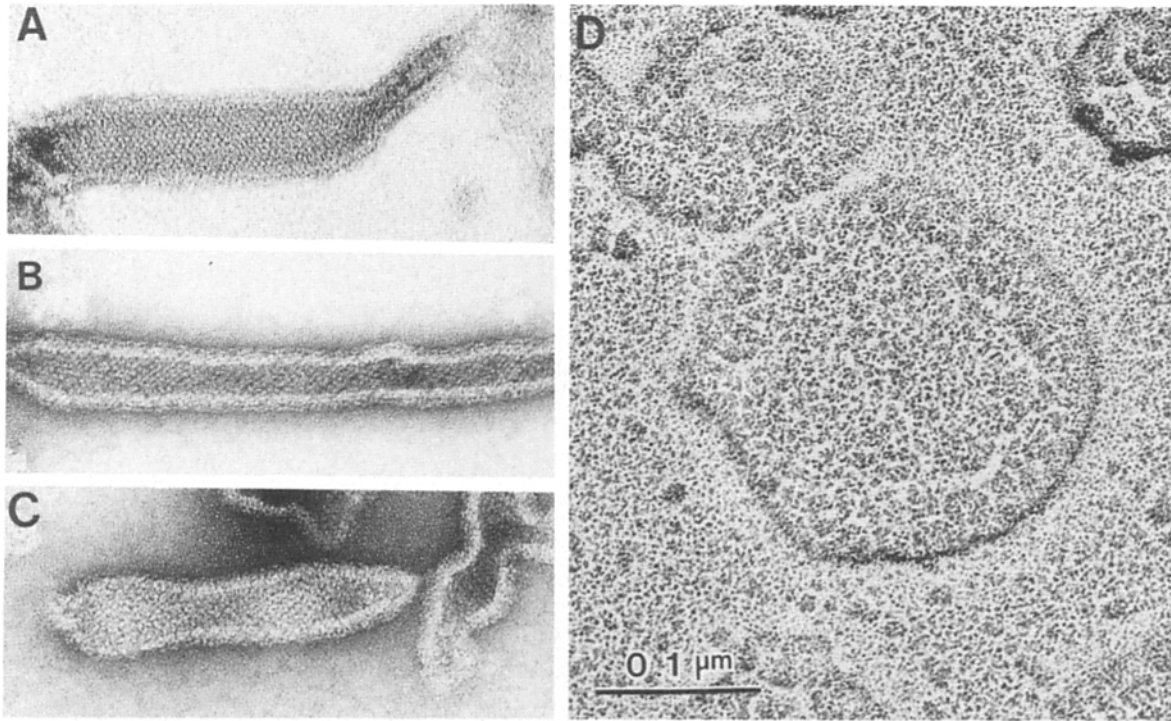
Figure 4. (*A–C*) Crystalline arrays of the Ca^{2+}-ATPase are observed by negative staining in heater tissue SR membranes treated with Na$_3$VO$_4$. SR vesicles were incubated in a solution containing 5 mM Na$_3$VO$_4$ for 2 d and then negatively stained with 2% uranyl acetate. (*D*) Rotary shadowed and freeze-dried SR vesicles isolated from heater cells. The entire surface of the vesicles are covered by a finely grained shadow which has previously been shown to represent the hydrophilic tails of the ATPase molecules (13).

brane and T tubule contaminants were present in this fraction. The affinity and number of binding sites of [^{3}H]ouabain were determined in the presence of saponin to make the vesicles more permeable (ouabain binding site is located on the luminal side of the isolated vesicle [21]). Scatchard plot analysis of [^{3}H]ouabain binding (summarized in Table I) in three species of fish with heater tissue indicated there was specific binding to a single class of sites and gives a value ranging from 31 to 57 pmol/mg protein for the maximum number of [^{3}H]ouabain binding sites (B_{max}) and a mean K_d of 29.7 nM for the three species examined. The number of binding sites (B_{max}) in heater tissue is threefold greater than the number measured in fish muscle controls but are comparable to the number of binding sites obtained in membrane fractions (prepared under different conditions) from rabbit fast-twitch skeletal muscle (B_{max} is 45 pmol/mg protein and the K_d is 35 nM).

Triads

The junction between the T and SR could be identified in heater tissue on the basis of four structural features that char-

acterize the triads of skeletal muscles. These are the presence of two enlarged cisternae of the SR containing an osmiophilic content (terminal cisternae), a constant gap between the junctional SR and T tubule membrane, evenly disposed junctional feet, and a slight scalloping of the junctional SR membrane.

The triads occur frequently throughout the heater cells and there is not any identifiable pattern to their distribution (see also Fig. 8 *A*, *arrowheads*). The terminal cisternae have a visible content, the junctional SR and T tubules are separated by a gap of ∼14 nm, containing a periodic disposition of junctional feet, and there is a scalloped edge of the junctional SR membrane (Fig. 8, *B* and *C*). The feet have a 30 ± 6 nm spacing (from 20 micrographs, 33 triads), similar to that reported for skeletal muscle (12), and often form long rows that occasionally are sharply curved (Fig. 8 *B*).

More precise quantitation of triad parameters (i.e., frequency of junctions, precise extent of the junctional surfaces, and corresponding number of feet per junction) are difficult to assess due to the random orientation of the triad in heater tissue and the difficulty of finding the junction in the extremely crowded cytoplasm. In skeletal muscle, this type of

Figure 3. (*A*) Freeze-fracture of a heater cell unidirectionally shadowed at 45°. Mitochondria (*M*) with their circular profile and cristae are readily distinguished from the SR and T tubules (*T*) that lie in between them. The SR (*arrows* and labeled SR on stacks) appears in flattened cisternal stacks and in tubules. The stacks appear pancake-like and the cytoplasmic leaflet of the SR is covered with numerous intramembranous particles which represent the Ca^{2+}-ATPase molecule while the convex luminal leaflet appears smooth surfaced. (*B*) Higher magnification of the cytoplasmic leaflets of the SR tubules revealing the pump particles crowding the surface of the SR. (*C* and *D*) Freeze-fracture of SR stacks shows alternating cytoplasmic (*C*) and luminal (*L*) leaflets. The cytoplasmic leaflet of the SR stack is crowded with pump particles. Stacking of the SR provides a large surface area–to-volume ratio that allows for a high pump density.
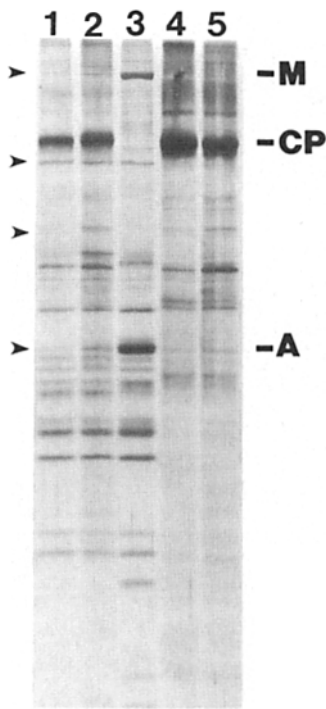
Figure 5. SDS-PAGE silver-stained gel comparing the protein profiles of the crude SR vesicles from marlin eye muscle (lanes *1* and *2*) with the protein profile of the crude SR vesicles from heater tissue (lanes *4* and *5*). Lane *3* is a skeletal muscle homogenate with prominent myosin (*M*) and actin (*A*) bands for reference. The most prominent protein in all fractions is the Ca^{2+} pump protein (*CP*) at ~100 kD. Heater tissue profiles have low amounts of the 200-kD band corresponding to myosin, the 44-kD band corresponding to actin, or the 97-kD band corresponding to glycogen phosphorylase. The location of molecular mass standards are marked by arrowheads and their values are, from top to bottom, 200, 97.4, 68, and 44 kD.

membrane area, most of which belongs to the free or calcium pumping SR.

In muscle, junctional SR and T membranes can also be distinguished using freeze-fracture techniques. On the luminal junctional SR leaflet, periodic bumps mark the location of the feet (11, 18). In heater tissue replicas the luminal leaflet of the junctional SR was occasionally apparent as a narrow, smooth-surfaced, 55-nm strip of SR membrane with two rows of raised bumps at a 30-nm center-to-center spacing between individual structures. The periodicity of the structures is indicative of the feet protein and recent work with toadfish muscles indicates that the bump in the SR membrane does represent a portion of the foot protein (presumably intramembranous) as first suggested by Kelly and Kuda (18). Junctional T membranes are distinguished in freeze-fracture by groups of particles that occasionally form diamond-shaped clusters on either the cytoplasmic or luminal leaflets (14). Such distinct clusters of T tubule particles have not been identified as of yet in the heater tissue replicas.

Transitional Muscle Cells

In billfish, the heat-producing portion of the muscle is continuous with a portion of the eye muscle that inserts on the back of the eye and contains normal skeletal muscle cells. Although there is little developmental work on this tissue, there is abundant structural evidence suggesting that heater cells arise from differentiated skeletal muscle cells. Many cells in the eye muscle have a transitional appearance between muscle and heater with numerous mitochondria, stacks of SR, and small regions of disorganized myofibrils (Fig. 9, *A* and *B*). Some cells clearly have a heater cell phenotype (numerous mitochondria and SR), but also contain small bundles of contractile filaments randomly scattered throughout the cytoplasm.

information can be obtained by examining the shape of T tubules in Golgi-infiltrated cells. In muscle, the portion of tubules forming junctions with the SR (junctional T tubules) are clearly distinguishable from the nonjunctional or free tubules. The former have flat cross sections, the latter a smaller, circular cross section. The extent of the junctional area can be determined by the size and shape of the flat T tubule surfaces which face the junctional SR membrane. A similar correlation has proven more difficult in heater tissue, because we cannot rely on landmarks from the myofibrils for the unequivocal identification of the junctional regions of the network which in muscle usually are positioned at either the A–I junction or Z line. From the number of feet seen in thin-section images of heater cell triads, and from the narrowness of the T tubule lumen, we surmise that the junctional areas should be fairly extensive and that the T tubule should have a flat shape. The expanded cisternal elements of the T tubule network seen with the Golgi stain, which have variable round shapes and are occasionally elongated, fulfill these criteria. With this in mind, the Golgi view of the T network can be used to assess the approximate frequency of triads in heater cells (see Fig. 7 *A*). Only a minor portion of the network participates in junction formation and the junctions are dispersed at random in the fiber. The junctional surfaces undoubtedly comprise a small fraction of the total nonmitochondrial

Discussion

In this study we have used standard ultrastructural techniques to identify the types of membranes found in heater cells and to determine their relationship with skeletal muscle SR and T tubules. This study confirms previous biochemical and early structural studies which indicated heater cells were modified muscle cells (1–3). Such structural and functional plasticity in fish muscle is not unprecedented as electric organs also develop from skeletal muscle (8, 12). Indeed, from the present study it is clear that heater cells are a unique type of muscle cell, which lacks contractile proteins yet retains the SR and T system. Contraction and relaxation cannot occur in these modified muscle cells. This emphasis on the membrane and mitochondrial volumes of the muscle cell at the expense of the contractile filaments is associated with the functional transition of these cells into furnaces. Heat pro-

Figure 6. (*A*) Intermediate voltage electron microscope image of a thick section (1.5 μm) through a heater cell of a blue marlin infiltrated with the Golgi stain to reveal the disposition of the T tubular network. The T system is represented by the black-staining tubules which are only excluded from the nuclear region (*N*) in the center of the cell. The mitochondria appear ghostlike in the background and the circular disposition of the elements of the T system occurs as the T courses around the mitochondria. The network has numerous expanded cisternae that may be associated with junctional areas. (*B*) Stereomicrograph of heater cell T system. The complicated three-dimensional structure of the T system in heater cells due primarily to the loss of the myofibrillar lattice is evident in this micrograph.
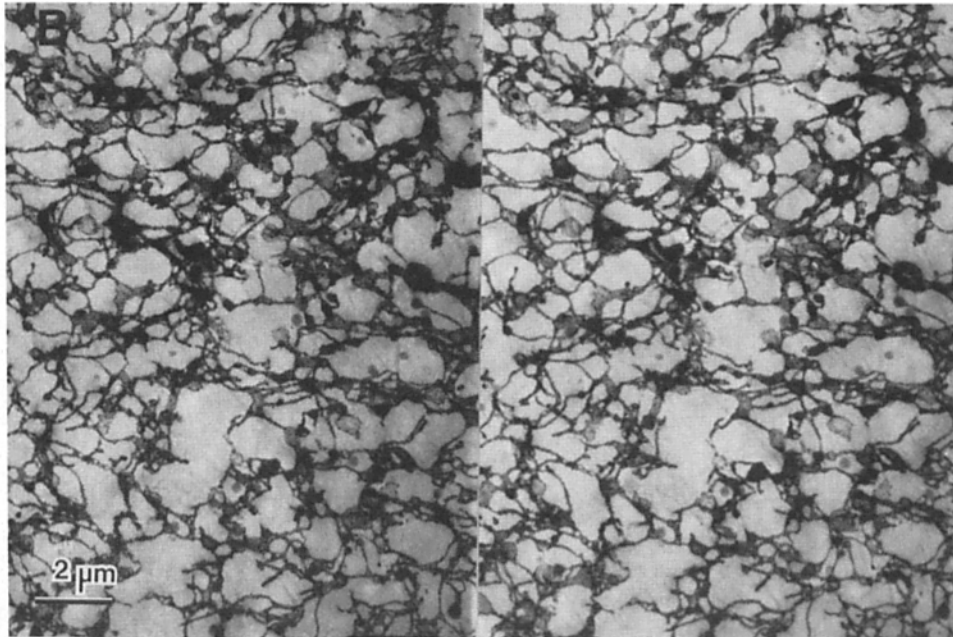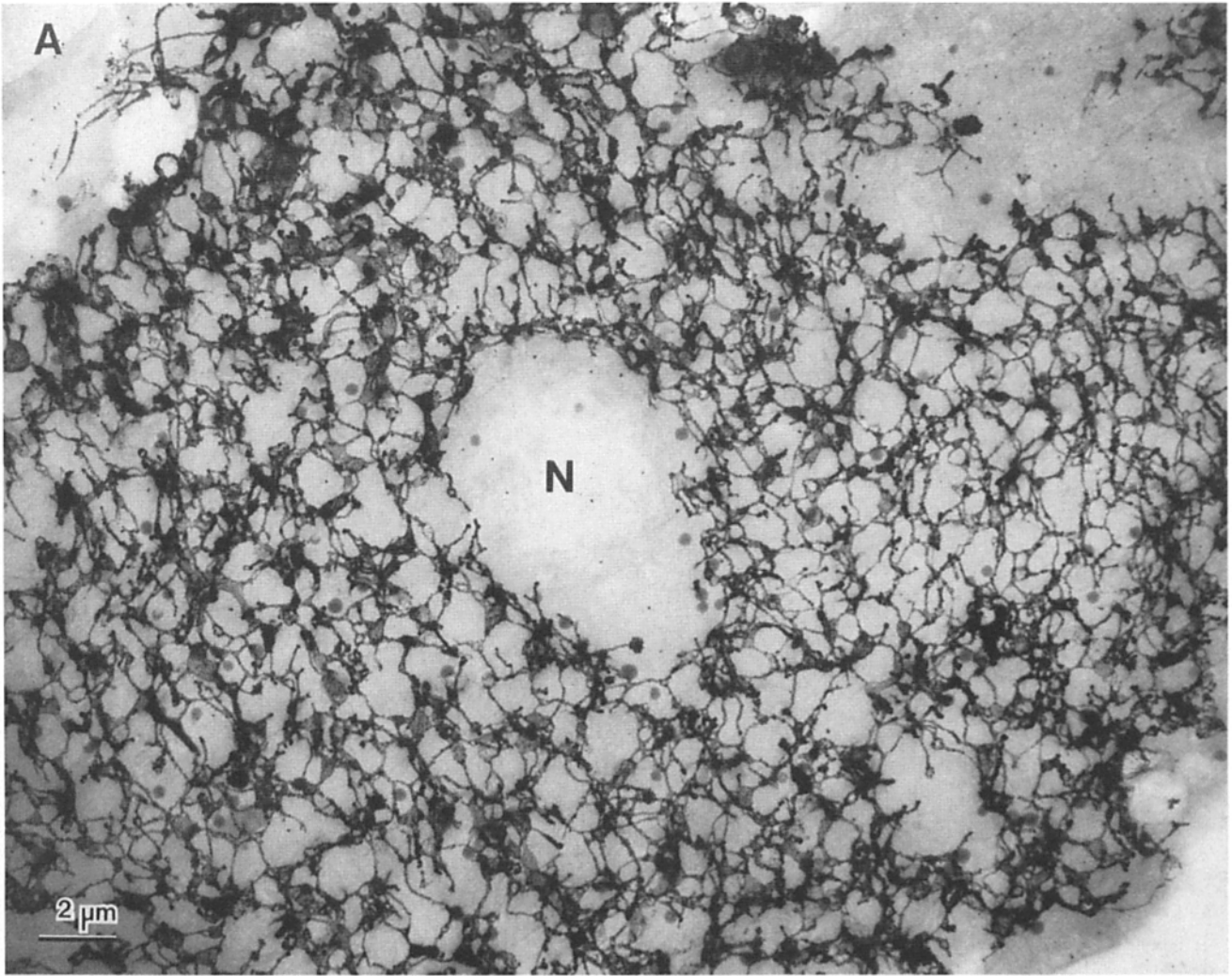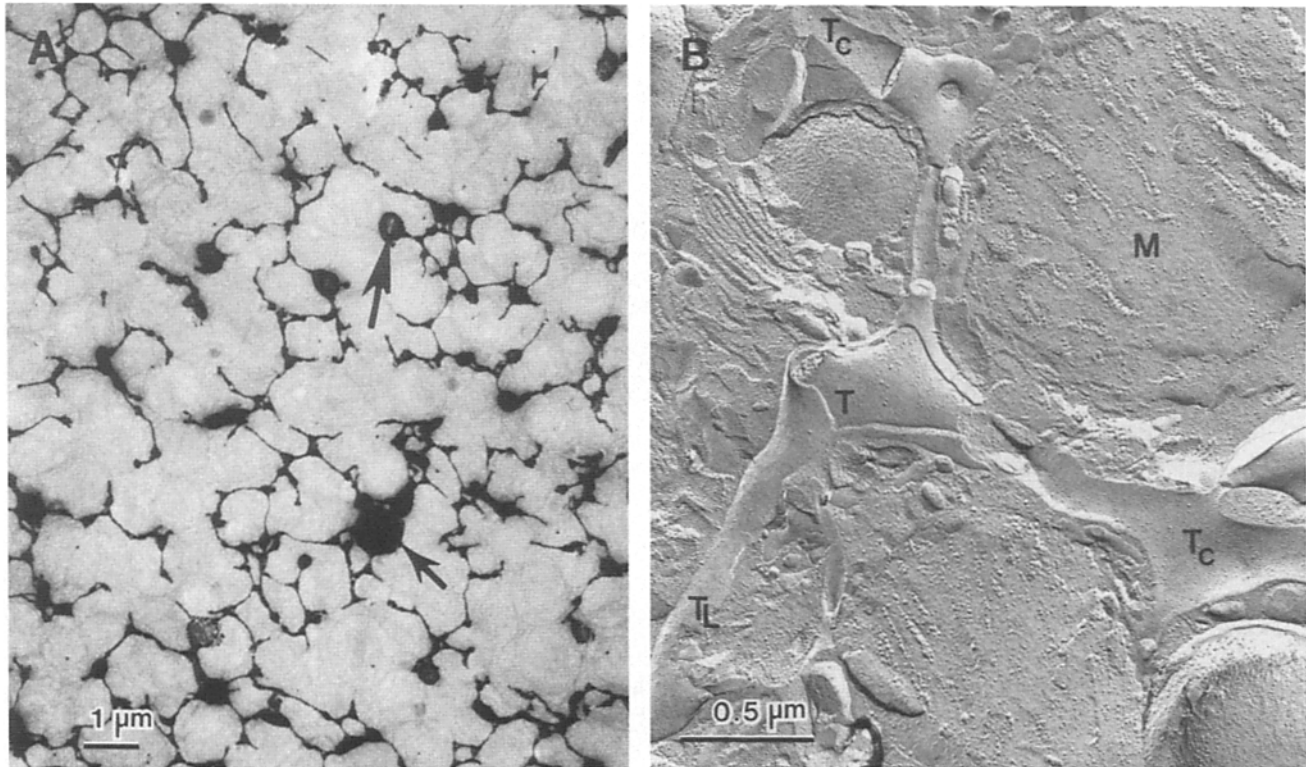
Figure 7. (A) Intermediate voltage electron microscope image of Golgi infiltrated heater cell revealing possible junctional regions of the T network. The expanded cisternae (*arrows*) are similar in their disc-shaped appearance to the junctional regions of tonic fibers of toadfish and frog. (B) High magnification of a T tubule (T) in a heater cell as seen in freeze-fracture. The tubule has a cylindrical shape as it courses between mitochondria (M) but often expands into wider regions as in the center of this micrograph. The expanded regions often have a disc shape and are probably involved with forming junctions with the SR. The cytoplasmic surface of the T can have randomly scattered particles and is easily distinguished from the cytoplasmic surface of the SR. The luminal leaflet is smooth surfaced. T_c, cytoplasmic leaflet of T tubule; T_L, luminal leaflet of T tubule.

duction is most likely associated with ATP-dependent cycling of Ca^{2+} ions at the SR which requires a large surface area of SR membrane to house Ca^{2+} pumps, and numerous mitochondria for oxidative phosphorylation. Thermogenesis in heater cells would begin with Ca^{2+} release from the SR and in this paper we provide strong evidence indicating that the components of the normal pathway for Ca^{2+} release in SR, the feet protein, are also present in heater cells.

The SR of skeletal muscle has three distinctive structural features, with a precise functional correlation. (a) Most of the SR membrane (the so-called nonjunctional or longitudinal SR) has a high content of Ca^{2+}-ATPase and a dense population of intramembranous particles on the cytoplasmic leaflet. This portion of the SR is primarily involved with Ca^{2+} uptake. (b) A small portion of the SR membrane (the

Table I. [³H]Ouabain Binding in Heater Tissue

Heater tissue*	Binding sites‡	K_d
	pmol/mg protein	*nM*
Marlin	57	27
Spearfish	56	31
Sailfish	31	31

* Measurements are from crude membrane vesicles: for the marlin $n = 4$, spearfish $n = 1$, and sailfish $n = 2$ individual preparations.
‡ Measured in the presence of 0.04% saponin and 10 mM Tris-ATP.

junctional SR) contains the feet protein, which has recently been identified as the actual Ca^{2+}-release channel of SR (14, 16, 20). The feet have a distinctive periodic distribution in thin sections and the intramembranous components of the feet protein make small quatrefoil protrusions in freeze-fracture. (c) The terminal cisternae, the portion of the SR immediately adjacent to the T tubules, are calcium storage sites that have a visible content that has been identified as the calcium binding protein, calsequestrin (4, 24, 26).
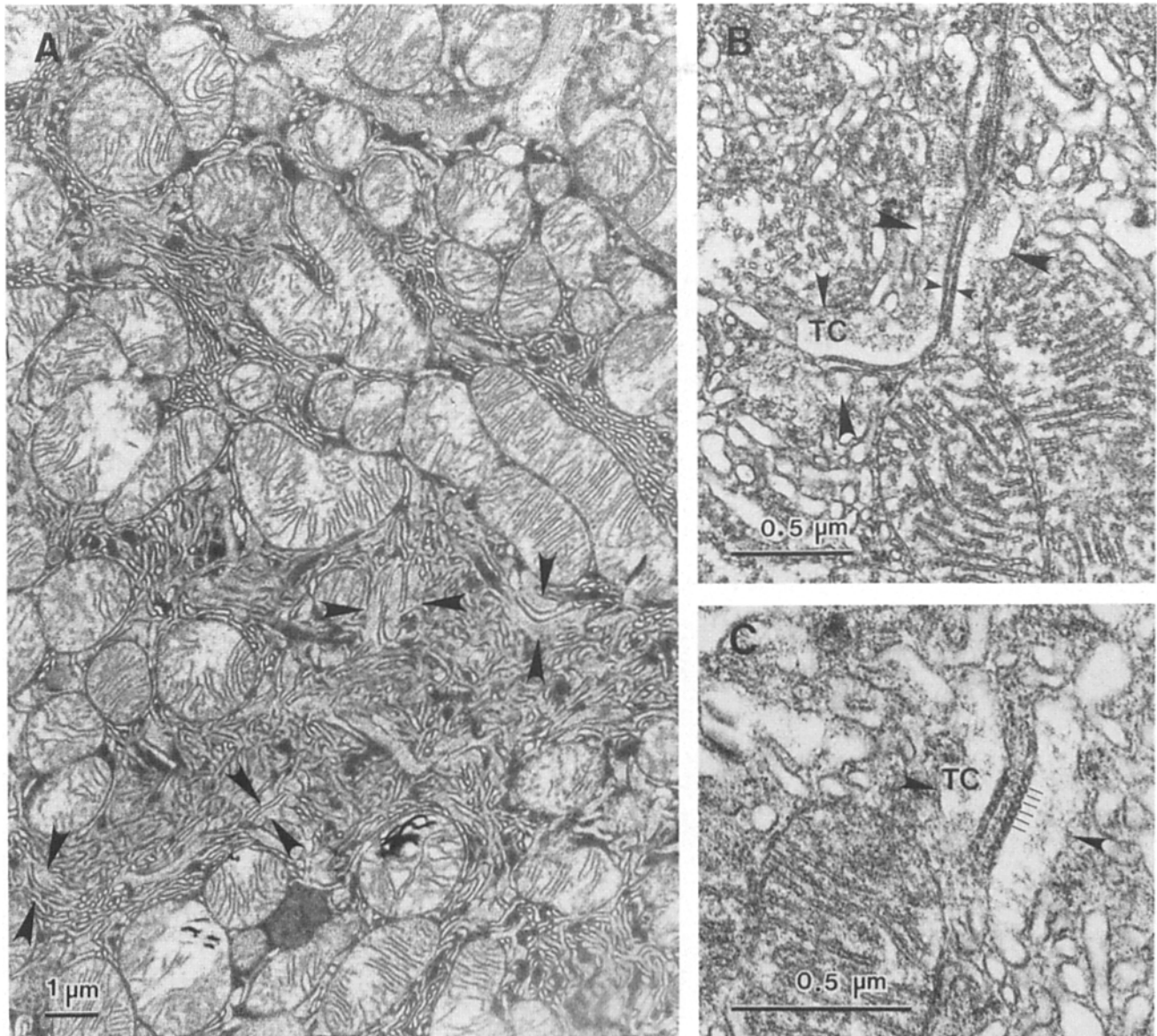
In heater cells, we have identified all of the above SR features and thus we consider the SR in heater cells to be structurally and functionally homologous with skeletal muscle SR. Freeze-fracture, vanadate crystallization, rotary shadowing, and SDS gels all indicate that the SR of heater cells has a high Ca^{2+}-ATPase content which corroborates earlier biochemical studies (2, 3). Most of the SR in heater cells is primarily composed of the Ca^{2+}-ATPase laden, pumping regions. One of the most conspicuous features of the heater cell SR are the numerous flat pancake-like cisternae that can be found throughout the cytoplasm in various degrees of stacking. Stacking of the SR in skeletal muscles has occasionally been reported and similarly arrayed tubular aggregates of SR have been reported in ischemic muscle (29). All of the species of fish examined in this study have this stacking arrangement of SR membranes. We have demonstrated that each individual stack is packed with Ca^{2+}-ATPase molecules. This unusual arrangement of the SR membranes ap-

Figure 8. (*A*) Tannic acid–stained thin section of a heater cell revealing triads between the arrowheads. Triads in heater cells as in skeletal muscle have two terminal cisternae flanking a T tubule. (*B*) Higher magnification of a heater cell triad situated in between several mitochondria. The terminal cisternae (*TC*) of the triad has a visible content that most likely is calsequestrin. This is a long curved triad with two rows of feet marked between the inner arrowheads. The periodicity of the feet can be seen along the rows. (*C*) Additional view of a triad with the 30-nm periodicity of the feet (*bars*) and the scalloped edge of the junctional SR clearly visible. Such features are standard characteristics of skeletal muscle triads.

pears to provide a maximum surface-to-volume ratio that allows for a high pump density and is an important parameter in a cell designed for heat production. Continuities often occur between the stacks and SR profiles that contain a calsequestrin-like content. The presence of such a Ca^{2+} binding protein contiguous with the lumen of the stack regions would be important for promoting Ca^{2+}-ATPase activity by lowering the free Ca^{2+} concentration in the narrow lumen of the stacks.

Why Do Heater Cells Have T Tubules?

The present study has clearly demonstrated that heater cells also have an elaborate T network that provides all regions of the heater cell with the cable system required to propagate the membrane depolarization signal from the plasmalemma. [³H]Ouabain binding is well recognized as a method for determining the presence of T tubular and sarcolemma membranes. The specific activity in the heater fractions is high and confirms the presence of the T tubular system. The visualization of the three-dimensional structure of the T tubule network with thick sections of Golgi-infiltrated heater cells indicated that the T system has a complex geometry when compared to the planar network of skeletal muscle. In normal skeletal muscle, the muscle filament lattice occupies most of the cytoplasmic space and provides a structural framework around which the T tubules must pass. The absence of the muscle filament lattice and the subsequent loss
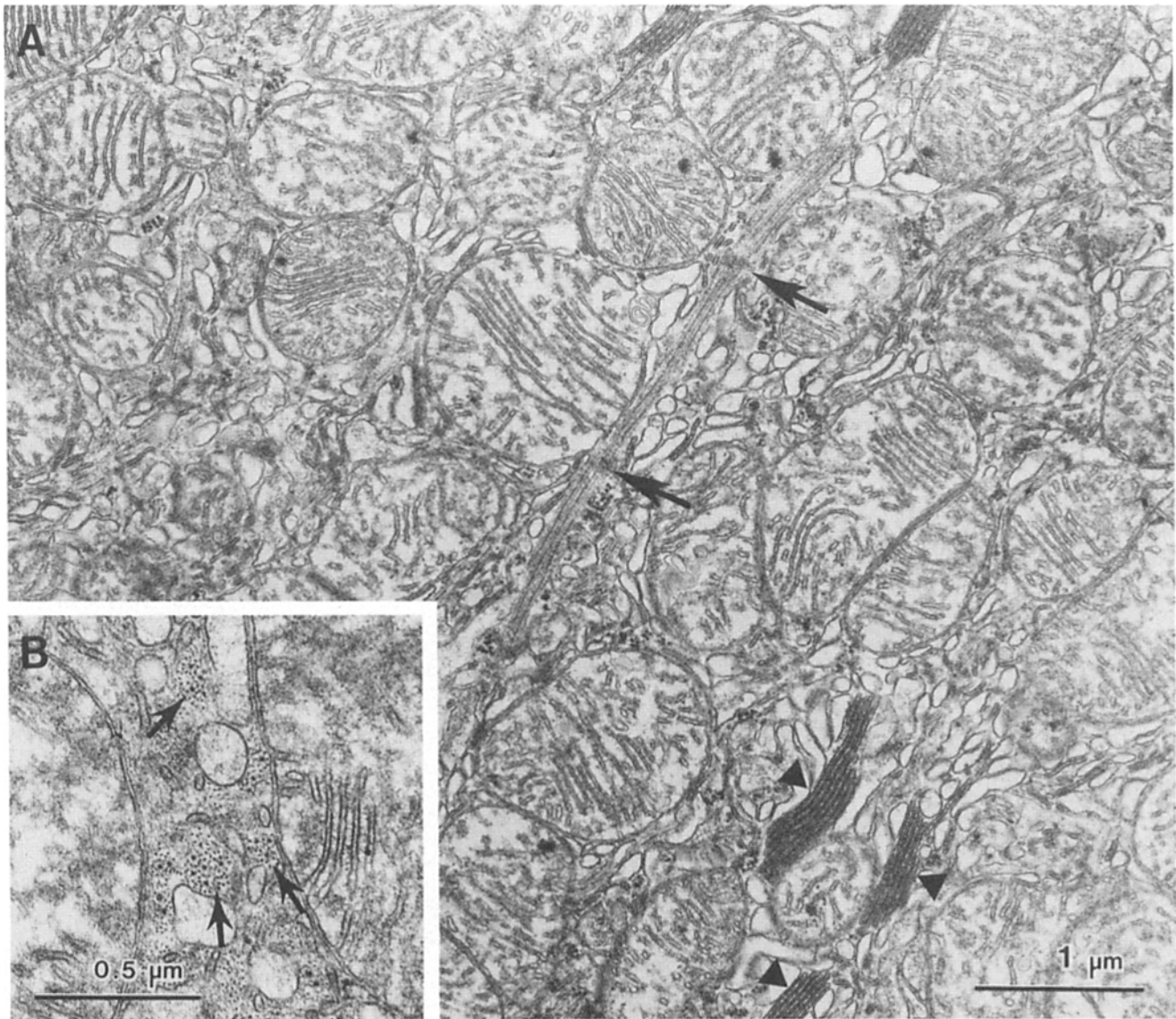
Figure 9. (*A*) A "transitional" cell with numerous mitochondria occupying >50% of the cell volume and with isolated myofibrils (*arrows*) in between. The arrows indicate Z bands providing markers for the myofibrils. Stacking of the SR is also apparent in this cell type (*arrowheads*). (*B*) Inset shows a similar transitional heater cell where isolated myofibrils with distinctive thick and thin filaments can be seen in cross section (*arrows*).

of anchoring sites for the T network most likely contribute to the disorderly appearance of the network.

Given that heat production is not as temporally dependent as muscle contraction and thus does not require a fast activation, why is there such a well-developed T system in the heater cells? Traditionally, T tubules are thought to be necessary for rapid transmission of the surface electrical signal to the fiber interior and the resultant, almost synchronous, release of calcium to the entire fiber cross section. Without the T system, the activator, calcium, would in most instances not have sufficient time to diffuse from the surface to the center of the fiber. Activation of the myofibrils should be as synchronous as possible in fibers with rapid rates of tension development. Rapidly contracting fibers may do without T tubules only when their diameter is very small (e.g., the fast scallop adductor muscle). The need for T tubules and triads

in heater cells may arise from a requirement other than speed of activation. The SR and mitochondria within the heater cells present a sizable high-affinity calcium sink capable of reducing the calcium concentration at short distances from its source (triad), in a manner even more dramatic than that illustrated by some classic calcium diffusion experiments in muscle (7). Hence the necessity of release sites spaced at relatively frequent intervals, in order to provide accessibility of calcium to the SR throughout the cell. This would then insure effective use of all SR pumps for heat production.

Triads in heater cells have the major constituents for the calcium-release mechanism in SR, the junctional feet, and we can thus assume that Ca^{2+} release from SR occurs at these junctions. So far we were unable to recognize the signature of the junctional T membranes: the prominent T tubule particles whose location correspond to that of feet subunits

(11). Such particles represent integral T tubular membrane proteins and possibly are dihydropyridine receptors (22), the presumed voltage sensor molecules involved in excitation–concentration coupling (28, 30, 31). The absence of these particles in heater cells is not alarming given that structural preservation of this information requires excellent primary fixation and this has not been an easy problem to overcome with the marlin due to the difficulty of perfusing the heater organ in such large oceanic fish at sea. Further studies are required to clarify this point.

Conservation of the Triad

Despite a striking rearrangement of the membrane systems and absence of the myofilament lattice, the triad structure is maintained in heater cells. The preservation of the structural components and shape of the triad in the heater cells emphasizes that a precise spatial relationship between the SR, feet protein, and T tubular junctional membranes and proteins is required for the performance of their function at this critical junction. The regular arrangement of the feet and the size of the junctional gap are constant throughout the animal kingdom in diverse types of muscle tissue. This suggests that proper signal transmission at junctional regions in muscles is dependent on the specific disposition of the feet protein which in turn lock the T (or surface membranes) and SR into a fixed position.

The molecular mechanism of excitation–contraction coupling or signal transduction at the junctional gap in muscle is presently being intensely investigated. Two proteins (a ryanodine and dihydropyridine receptor), which are thought to have a major role in this process have been isolated on respective sides of the SR and T junctional membranes. The SR Ca^{2+}-release channel has been purified using [^{3}H]ryanodine as a probe and identified with the junctional feet (11, 17, 20). The isolated channel has a tetrafoil or clover-leaf structure similar to that of the junctional feet (11) and is thought to be composed of four polypeptides of M_r 400,000–450,000. Using a protocol similar to Lai et al. (20), a ryanodine binding protein of M_r 400,000 that also has the tetragonal substructure of the foot protein has been isolated from heater tissue suggesting that the heater cell has a functional SR Ca^{2+}-release channel. Preliminary ryanodine binding in heater tissue SR vesicles indicates that these cells may have fewer feet protein and hence Ca^{2+}-release channels than mammalian skeletal muscle. Not much is known yet concerning the abundance of dihydropyridine receptors in the heater tissue but knowledge of the level of dihydropyridine binding will be important for establishing if the heater cells use these voltage-sensing molecules in excitation–thermogenic coupling.

Transitional Cells

Nothing is known about the development of heater cells. The location of heater tissue in continuity with the fiber bundles of the extraocular muscle, the presence of intermediate cells with small myofibrils, and the similarity of the membrane components of heater with muscle cells, indicate a muscle origin. However, it remains to be determined whether the cell derives directly from myoblasts with a specified density or from a myoblast that goes through an obligatory phase of expression of muscle-specific proteins during differentiation before expressing the heater cell phenotype.

Much of the structural evidence gathered on heater tissue in adult and juvenile fishes suggests that there may be a particular muscle fiber type that gives rise to the heater cell phenotype at some stage in the life cycle of the billfish. A relevant question is what fiber type gives rise to a heater cell. On an ultrastructural basis we can recognize two distinct fiber types in the intact portion of the superior rectus muscle. One fiber appears to be a fast fiber type with few mitochondria and large amounts of SR and T tubules. The other fiber is a very oxidative type with numerous mitochondria, a moderate amount of SR and T tubules, a diffuse M line, and a thick Z line. Triads in both fibers appear at the A–I region. The mitochondria-rich fiber appears to be more closely associated with the heater cells.

The heater cell has requirements that set the stage for selection from an oxidative fiber type. Foremost is the fact that these cells have an extraordinarily high capacity for oxidative phosphorylation. Thus, the metabolic requirements of the heater cell lean toward an oxidative fiber type. Whether the precursor fiber needs to be of a slow fiber type or fast fiber is not clear. The fact that swordfish, in which the heater organ is most developed, spend up to 12 h on a diurnal cycle in deep, cold (4°C) waters (6) suggests that the properties best suited for the heater cell arise from a fatigue-resistant fiber type.

References

1. Block, B. A. 1986. Structure of brain and eye heater tissue in marlins, sailfish and spearfish. J. Morphol. 190:169–189.
2. Block, B. A. 1986. Brain and eye warming in billfishes (istiophoridae): the modification of muscle into a thermogenic tissue. Doctoral Dissertation. Duke University, North Carolina. 150 pp.
3. Block, B. A. 1987. The billfish brain and eye heater: a new look at non-shivering heat production. News Physiol. Sci. 2:208–213.
4. Campbell, K. P., C. Franzini-Armstrong, and A. E. Shamoo. 1980. Further characterization of light and heavy sarcoplasmic reticulum vesicles. Identification of the "sarcoplasmic reticulum feet" associated with the heavy sarcoplasmic reticulum. Biochim. Biophys. Acta. 602:97–116.
5. Carey, F. G. 1982. A brain heater in the swordfish. Science (Wash. DC). 216:1327–1329.
6. Carey, F. G., and B. H. Robison. 1981. Daily patterns in the activities of swordfish, Xiphias gladius, observed by acoustic telemetry. Fish. Bull. (Dublin). 79:277–292.
7. Constantin, L. L., and R. J. Podolsky. 1964. Regulation by calcium of the contraction and relaxation of muscle fibers. Fed. Proc. 23:933–939.
8. Dahlgren, U. 1910. Origin of the electric tissue in fishes. Am. Nat. 44:193–202.
9. Dux, L., and A. Martonosi. Two-dimensional arrays of proteins in sarcoplasmic reticulum and purified Ca^{2+}-ATPase vesicles treated with vanadate. J. Biol. Chem. 258:2599–2603.
10. Ferguson, D. G., C. Franzini-Armstrong, L. Castellani, P. M. Hardwicke, and L. J. Kenny. 1985. Ordered arrays of Ca^{2+}-ATPase on the cytoplasmic surface of isolated sarcoplasmic reticulum. Biophys. J. 48:597–605.
11. Ferguson, D. G., H. A. Schwartz, and C. Franzini-Armstrong. 1984. Subunit structure of junctional feet in triads of skeletal muscle. A freeze drying and rotary shadow study. J. Cell. Biol. 99:1735–1742.

12. Fox, G. Q., and G. P. Richardson. 1978. The developmental morphology of Torpedo marmorata electric organ-myogenic phase. J. Comp. Neurol. 179:677–697.

13. Franzini-Armstrong, C., and D. G. Ferguson. 1985. Density and disposition of Ca^{2+}-ATPase in sarcoplasmic reticulum membrane as determined by shadowing techniques. Biophys. J. 48:607–615.

14. Franzini-Armstrong, C., and G. Nunzi. 1983. Junctional feet and particles in the triads of a fast-twitch muscle fibre. J. Muscle Res. Cell Motil. 4:233–252.

15. Franzini-Armstrong, C., and L. D. Peachey. 1982. A modified Golgi black reaction method for light and electron microscopy. J. Histochem. Cytochem. 30:99–105.

16. Imagawa, T., J. S. Smith, R. Coronado, and K. P. Campbell. 1987. Purified ryanodine receptor from skeletal muscle is the Ca^{2+} permeable pore of the calcium release channel. J. Biol. Chem. 262:16636–16643.

17. Inui, M., A. Saito, and S. Fleischer. 1987. Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from skeletal muscle. J. Biol. Chem. 262:1740–1747.

18. Kelly, D. E., and A. M. Kuda. 1979. Subunits of the triadic junction in fast skeletal muscle as revealed in freeze-fracture. J. Ultrastruct. Res. 68:220–233.

19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.

20. Lai, F. A., H. P. Erickson, E. Rousseau, Q. Liu, and G. Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. Nature (Lond.) 331:315–319.

21. Lau, Y. H., A. H. Caswell, and J. P. Brunschwig. 1977. Isolation of transverse tubules by fractionation of triad junctions of skeletal muscle. J. Biol. Chem. 252:5565–5574.

22. Leung, A. T., T. Imagawa, and K. P. Campbell. 1987. Structural characterization of the 1,4 dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from rabbit skeletal muscle. J. Biol. Chem. 262:7943–7946.

23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265–275.

24. Martonosi, A., K. A. Taylor, S. Varga, and H. Ping Ting-Beall. 1987. The molecular structure of sarcoplasmic reticulum. In Membranous Structures. Academic Press, London. 256–376.

25. Meissner, G. 1984. Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum. J. Biol. Chem. 259:2365–2374.

26. Meissner, G., G. Conner, and S. Fleischer. 1973. Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+} pump and Ca^{2+} binding proteins. Biochim. Biophys. Acta. 298:246–269.

27. Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science (Wash. DC). 211:1437–1438.

28. Rios, E., and G. Brum. 1987. Involvement of dihydropyridine receptors in excitation–contraction coupling in skeletal muscle. Nature (Lond.). 325:717–720.

29. Schiaffino, M. D., M. D. Severin, M. Cantini, and S. Sartore. 1977. Tubular aggregates induced by anoxia in isolated rat skeletal muscle. Lab. Invest. 37:223–228.

30. Schneider, M. F., and W. K. Chandler. 1973. Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. Nature (Lond.). 242:244–246.

31. Schwartz, L. M., E. W. McCleskey, and W. Almers. 1985. Dihydropyridine receptor in muscle are voltage-dependent but most are not functional calcium channels. Nature (Lond.). 314:747–751.

32. Veratti, E. 1902. Ricerche sulla fine struttura della fibra muscolare striata. Mem. 1st Lombardo Classe Sci. Mat. Nat. 19:87–133. Translated 1961. J. Biophys. Biochem. Cytol. 10(Suppl.):3–59.