

Evolution of skeletal type e–c coupling: a novel means of controlling calcium delivery

Valentina Di Biase^{1,2} and Clara Franzini-Armstrong¹

¹Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104

²Department of Physiology and Medical Physics, Innsbruck Medical University, A-6020 Innsbruck, Austria

The functional separation between skeletal and cardiac muscles, which occurs at the threshold between vertebrates and invertebrates, involves the evolution of separate contractile and control proteins for the two types of striated muscles, as well as separate mechanisms of contractile activation. The functional link between electrical excitation of the surface membrane and activation of the contractile material (known as excitation–contraction [e–c] coupling) requires the interaction between a voltage sensor in the surface membrane, the dihydropyridine receptor (DHPR), and a calcium release channel in the

sarcoplasmic reticulum, the ryanodine receptor (RyR). Skeletal and cardiac muscles have different isoforms of the two proteins and present two structurally and functionally distinct modes of interaction.

We use structural clues to trace the evolution of the dichotomy from a single, generic type of e–c coupling to a diversified system involving a novel mechanism for skeletal muscle activation. Our results show that a significant structural transition marks the protochordate to the Craniate evolutionary step, with the appearance of skeletal muscle-specific RyR and DHPR isoforms.

Introduction

A major step in the evolution of vertebrates seems to have been the duplication of genes that allowed for the differentiation of muscle-specific myosin II into separate cardiac and skeletal isoforms, and thus the appearance of a separate cardiac system (McGuigan et al., 2004). Skeletal and cardiac muscles of higher vertebrates differ not only in myosin composition but also in the mechanism of muscle activation known as excitation–contraction (e–c) coupling and in the proteins that are involved in this mechanism. The evolution from a single mechanism of e–c coupling for all muscle fibers to one that differentiates between cardiac and skeletal muscles appears to have occurred at the transition between chordates and vertebrates, in parallel to the myosin II dichotomy (Inoue et al., 1994). The transition involved the acquisition by skeletal muscle of a novel mechanism for controlling the initiation of contraction, which was driven by the appearance of channels responsible for releasing calcium from the sarcoplasmic reticulum (SR) that were different from the channels of invertebrates and cardiac muscle (Chugun et al., 2003). The new mechanism offers the advantage of a more stringent control and a tighter energy metabolism, and it is based on a totally new protein–protein interaction, as detailed in the following paragraphs.

The signal for the initiation of muscle contraction is the rapid rise of intracellular calcium in response to an electrical signal at the plasmalemma, via a series of events called e–c coupling. The key structures involved are the calcium release units (CRUs; Flucher and Franzini-Armstrong, 1996), which are large macromolecular complexes that allow a specific, localized interaction between the plasmalemma and the SR. Within the CRU, the dihydropyridine receptor (DHPR, or L-type Ca²⁺ channel) sends a signal to the calcium release channels of the SR, the ryanodine receptors (RyRs, or feet), thus initiating calcium release. In skeletal muscle, DHPRs are linked to RyRs and control their activity via a direct molecular interaction in a unique, direct coordination of the activity of two separate membrane systems within the same cell (Schneider and Chandler, 1973; Nakai et al., 1996). The structural link is made evident by the observation that DHPRs, as detected by the freeze-fracture technique, are organized in tetrads, i.e., groups of four intramembrane particles (each representing a single DHPR) located at the corners of small squares (Block et al., 1988; Takekura et al., 1994). The disposition of DHPR into tetrads is dictated by a specific attachment of four DHPRs at the corners of the four equal subunits of an RyR (Protasi et al., 1997).

On the other hand, in muscles from invertebrates and in cardiac and smooth muscles of vertebrates, DHPRs, although equally located in CRUs, are not arranged into tetrads, and there is no evidence for a molecular link between them and

Correspondence to Valentina Di Biase: Valentina.Di-Biase@uibk.ac.at

Abbreviations used in this paper: CRU, calcium release unit; DHPR, dihydropyridine receptor; e–c, excitation–contraction; jSR, junctional SR; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; T, transverse.

RyRs (Sun et al., 1995; Protasi et al., 1996; Takekura and Franzini-Armstrong, 2002; Tijssens et al., 2003; Moore et al., 2004). In these muscles, DHPR–RyR interaction is indirect, probably involving calcium as a short-range transmitter (Zacharova and Zachar, 1967; Scheuer and Gilly, 1986; Nabauer et al., 1989; Gyorke and Palade, 1993). These structural and functional differences, between skeletal muscle on one side and cardiac/invertebrate muscle on the other, are uniquely dependent on the isoform composition of CRUs. The assembly of DHPRs into RyR-linked tetrads requires either the two skeletal-specific isoforms ($\alpha 1$ sDHPR and RyR1) or the presence of essential domains derived from these two isoforms (Protasi et al., 2000, 2002; Takekura et al., 2004). Thus, the presence of tetrads gives an unequivocal structural signature for the concerted expression of skeletal muscle-specific isoforms of both DHPRs and RyRs.

This study focuses on the transition from random to tetradic positioning of DHPRs, indicative of the absence or presence of a direct DHPR–RyR link, and its correlation to e–c coupling modes in the course of evolution. The hypothesis to be tested is that one of the evolutionary steps leading to vertebrates was the development of a novel means of activating muscle that is based on a specific coupling of DHPRs to RyRs offering tight control. This could be considered one of the “steps which are necessary to achieve a developmental fate” as proposed in the review by Langille and Hall (1989). Four animal models (Amphioxus, hagfish, lamprey, and garfish) were selected based on their position in the phylogenetic tree (Fig. 1) and, importantly, on the knowledge that their e–c coupling properties have been well established (see Discussion). Amphioxus, a protochordate, is appropriately placed in a transitional position between invertebrates and vertebrates. The hagfish, one of the most primitive craniates, is considered to be either a transitional species between low chordates and vertebrates or the most primitive living vertebrate (Forey and Janvier, 1993; Jorgensen et al., 1998; Mallat et al., 2001). The lamprey is an early vertebrate, and the garfish is an ancient bony fish. Data about more advanced fish are available in previous studies (Franzini-Armstrong and Nunzi, 1983; Block et al., 1988). Our results show that a significant structural transition marks the evolutionary step from protochordate to craniate.

Results

Correlation between thin-section and freeze-fracture images

In all muscles examined, freeze fractures of the plasmalemma show special domains that are distinguished by clusters of large intramembranous particles visible in the fractured cytoplasmic leaflet. We identify the clusters as belonging to peripheral couplings, and thus as representing clusters of DHPRs associated with RyRs, based on correlations with the thin-section images, as follows.

Amphioxus

Amphioxus myotomes are composed of extremely thin (1–2 μm) sheetlike cells, called lamellae, containing a single myofibril

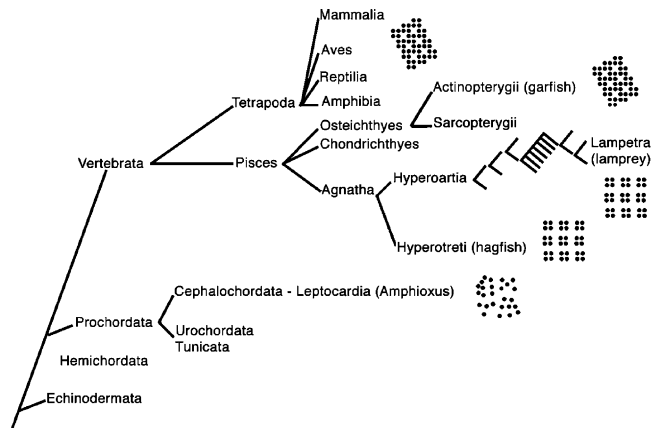


Figure 1. **Phylogenetic tree showing details of the transition between low chordates and Craniata.** At the right of the phylogenetic tree, the dispositions of the DHPR particles found in this study are indicated for the animal models used.

and no transverse (T) tubules (Peachey, 1961). The ultrastructure of the lamellae has been extensively described (see references below), but some images are shown here to be used in direct comparison with those of the more advanced chordates. The junctional SR (jSR) is located at the periphery between the myofibril and the plasmalemma (Hagiwara et al., 1971; Henkart et al., 1976; Flood, 1977; Melzer, 1982a,b). The jSR vesicles are associated with the RyRs (Fig. 2 B, between arrows; Henkart et al., 1976; for other muscles see Franzini-Armstrong, 1970; Block et al., 1988), have an electron-dense content, presumably calsequestrin (Fig. 2 B), and form peripheral couplings by associating with the plasmalemma, thus constituting the CRUs of Amphioxus lamellae. The jSR vesicles usually have a large lumen and push junctional domains of plasmalemma outward, giving them a domelike shape. The peripheral couplings are clustered in proximity of the Z lines (Fig. 2 A, arrows) at each sarcomere, extending from the immediate proximity of the Z line up to the edges of the A band.

The fractured plasmalemma of Amphioxus shows parallel rows of dome-shaped protrusions located in circumferential belts centered over the Z lines (Fig. 2 C). The rows of protrusions are two to three (Fig. 2 A, arrows), but additional protrusions may also be present (Fig. 2 C, asterisks). The size, positioning, and domed shape of these membrane domains closely correspond to those of the small domains of plasmalemma that face the jSR vesicles at peripheral couplings, as seen in thin sections (Fig. 2, compare A and C; Henkart et al., 1976). The raised membrane domains are differentiated from the rest of the plasmalemma by their content of large intramembranous particles and the exclusion of smaller particles (Fig. 2, D and inset). No openings of caveolae are visible in Amphioxus muscle, which corresponds to the absence of caveolae in thin sections.

Hagfish

Unlike Amphioxus, all of the muscles from lower vertebrates that we examined have larger muscle fibers and contain T tubules. The jSR in these muscles forms both peripheral couplings

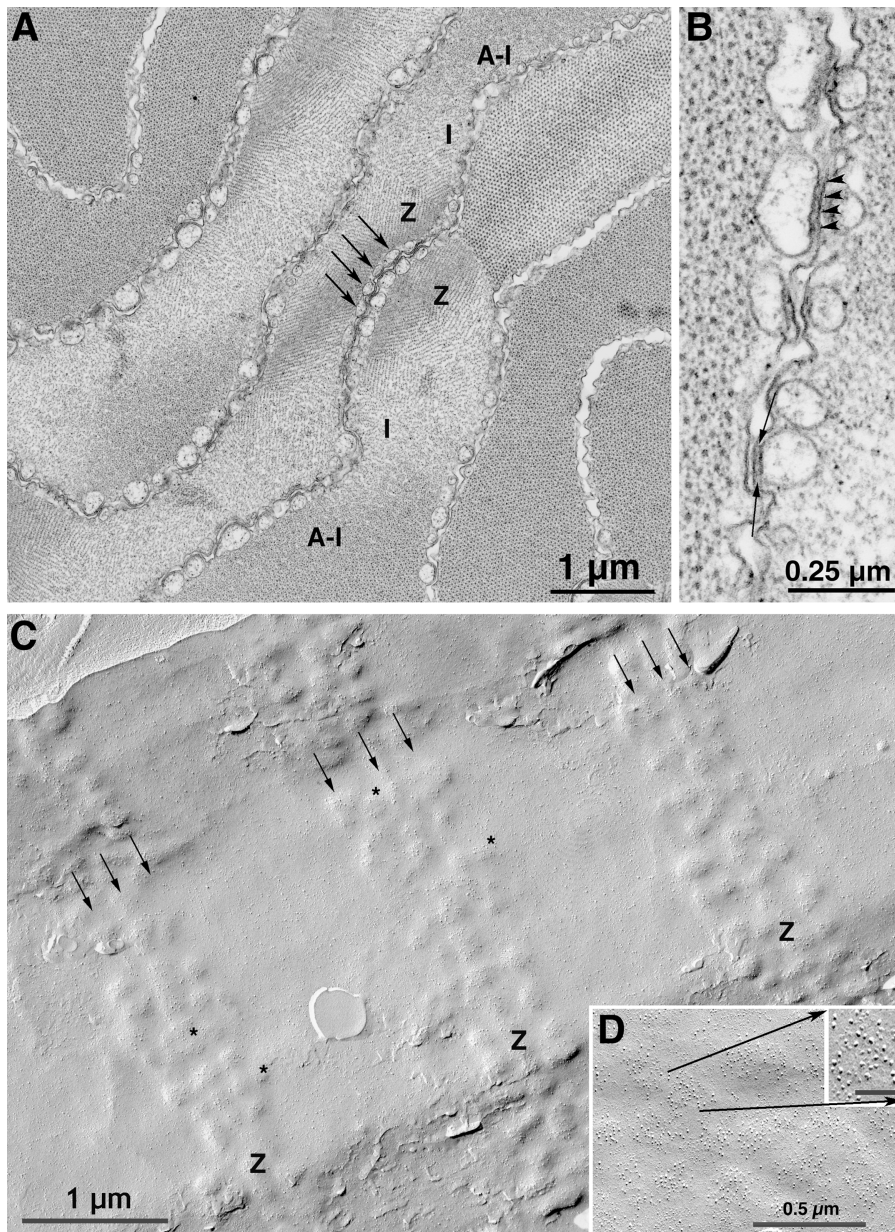


Figure 2. Peripheral couplings in *Amphioxus* muscle. (A and B) Cross-sections of myotomes in *Amphioxus* show series of lamellae, each containing only a single myofibril. (A) At the fiber periphery several jSR sacs associate with the plasmalemma, forming peripheral couplings (arrows) opposite the Z line, I band, and the edges of the A band. (B) Feet (RyRs; arrows) are present in the junctional gap. (C and D) Freeze-fracture replicas of the cytoplasmic leaflet from the plasmalemma in *Amphioxus* lamellae. Dome-shaped plasmalemma domains are arranged in three circumferential rows (arrows). Additional domes are occasionally present (asterisks). (A) The rows are centered on the Z lines (Z) but cover a broad band of the surface. The shape and positioning of these domains are consistent with those of peripheral couplings (compare with A). (D, inset) The plasmalemmal domes are decorated by clusters of particles consistently larger than those decorating the rest of cytoplasmic leaflet. Bar, 100 nm.

at the periphery and dyads/triads (SR-T junctions) internally. The hagfish has well developed T tubule networks located at the A-I junction (Fig. 3 A). Extensive triads have transverse (Fig. 3 A) and, less frequently, longitudinal (Fig. 3 C) orientation. Triads have two rows of feet, each comprised of 25–30 feet, with an orderly arrangement (Fig. 3 C, arrows). Peripheral couplings, on the other hand, are quite small, and less frequent than triads: only two to three feet (Fig. 3 B, arrows) occupy the junctional gap.

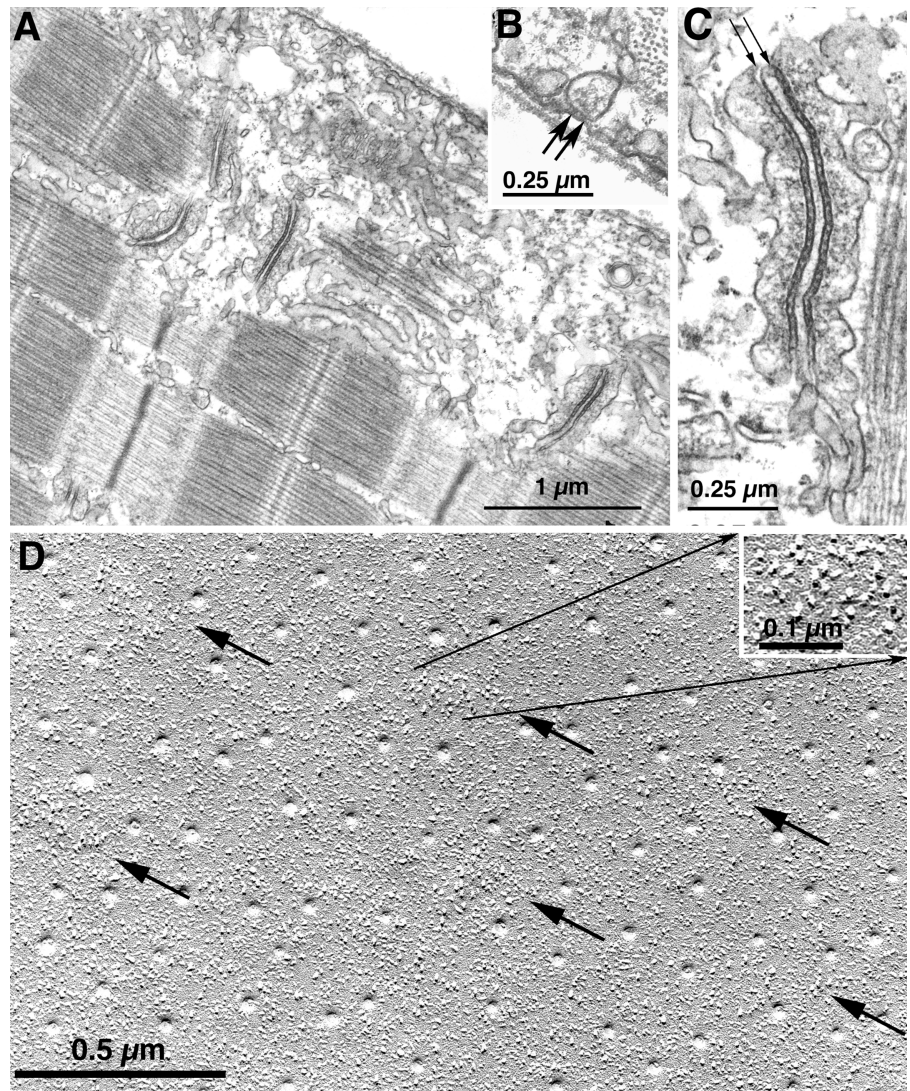
The plasmalemma of hagfish has small patches of unusually large particles (Fig. 3 D, arrows and inset). The positioning of the patches relative to the bands of the underlying myofibrils is not always clear because the plasmalemma has a flat shape. However, in some instances it is clear that the patches are aligned along two closely spaced circumferential lines (Fig. 3 D), presumably corresponding to the edges of the I bands, indicating a position of peripheral couplings equivalent

to that of the internal triads. The small size of the patches and their low frequency correspond well with the paucity and small size of peripheral couplings in this muscle (Fig. 3, compare B and D). Numerous evenly distributed openings of caveolae are present.

Lamprey

In lamprey muscle, extensive triads are located at the Z line and have two different orientations, transverse (Fig. 4 A) and longitudinal (Fig. 4 B), often near each other in the same fiber. Notice that in the latter case the jSR cisternae associated with T tubules are located between the T tubule and the myofibrils and the junctional surfaces are longitudinally oriented. Unlike hagfish, the lamprey has numerous large peripheral couplings, located at the Z line (Fig. 4 B, between arrows). All types of CRUs in lamprey have extensive arrays of periodically disposed feet in the junctional gap.

Figure 3. **Longitudinal section of myotome muscle in hagfish.** At the A-I band junctions, jSR associates with T tubules to form triads (A) with numerous feet (C, arrows). In this muscle, peripheral couplings between SR and plasmalemma are small, have few feet (B, arrows), and are not frequent. The cytoplasmic leaflet of plasmalemma in hagfish myotomes shows a flat membrane rich in particles and in caveolae; its neck openings appear as small circles (D). Small patches of membrane contain a population of large particles (D, arrows) that are clustered into tetrads (inset).



The lamprey's plasmalemma shows frequent clusters of large particles occupying elongated, rectangular patches of surface membrane (Fig. 4 C, arrows) in proximity of the Z lines. The particle clusters are larger (Fig. 4 D) and more frequent than those found in the hagfish, and their long axis is oriented along the circumference of the fiber. This fits very well with the observation that peripheral couplings are located at the Z lines; they are frequent and they have extensive rows of feet when seen in cross-sections of the muscle fibers, i.e., when observed in the direction of the fiber's circumference (Fig. 4, compare B and C). Caveolae are unevenly distributed; they are more frequent along longitudinal bands marking the spaces between myofibrils and in circumferential bands covering the I-Z-I regions.

Garfish

The SR in garfish has a somewhat unusual geometry; the jSR cisternae are in the form of longitudinally oriented fingerlike extensions (Fig. 5 A, asterisks) that wrap around the T tubule (T) surfaces facing the myofibrils at the level of the Z lines (Z). In cross-sections (Fig. 5 B), each fingerlike jSR cisterna appears as a small vesicle interposed between the T tubule sur-

face and the myofibrils. Often the vesicles are present on both sides of the T tubule. Similar fingerlike cisternae are located at the fiber's edge, forming peripheral couplings that are also positioned at the level of the Z lines (Fig. 5 C), are very frequent, and accommodate two to three rows of feet (Fig. 5 C, arrowheads). The facing junctional domains of SR and T tubules and of SR and plasmalemma are oriented in a longitudinal direction. Note also that the plasmalemma is pushed slightly outward opposite the jSR vesicles forming the peripheral couplings and slightly invaginated between them (Fig. 5 C).

In garfish muscle fibers, membrane domains occupied by large particles are located at the Z line level (Fig. 5 D). The domains are narrow, elongated in the direction parallel to the fiber's long axis, arranged into groups of five to eight (Fig. 5 D, arrows), and separated from each other by narrow strips of membrane. The particle-occupied membrane domains are slightly raised and the membrane in between is slightly depressed (Fig. 5 E), which is in close correspondence with the shape of the plasmalemma at and between peripheral couplings (Fig. 5, compare C and D). Caveolae are located along the lateral two-thirds of the A bands and are mostly excluded from the Z line and its vicinity.

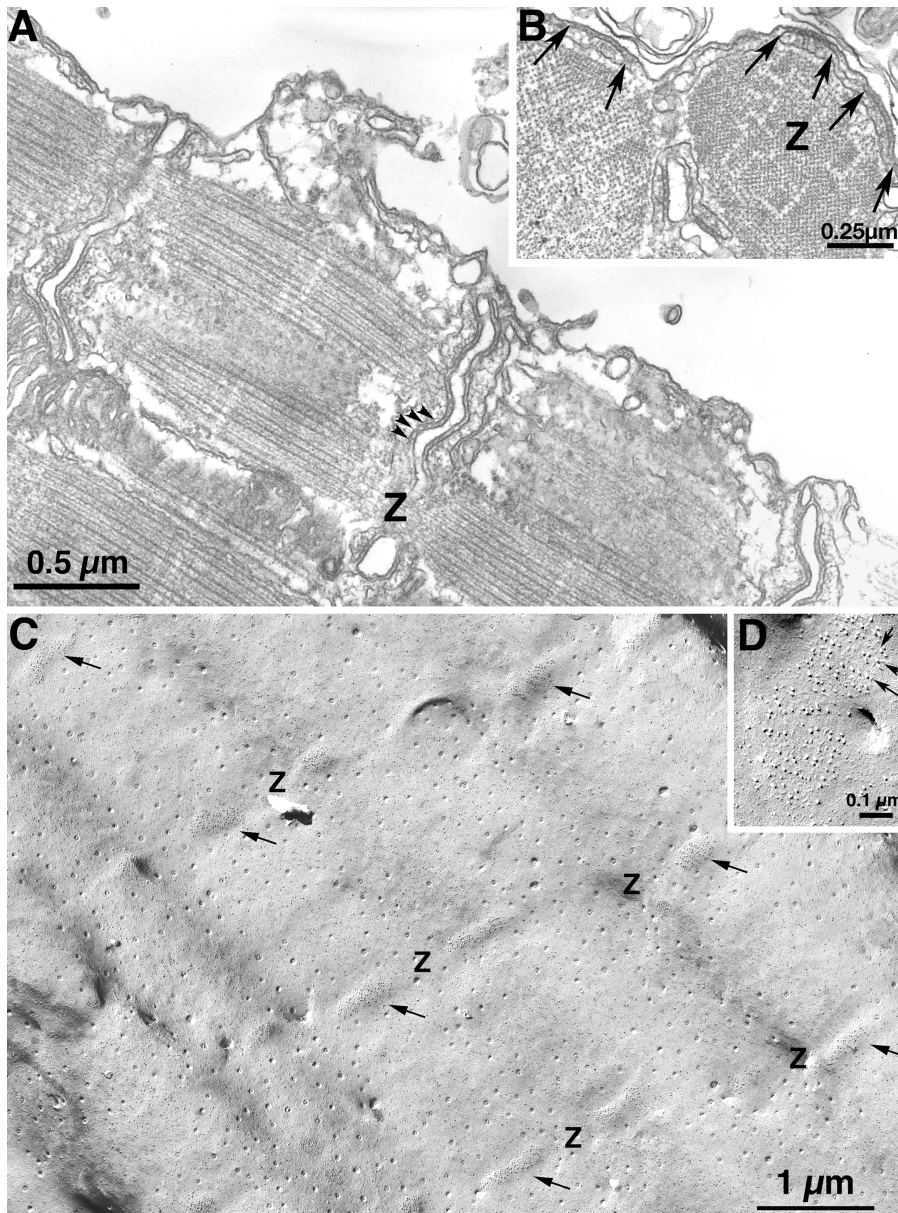


Figure 4. **Triads and peripheral couplings in a longitudinal section of myotome muscle in lamprey.** (A) Triads located at the Z line (Z). In all cases feet occupy the junctional gap (arrowheads). (C and D) In freeze-fracture, the cytoplasmic leaflet of the plasmalemma shows groups of large particles located at the Z lines (Z). The groups are frequent, large, and often elongated in the circumferential direction. (B) This corresponds to the location of large peripheral couplings seen in thin sections.

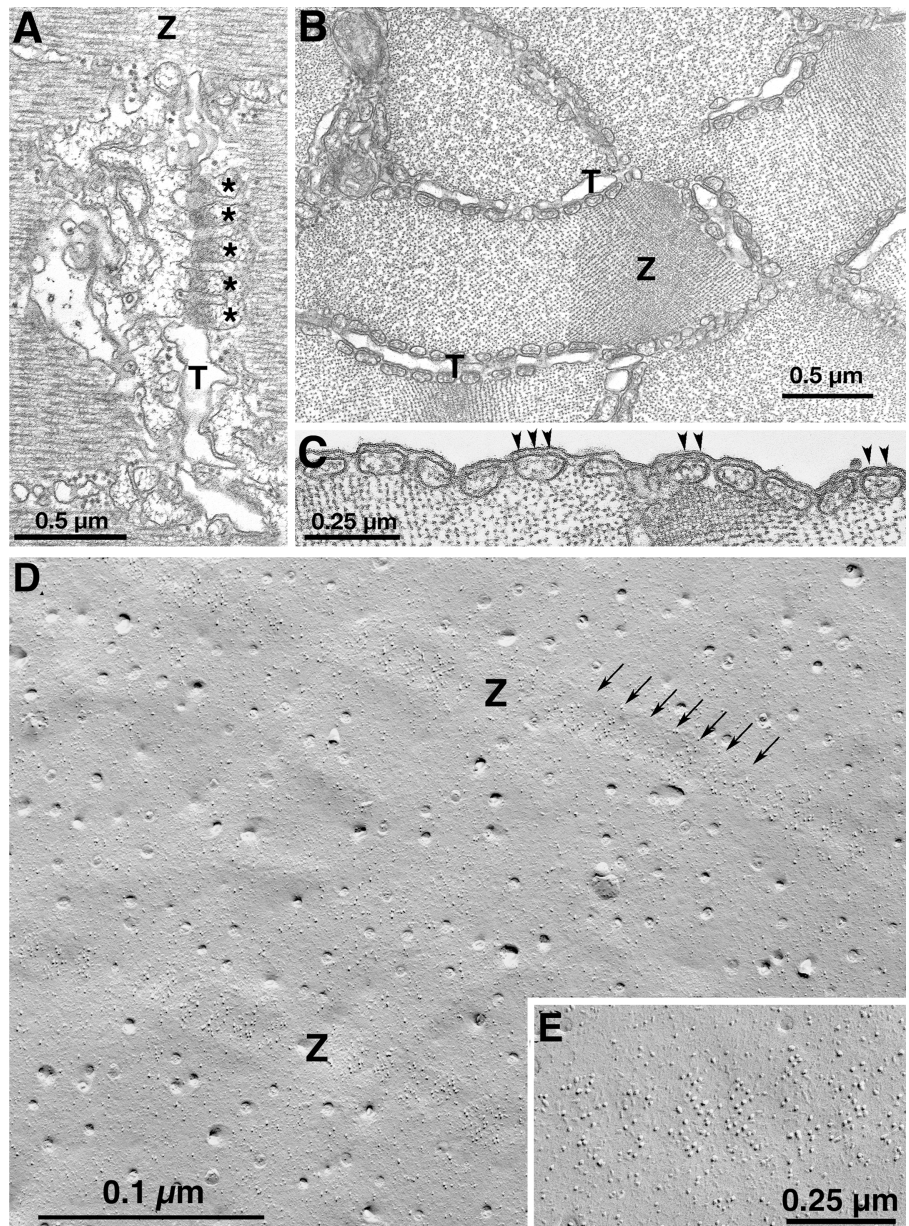
Particle arrangements

The arrangements of large particles within the aforementioned clusters fall into two major categories. In *Amphioxus*, the large particles are clustered in fairly close proximity to each other, but there is no detectable order in their disposition within the clusters (Fig. 6, A–D); the particles are neither aligned with each other along straight lines nor consistently grouped into a recognizable pattern. Muscle fibers from all other samples (Fig. 6, E–H for hagfish; I–L for lamprey; and M–P for garfish) show grouping of the particles into tetrads, as first seen in bony fish (Franzini-Armstrong and Nunzi, 1983; Block et al., 1988). However, the tetrads are often incomplete and are distorted by the fracturing process. So each tetrad may be missing one or more particles, and the position of the particles may not define a perfect square. This is attributed to a combination of incomplete fracturing and/or a lack of components within the tetrads. Incomplete and distorted tetrads are

recognized as such because they form part of ordered arrays in which the position of each tetrad is specified and the particles of adjacent tetrads tend to be aligned in two orthogonal directions. Thus, in areas where tetrads are less complete, their overall arrangement is detected by the alignment of the remaining particles (Block et al., 1988; Protasi et al., 2000, 2002). A group of either three or four particles is also recognized as a tetrad when the lines connecting adjacent particles intersect at $\sim 90^\circ$ and the particles are located in fairly close proximity to each other (~ 20 nm; Paolini et al., 2004). In clusters where particles do not have a specific arrangement, such as in *Amphioxus*, tetrads as we have defined them are seen extremely rarely (as may be expected from a random event) and particles are only aligned over very short distances.

One way of determining the parameters of a tetrad array is to outline the tetrads with a square tangent to their outer profile (Fig. 6, F, H, J, L, N, and P). When this is done, it is

Figure 5. **The unusual jSR in myotome muscles of garfish.** (A) Longitudinal section shows a peculiar fingerlike shape of the jSR (asterisks) facing the T tubules (T) at the Z line (Z). (B) When cut in cross-section the fingers appear as small vesicles facing the T tubules. (C) Similarly elongated jSR fingers are associated with the plasmalemma, appearing as closely spaced jSR vesicles in cross-section. Regularly spaced feet are present in the junctional gap (arrowheads). (D and E) In freeze fracture of the surface membrane, groups of large particles are located in numerous parallel domains at the Z lines (arrows). The domains are elongated in the longitudinal direction and correspond in position and shape to the areas of junctions between the fingerlike jSR and the plasmalemma (compare with A). (E) The membrane domains occupied by large particles are slightly raised, whereas the membrane between is slightly depressed.



clear that two different arrangements of tetrads are displayed, with the tetrads of lamprey and hagfish differing from those of garfish.

In lamprey and hagfish, the squares delimiting tetrads lie side by side, so that the lines connecting the centers of adjacent tetrads are parallel to the lines connecting the subunits of the tetrads along the edges of the square. In garfish, on the other hand, the tetrads are displaced by one-half width relative to each other, and the lines connecting the centers of adjacent tetrads are at an angle relative to the lines delimiting the tetrad squares.

Models of the relationship between feet and tetrad arrays

The parameters of feet and tetrad arrays were measured in thin sections and in freeze-fracture replicas, respectively. The inter-foot distances, measured as the center-center distances between

adjacent feet in sections cutting across the junctional gap, as illustrated in Figs. 1–4, are 27.9 ± 3.6 (mean \pm SD; n [number of measurements] = 30) for *Amphioxus*; 37.2 ± 5.3 nm ($n = 44$) for lamprey; 30.7 ± 2.0 ($n = 28$) for hagfish; and 28.7 ± 3.1 ($n = 25$) for garfish. The center-center distances between adjacent tetrads is 42.6 ± 3.9 ($n = 96$) for lamprey; 41.5 ± 3.8 ($n = 66$) for hagfish; and 42.6 ± 4.4 ($n = 39$) for garfish.

Tetrads and feet are observed by two different techniques and thus the only way of correlating the two is by modeling. We have modeled the possible relationships between arrays of feet and tetrads based on the aforementioned data and on the belief that the grouping of DHPRs into tetrads and the arrangements of tetrads into arrays are entirely dependent on the presence of RyRs, with which the DHPRs associate (Protasi et al., 2000). Thus, we can simply use the geometry of the tetrad arrays that we observed in freeze-fracture replicas to predict the geometry of the feet arrays in the same specimens and to see if

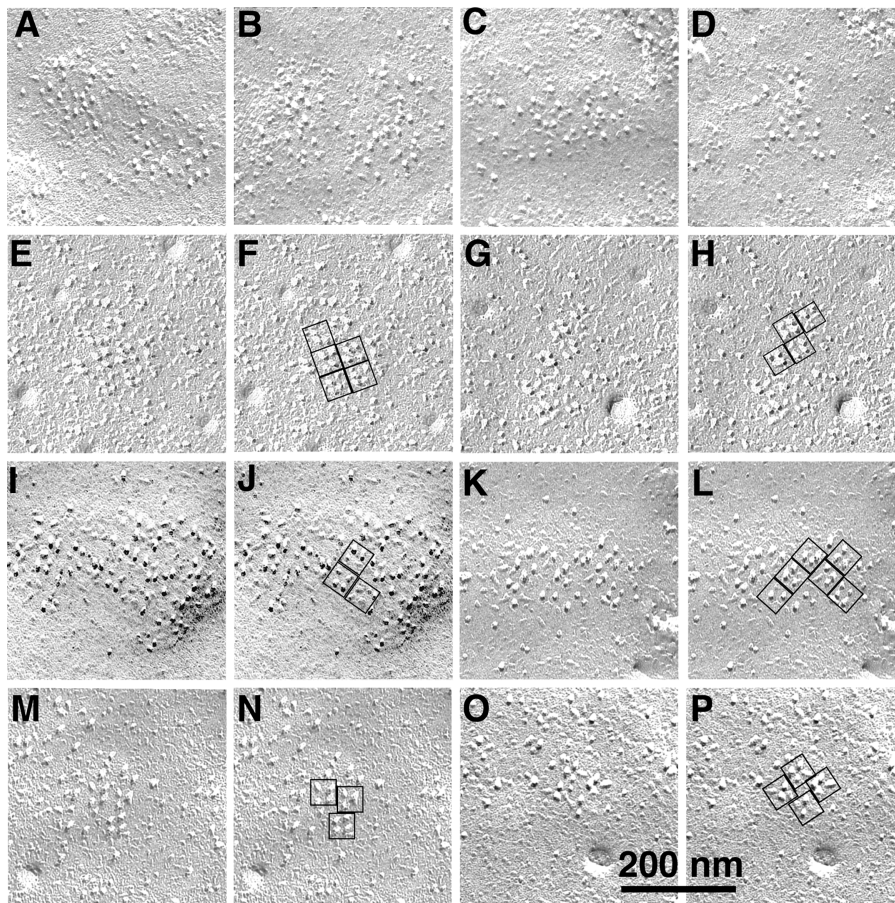


Figure 6. Particle clusters illustrate DHPR disposition. Details of large particle clusters in Amphioxus (A–D), hagfish (E–H), lamprey (I–L), and garfish (M–P). In Amphioxus, the particles are clustered, but show no special arrangement within the clusters. In hagfish, lamprey, and garfish, the particles tend to be grouped into small orthogonal arrangements of four (tetrads, within squares). The tetrads are not always complete, but they clearly form part of a larger array. Two arrangements are visible. In hagfish (E–H) and lamprey (I–L), the tetrads abut side by side; in garfish (M–P), on the other hand, the tetrads are in proximity to their corners. The latter arrangement is typical of most vertebrates.

the predictions fit the data. The predicted arrays must be consistent with the measured feet and tetrad spacings, as well as with the known size of the cytoplasmic domains of RyRs (Radermacher et al., 1994; Serysheva et al., 1999). In preparing the model, we rely on the observation that the size of RyRs does not vary by much in different species (Loesser et al., 1992).

In Fig. 7, tetrads are represented by groups of four pink circles. The center of the circle corresponds to the center of the freeze-fracture particle, but the diameter of the circle is not the same as the diameter of the particle. Fig. 7 A shows the array of tetrads in primitive fish such as lamprey and hagfish, in which the tetrads are aligned with parallel sides. Associated with it is a hypothetical array of feet, in light green, that was assembled based on the known basic structure of the RyR cytoplasmic domain (Radermacher et al., 1994; Serysheva et al., 1999). When related to the intertetrad distances (42 nm, obtained by averaging the intertetrad distances of lamprey and hagfish) the predicted size and shape of the RyR in the model is a 26×26 -nm square, which is quite consistent with the published data for mammalian RyR (Radermacher et al., 1994; Serysheva et al., 1999).

Fig. 7 B illustrates the RyR-tetrad relationship for garfish, which is the same as that of other higher vertebrates (Block et al., 1988). The arrangement of feet and tetrads along the triads in Fig. 7 D would result in a 1.41 ratio of tetrad to feet distances, which is sufficiently consistent with the measured 1.48 ratio.

Fig. 7 C illustrates the possible arrangement of feet and tetrads in the hagfish triads. In this case, the predicted ratio between interfeet (blue arrow) and intertetrad (yellow arrow) distance is the square root of two, or 1.41. The measured ratio is 1.35.

In lamprey, the ratio between measured interfeet and intertetrad distances is 1.1. Fig. 7 E illustrates how the model shown in Fig. 7 A can predict a unitary ratio between the two distances by showing the possible orientation of two rows of feet and one row of tetrads along a triad. Given the orientation shown, the interfeet distance along the two rows of feet (Fig. 7 E, blue arrows) would be the same as the intertetrad distance (yellow arrows). Indeed, a rare fracture along the surface of a T tubule in this fish showed a single row of tetrads arranged as in Fig. 7 E (not depicted).

Given the knowledge that the intertetrad distances are subject to changes due to fracturing distortions, that the interfeet distances are subject to changes during the embedding, and that some uncertainty exists when measuring the center–center distances between feet, the 4–10% of discrepancies between the predicted and measured ratios are minor, and thus the models are consistent with the data.

Discussion

In all muscles examined, freeze-fracturing reveals clusters of large intramembrane particles that are located in correspondence with peripheral couplings. In all Craniata, the particles

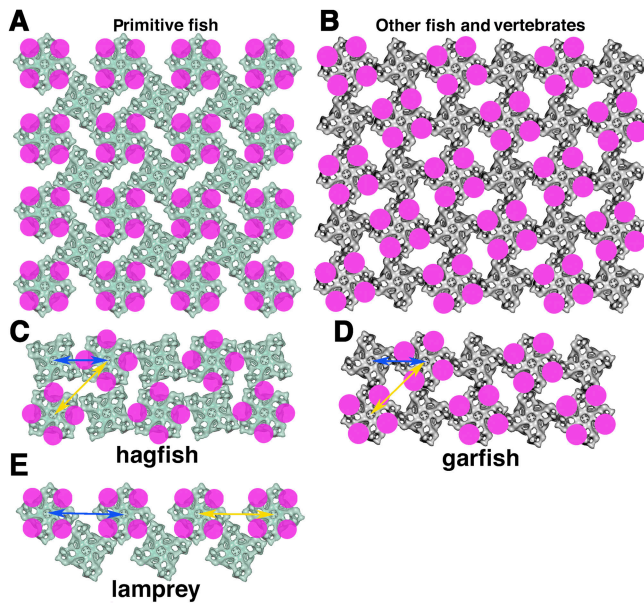


Figure 7. The arrangements of DHPR tetrad particles described in the text are superimposed on a possible arrangement of RyRs that may account for the tetrad disposition. Each pink circle indicates one DHPR molecule, and RyRs are green or gray tetrameric molecules. Center-center distances (arrows) between tetrad particles are drawn in the appropriate scale relative to RyR size, but the size of the particle is arbitrary. The RyR array in B has been directly observed; the others are modeled on the basis of the tetrad arrays.

are arranged in tetrads, which directly identifies them as representing DHPRs, as previously established (Takekura et al., 1994). Isoform expression for DHPRs and RyRs has not been determined for the muscles used in this study, but the structural information presented here allows for some specific conclusions. The arrangement of DHPRs into tetrads directly identifies them as having the molecular determinants of the skeletal type-specific $\alpha 1$ s because this isoform is the only one that assembles into tetrads in the presence of RyR (Takekura et al., 2004). In addition, the presence of tetrads indicates that the muscles contain a variant of type 1 RyR because this is the only isoform that can associate with $\alpha 1$ s and induce tetrad formation (Franzini-Armstrong and Protasi, 1997; Protasi et al., 2002). The presence of an additional RyR type (e.g., type 3) is not excluded. In the case of Amphioxus, even though the particles do not have a specific arrangement we rely on the analogy with muscles of invertebrates and cardiac muscle (Protasi et al., 1996; Takekura and Franzini-Armstrong, 2002; Tijssens et al., 2003) to propose that they also represent the calcium channels responsible for the large inward calcium current in these muscles (Hagiwara et al., 1971; Melzer, 1982a). Because the DHPRs do not assemble into tetrads, this means that either one or both of the DHPR-RyR tandem are not skeletal muscle specific. The appearance of tetrads in the Craniata is thus a direct indication that the new acquisition of the skeletal muscle-specific isoforms of RyR (RyR1 or homologous) and $\alpha 1$ sDHPR (SkDHPR or homologous) occurs at the transition between low chordates and vertebrates (Fig. 1).

The acquisition of DHPR tetrads by low Craniata corresponds quite well with the acquisition of a DHPR-RyR interac-

tion that does not require calcium permeation through the L-type channels by the same species (Inoue et al., 1994, 2002). This then represents a transition to a more advanced form of e-c coupling that was made possible by the ability of the two molecules to form a supramolecular complex and to directly influence each other. In addition to allowing a direct interaction with RyR, the skeletal muscle DHPRs carry little calcium current (Tanabe et al., 1988), thus reducing the metabolic burden on the muscle fiber.

Amphioxus represents a transition. The plasmalemmal intramembrane particles associated with peripheral couplings in this muscle are not arranged into tetrads (Henkart et al., 1976). This occurs under the same fixation treatment that consistently preserves the tetrad arrangement of DHPRs in all vertebrate muscles. Thus, similar to cardiac muscle, DHPRs in Amphioxus are located in the proximity of RyRs, but are not capable of forming a specific direct link with them. Amphioxus calcium channels also share the cardiac muscle property of carrying a large current. Hagiwara et al. (1971) and Inoue et al. (1994) have shown that e-c coupling in *Branchiostoma lanceolatum* requires calcium permeation and thus has the more primitive characteristics of the invertebrate and cardiac e-c coupling. Under some conditions, contraction can be initiated in the absence of extracellular calcium (Melzer, 1982b). The lack of apparent linkage between RyR and DHPR dispositions in this muscle, however, would indicate that true skeletal-type e-c coupling cannot be present in Amphioxus and that this novel mode of calcium release induction occurred with the advent of craniates.

Interestingly, all tetrads are arranged into ordered arrays, but the arrays in hagfish and lamprey, the more primitive of the three fish examined, are distinctly different from those of more highly evolved fish (including garfish). In hagfish and lamprey, the model predicts that feet have two possible dispositions within the array, and thus interact with each other at two different sites, whereas in other vertebrate muscles all RyR-RyR interactions in the array are identical. It is possible that the two geometrically distinct types of RyRs in the lower vertebrates represent the two RyR isoforms (RyR1 and RyR3 or α and β isoforms) that are known to be present in fish as well as higher vertebrates (O'Brien et al., 1995; for review see Murayama and Ogawa, 2002; Ogawa et al., 2002). In higher vertebrates, the two isoforms are spatially separated (Felder and Franzini-Armstrong, 2002) and the RyR1 array, containing a single isoform, has the form shown in Fig. 7. In the more primitive fish, RyR1 may mingle either with RyR3 (or its equivalent) or with a form of RyR2 (the cardiac muscle-specific isoform) that may still be expressed in the skeletal muscle. This needs to be explored with molecular biology. Interestingly, the modeled disposition of RyRs in the two more primitive fish resembles that found in some invertebrate muscles (Loesser et al., 1992), indicating that in these two organisms evolution of e-c coupling has diverged from that of the line leading to fish and higher vertebrates.

T tubules have variable dispositions in the muscles examined (including their absence in Amphioxus). However, this does not seem to have evolutionary significance because equivalent variabilities in the presence and/or disposition of T tubules are found throughout the invertebrates (Veratti, 1902, 1961; Franzini-Armstrong, 2002).

Materials and methods

Branchiostoma floridae (Amphioxus) were purchased from Gulf Specimen Marine Laboratories, Inc.; *Lampetra planari* larvae (lamprey) were obtained from Carolina Biological Supply; *Eptatretus stouti* (hagfish) were purchased from a private fisherman in California; and *Lepisosteus osseus* (garfish) were purchased from EkkWill Waterlife Resources. Two or more animals were examined for each species.

After skinning the fish, the muscles were fixed at RT in 6% glutaraldehyde in 0.5 M (Amphioxus and hagfish) or 0.1 M (lamprey and garfish) cacodylate buffer and kept at 4°C for varying periods of time. For thin sections, the muscles were postfixed in 2% OsO₄ in either 0.25 or 0.1 M cacodylate buffer, contrasted en bloc with saturated uranyl acetate, and embedded in Epon. The sections were contrasted in saturated uranyl acetate in 50% ethanol and in a solution of lead salts (Sato, 1968). Small groups of fibers were dissected from the fixed muscle, cryoprotected in 30% glycerol, mounted between two copper holders covered with a thin layer of 20% polyvinyl alcohol in 30% glycerol, and frozen in liquid nitrogen-cooled propane. The bundles were freeze fractured by separating the two holders under vacuum, shadowing with platinum at 45°C, and replicating with carbon in a freeze-fracture unit (model BFA 400; Balzers SpA). Sections and replicas were observed in an electron microscope (model 410; Philips). Images for publication were prepared using Adobe Photoshop on images digitized from the photographic negatives.

This study was supported by National Institutes of Health grant RO1 HL-48093 to C. Franzini-Armstrong.

Submitted: 15 March 2005

Accepted: 11 October 2005

References

- Block, B.A., A. Leung, K.P. Campbell, and C. Franzini-Armstrong. 1988. Structural evidence for direct interaction between the molecular components of the transverse tubules/sarcoplasmic reticulum junction in skeletal muscle. *J. Cell Biol.* 107:2587–2600.
- Chugun, A., K. Taniguchi, T. Murayama, T. Uchide, Y. Hara, K. Temma, Y. Ogawa, and T. Akera. 2003. Subcellular distribution of ryanodine receptors in the cardiac muscle of carp (*Cyprinus carpio*). *Am. J. Physiol.* 285:R601–R609.
- Felder, E., and C. Franzini-Armstrong. 2002. Type 3 ryanodine receptors of skeletal muscle are segregated in a parajunctional position. *Proc. Natl. Acad. Sci. USA.* 99:1695–1700.
- Flood, P.R. 1977. The sarcoplasmic reticulum and associated plasma membrane of trunk muscle lamellae in *Branchiostoma lanceolatum* (pallas). A transmission and scanning electron microscopic study including freeze-fractures, direct replicas and x-ray microanalysis of calcium oxalate deposits. *Cell Tissue Res.* 181:169–196.
- Flucher, B.E., and C. Franzini-Armstrong. 1996. Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. *Proc. Natl. Acad. Sci.* 93:265–278.
- Forey, P., and P. Janvier. 1993. Agnathans and the origin of jawed vertebrates. *Nature.* 361:129–134.
- Franzini-Armstrong, C. 1970. Studies of the triad: I. Structure of the junction in frog twitch fibers. *J. Cell Biol.* 47:488–499.
- Franzini-Armstrong, C. 2002. Veratti and beyond: structural contributions to the study of muscle activation. *Rendiconti Lincei Scienze Fisiche e Naturale.* 13:289–323.
- Franzini-Armstrong, C., and G. Nunzi. 1983. Junctional feet and membrane particles in the triads of a fast twitch muscle fiber. *J. Muscle Res. Cell Motil.* 4:233–252.
- Franzini-Armstrong, C., and F. Protasi. 1997. The ryanodine receptor of striated muscles, a complex channel capable of multiple interactions. *Physiol. Rev.* 77:699–729.
- Gyorke, S., and P. Palade. 1993. Role of local Ca²⁺ domains in activation of Ca²⁺-induced Ca²⁺ release in crayfish muscle fibers. *Am. J. Physiol.* 264:C1505–C1512.
- Hagiwara, S., M.P. Henkart, and Y. Kidokoro. 1971. Excitation contraction coupling in Amphioxus muscle cells. *J. Physiol.* 219:233–251.
- Henkart, M., D.M. Landis, and T.S. Reese. 1976. Similarity of junctions between plasma membranes and endoplasmic reticulum in muscle and neurons. *J. Cell Biol.* 70:338–347.
- Inoue, I., I. Tsutsui, Q. Bone, and E.R. Brown. 1994. Evolution of skeletal muscle excitation-contraction coupling and the appearance of dihydropyridine-sensitive intramembrane charge movement. *Proc. R. Soc. Lond. B. Biol. Sci.* 255:181–187.
- Inoue, I., I. Tsutsui, and Q. Bone. 2002. Excitation contraction coupling in skeletal and caudal heart muscle of the hagfish *Eptatretus burgeri* Girard. *J. Exp. Biol.* 205:3535–3541.
- Jorgensen, J.M., J.P. Lomholt, R.E. Weber, and H. Malte, editors. 1998. The Biology of Hagfishes. Chapman and Hall, London. 600 pp.
- Langille, R.M., and B.K. Hall. 1989. Developmental processes, developmental sequences and early vertebrate phylogeny. *Biol. Rev. Camb. Philos. Soc.* 64:73–91.
- Loesser, K.E., L. Castellani, and C. Franzini-Armstrong. 1992. Disposition of junctional feet in muscles of invertebrates. *J. Muscle Res. Cell Motil.* 13:161–173.
- Mallat, J., J. Sullivan, and C.J. Winchell. 2001. The relationships of lamprey to hagfishes: a spectral analysis of ribosomal DNA sequences. In Major Events in Early Vertebrate Evolution. Paleontology, Phylogeny, Genetics and Development. Systematics Association Special Volume Series, vol. 61. P.E. Ahlberg, editor. Taylor and Francis, London. 106–118.
- McGuigan, K., P.C. Phillips, and J.H. Postlethwait. 2004. Evolution of sarcomeric myosin heavy chain genes: evidence from fish. *Mol. Biol. Evol.* 21:1042–1056.
- Melzer, W. 1982a. Electrical properties of the muscle lamellae in *Branchiostoma* myotomes. *Eur. J. Cell Biol.* 28:213–218.
- Melzer, W. 1982b. Twitch activation in Ca²⁺-free solutions in the myotomes of the lancelet (*Branchiostoma lanceolatum*). *Eur. J. Cell Biol.* 28:219–225.
- Moore, E.D., T. Voigt, Y.M. Kobayashi, G. Isenberg, F.S. Fay, M.F. Gallitelli, and C. Franzini-Armstrong. 2004. Organization of Ca²⁺ release units in excitable smooth muscle of the guinea-pig urinary bladder. *Biophys. J.* 87:1836–1847.
- Murayama, T., and Y. Ogawa. 2002. Roles of two ryanodine receptor isoforms coexisting in skeletal muscle. *Trends Cardiovasc. Med.* 12:305–311.
- Nabauer, M., G. Gallewaert, L. Cleemann, and M. Morad. 1989. Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science.* 244:800–803.
- Nakai, J., R.T. Dirksen, H.T. Nguyen, I.N. Pessah, K.G. Beam, and P.D. Allen. 1996. Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature.* 380:72–75.
- O'Brien, J., H.H. Valdivia, and B.A. Block. 1995. Physiological differences between the alpha and beta ryanodine receptors of fish skeletal muscle. *Biophys. J.* 68:471–482.
- Ogawa, Y., T. Murayama, and N. Kurebayashi. 2002. Ryanodine receptor isoforms of non-mammalian skeletal muscle. *Front. Biosci.* 7:1184–1194.
- Paolini, C., J.D. Fessenden, I.N. Pessah, and C. Franzini-Armstrong. 2004. Evidence for conformational coupling between two calcium channels. *Proc. Natl. Acad. Sci. USA.* 101:12748–12752.
- Peachey, L.D. 1961. Structure of the longitudinal body muscles of amphioxus. *J. Biophys. Biochem. Cytol.* 10:159–176.
- Protasi, F., X.-H. Sun, and C. Franzini-Armstrong. 1996. Formation and maturation of calcium release units in developing and adult avian myocardium. *Dev. Biol.* 173:265–278.
- Protasi, F., C. Franzini-Armstrong, and B. Flucher. 1997. Coordinated incorporation of skeletal muscle dihydropyridine receptors and ryanodine receptors in peripheral couplings of BC₃H1 cells. *J. Cell Biol.* 137:859–870.
- Protasi, F., H. Takekura, Y. Wang, S.R.W. Chen, G. Meissner, P.D. Allen, and C. Franzini-Armstrong. 2000. RYR1 and RYR3 have different roles in the assembly of calcium release units of skeletal muscle. *Biophys. J.* 79:2494–2508.
- Protasi, F., C. Paolini, J. Nakai, K.J. Beam, C. Franzini-Armstrong, and P.D. Allen. 2002. Multiple regions of RYR1 mediate functional and structural interactions with α_{1S} -DHPR in skeletal muscle. *Biophys. J.* 83:3230–3244.
- Radermacher, M., V. Rao, R. Grassucci, J. Frank, A.P. Timerman, S. Fleischer, and T. Wagenknecht. 1994. Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. *J. Cell Biol.* 127:411–423.
- Sato, T. 1968. A modified method for lead staining of thin sections. *J. Electron Microsc. (Tokyo).* 17:158–159.
- Scheuer, T., and W.F. Gilly. 1986. Charge movement and depolarisation-contraction coupling in arthropod vs. vertebrate skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 83:8799–8803.
- Schneider, M.F., and W.K. Chandler. 1973. Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature.* 242:244–246.
- Serysheva, I.I., N.M. Scatz, M. vanHell, W. Chou, and S.L. Hamilton. 1999. Structure of the skeletal muscle calcium release channel. *Biophys. J.* 77:1936–1944.
- Sun, X.H., F. Protasi, M. Takahashi, H. Takeshima, D.G. Ferguson, and C.

- Franzini-Armstrong. 1995. Molecular architecture of membranes involved in excitation-contraction coupling of cardiac muscle. *J. Cell Biol.* 129:659-671.
- Takekura, H., and C. Franzini-Armstrong. 2002. The structure of Ca²⁺ release units in arthropod body muscle indicates an indirect mechanism for excitation-contraction coupling. *Biophys. J.* 83:2742-2753.
- Takekura, H., L. Bennett, T. Tanabe, K.G. Beam, and C. Franzini-Armstrong. 1994. Restoration of junctional tetrads in dysgenic myotubes by dihydropyridine receptor cDNA. *Biophys. J.* 67:793-804.
- Takekura, H., C. Paolini, C. Franzini-Armstrong, M. Grabner, and B.E. Flucher. 2004. Differential contribution of skeletal and cardiac II-III loop sequences to the assembly of DHP-receptor arrays in skeletal muscle. *Mol. Biol. Cell.* 15:5408-5419.
- Tanabe, T., K.G. Beam, J.A. Powell, and S. Numa. 1988. Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature.* 336:134-139.
- Tijsskens, P., G. Meissner, and C. Franzini-Armstrong. 2003. Location of ryanodine and dihydropyridine receptors in frog myocardium. *Biophys. J.* 84:1079-1092.
- Veratti, E. 1902. Ricerche sulla fine struttura della fibra muscolare striata. Mem. R. Ist. Lombardo. *Classe Scienze Matematiche e Naturali.* 19:87-133.
- Veratti, E. 1961. Investigations on the fine structure of striated muscle. *J. Biophys. Biochem. Cytol.* 10:3-59.
- Zachrova, D., and J. Zachar. 1967. The effect of external calcium ions on the excitation contraction coupling in single muscle fibers of the crayfish. *Physiol. Bohemoslov.* 16:191-207.