The Structure of Ca^{2+} Release Units in Arthropod Body Muscle Indicates an Indirect Mechanism for Excitation-Contraction Coupling

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ABSTRACT The relative disposition of ryanodine receptors (RyRs) and L-type Ca^{2+} channels was examined in body muscles from three arthropods. In all muscles the disposition of ryanodine receptors in the junctional gap between apposed SR and T tubule elements is highly ordered. By contrast, the junctional membrane of the T tubule is occupied by distinctive large particles that are clustered within the small junctional domain, but show no order in their arrangement. We propose that the large particles of the junctional T tubules represent L-type Ca^{2+} channels involved in excitation-contraction (e-c) coupling, based on their similarity in size and location with the L-type Ca^{2+} channels or dihydropyridine receptors (DHPRs) of skeletal and cardiac muscle. The random arrangement of DHPRs in arthropod body muscles indicates that there is no close link between them and RyRs. This matches the architecture of vertebrate cardiac muscle and is in keeping with the similarity in e-c coupling mechanisms in cardiac and invertebrate striated muscles.

INTRODUCTION

The functional, structural, and molecular basis for excitation-contraction (e-c) coupling is essentially the same in all cross-striated muscles, with few exceptions. The initial event is a depolarization of the exterior membranes (plasmalemma/transverse, T tubules) and this is followed by release of Ca^{2+} from elements of the sarcoplasmic reticulum (SR) that are immediately adjacent to the exterior membranes (see Ebashi, 1991; Schneider, 1994 for reviews). The geometry of the Ca^{2+} release units (CRUs) formed by an association of the junctional domains of SR (jSR) and of the plasmalemma/T tubules varies considerably from one muscle to the other. Some muscles have junctions between jSR and jT domains (in the form of either triads or dyads) and these junctions are oriented either transversely or longitudinally in the muscle fiber. Other muscles have junctions between jSR and the plasmalemma, either exclusively or in addition to triads and dyads. These geometrical variations between various CRUs do not affect their intrinsic architecture, which is the same in body muscles of arthropods and in skeletal and cardiac muscles of vertebrates (Smith, 1966, 1968, 1972; Huddart and Oates, 1970; Smith and Aldrich, 1971; Sherman, 1973; Sherman and Luff, 1971; Hoyle, 1973; Eastwood et al., 1982; Franzini-Armstrong, 1974; Franzini-Armstrong et al., 1986; Loesser et al., 1992). The geometry of the junctions and their position does, however, affect the density of the Ca^{2+} release sites within the fiber.

In muscles of vertebrates (skeletal and cardiac), dihydropyridine sensitive L-type Ca^{2+} channels (DHPRs) in the surface membrane initiate e-c coupling by responding to the surface membrane depolarization (Rios and Brum, 1987; Tanabe et al., 1988; Adams et al., 1990). Ryanodine receptors (RyRs), large Ca^{2+} channels in the jSR or SR Ca^{2+} release channels, act as the conduits for Ca^{2+} release from the SR lumen to the myofibrils (Kawamoto et al., 1986; Campbell et al., 1987; Inui et al., 1987; Lai et al., 1988, reviewed by McPherson and Campbell, 1993; Meissner, 1994; Coronado et al., 1994; Sutko and Airey, 1997; Franzini-Armstrong and Protasi, 1997). The cytoplasmic domains of RyRs are visible as feet in electron micrographs (Block et al., 1988).

In muscles from vertebrates, RyRs are located mostly, but not exclusively, at CRUs; that is, at the junctional sites between jSR and the exterior membranes (plasmalemma and/or T tubules). DHPRs are also located at CRUs, in the immediate proximity of RyRs (Block et al., 1988; Jorgensen et al., 1989; Flucher et al., 1991, 1993; Yuan et al., 1991; Carl et al., 1995a, b; Sun et al., 1995; Protasi et al., 1996, 1997; Gathercole et al., 2000). Thus DHPRs are appropriately positioned for transmitting a signal to RyRs following their own activation by the surface membrane depolarization.

RyRs have been detected by biochemical, molecular, and structural approaches in body muscles of arthropods and other invertebrates, where they are located in the junctional gap of CRUs and immediately adjacent to it (Smith, 1966; Huddart and Oates, 1970; Sherman and Luff, 1971; Smith, 1972; Hoyle, 1973; Sherman, 1973; Franzini-Armstrong, 1974; Eastwood et al., 1982; Formelova et al., 1990; Hurnak et al., 1990; Hasan and Rosbash, 1992; Kim et al., 1992; Seok et al., 1992). Feet (RyRs) of invertebrate CRUs (Sherman and Luff, 1971; are disposed in orthogonal arrays that resemble those of skeletal muscle, with only minor differences (Loesser et al., 1992).

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Body muscles from arthropods have large Ca^{2+} currents (Hencek and Zachar, 1977; Zahradnik and Zachar, 1987; Gilly and Scheuer, 1984), carried by channels that have activation and pharmacological characteristics related to those of L-type, dihydropyridine-sensitive Ca^{2+} channels of vertebrate muscles (Araque et al., 1994; Hurnak et al., 1990; Gielow et al., 1995; Castellote et al., 1997; Erxleben and Rathmayer, 1997). α1 subunits of Ca^{2+} channels with sequence homology to the L-type channels of vertebrates have been sequenced in muscles from insects (Grabner et al., 1994; Inagaki et al., 1998). These channels are part of a family that can be traced back to the jellyfish (Jeziorski et al., 1998, 2000). Although the L-type channels of arthropods are not identical to vertebrate DHPRs, the similarities are sufficient to allow classification within the same type and to expect the channels to perform equivalent roles in excitation-contraction coupling.

The disposition of Ca^{2+} channels in arthropod body muscles has not been well defined. Here we describe the disposition of intramembrane particles in T tubules of body muscles from three different arthropods and identify a population of particles probably representing Ca^{2+} channels. The significance of their location and arrangement is discussed in terms of the e-c coupling mechanism.

MATERIALS AND METHODS

Direct flight muscles from dragonflies and damselflies of unknown species, tail or pedipalp muscles from a scorpion (*Centuroides sculpturatus*), and the TDT (tergal depressor of trochanter) from a fly (*Calliphora sp.*) were used in the experiments. The muscles were fixed with 2.5–3.5% glutaraldehyde in 100 mM sodium-cacodylate buffer at room temperature for ~2 h, and then stored in the fixative solutions at 4°C for further use. For thin sectioning, the muscle bundles were rinsed in 100 mM sodium-cacodylate buffer, post-fixed in 2% OsO_4 in the same buffer, both with and without 0.8% $K_3Fe(CN)_6$ for 1–2 h. After washing with H_2O several times, the tissue was en block stained with aqueous saturated uranyl acetate at 60°C for 4 h, dehydrated, and embedded in Epon or Spur. Thin sections were stained with uranyl acetate and Sato lead solutions, and examined at 60 kV in a Philips 410 electron microscope (Philips Electron Optics, Mahwak, NJ).

For freeze fracture, the glutaraldehyde-fixed muscles were infiltrated with 30% glycerol, frozen by immersion in liquid nitrogen-cooled propane and then fractured, shadowed at 45°, and replicated in a 400D Balzers freeze-fracture apparatus (Balzers, Hudson, NH). The replicas from insect muscles were initially cleaned in hydrofluoric acid to dissolve the tracheolae, and then in Clorox. Fixation and freeze-fracture protocols are the same as used in previous studies of skeletal and cardiac muscles of vertebrates.

RESULTS

Thin sections: general disposition of the membrane system

Fast-acting muscles have highly ordered structure due to the closely interrelated disposition of membranes and myofibrils. In the dragonfly flight muscles, as described in detail by D. S. Smith (1968, 1972), the fibrils are in the shape of flat ribbons and the mitochondria as well as SR and T tubules are disposed in a stereotyped manner throughout the fiber (Fig. 1 *A*). Myofibrils are completely separated from each other by a layer of large mitochondria, each spanning exactly the length of a sarcomere, from Z line to Z line. The mitochondria are in turn separated from the myofibrils, on either side, by sheets of fenestrated SR. T-tubules lie between the SR and the mitochondria, within grooves on either side of the mitochondrial surface, and are precisely located slightly past midway between the center of the sarcomere and the edge of the A band (*arrows*, Fig. 1 *A*). Flat T tubule profiles are closely associated with flat jSR cisternae, forming dyads (Fig. 1 *B*). jSR is recognizable on the basis of luminal density due to the presence of calsequestrin (Meissner, 1975). The apposed junctional domains of both the T-tubules and SR cisternae in the dyads are flat and wide and are separated by evenly spaced feet (*arrows*, Fig. 1 *B*). The junctional domains are oriented in a longitudinal direction; that is, they are parallel to the long axis of the muscle fiber. Each T tubule profile has a free surface facing toward the mitochondria and a junctional surface facing toward the SR and away from the mitochondria. A shallower mitochondrial groove opposite the H-band zone usually houses a double layer of SR (*arrowhead*, Fig. 1 *A*).

The TDT muscle of diptera (Fig. 1 *C*) is also fast acting, but it usually performs a single rapid contraction at the beginning of flight and thus its activity is not repetitive. The mitochondria (M) are few and are relegated to few selected regions in the cell. Similarly to those of the dragonfly flight muscle, T-SR junctions are discrete, numerous, and in the form of dyads (*arrows*, Fig. 1 *C*). The SR cisternae are located on one side of the T tubule profiles relative to the myofibrils (*arrows*) and sometimes the position of the SR changes from one side to the other of the T tubule in adjacent dyads (*arrowheads*, Fig. 1 *C*). The junctional surface of SR and T tubules face each other and the free surfaces face the myofibrils. Feet occupy the junctional gap. Scorpion body muscles (not shown) have a very similar arrangement.

Grazing views of the superimposed jSR and T tubule membranes in the dragonfly (Fig. 2 *A*) and the scorpion (Fig. 2, *B* and *C*; see also Loesser et al., 1992) show that junctional sites between SR and T tubules are discrete and oval in shape. Semicrystalline arrays of feet aligned along two orthogonal directions (*arrows*, Fig. 2, *B* and *C*) fill the junctional gap between SR and T tubules. In the dragonfly (Fig. 2 *A*) the feet-covered surfaces of the junctional SR cisternae are of the same size as the flat surfaces of the facing T tubules. One T tubule profile is outlined in Fig. 2 *A* to show that the array of feet does not extend beyond the T tubule profile. Equality in size of the feet-covered jSR domains and of the flat T tubule cisternae is confirmed by the equal length of junctional SR and T tubule profiles in sections cutting at right angle to the junctional surfaces (Fig. 1 *A*). In *Calliphora* TDT (Fig. 1 *B*) the SR profiles bearing

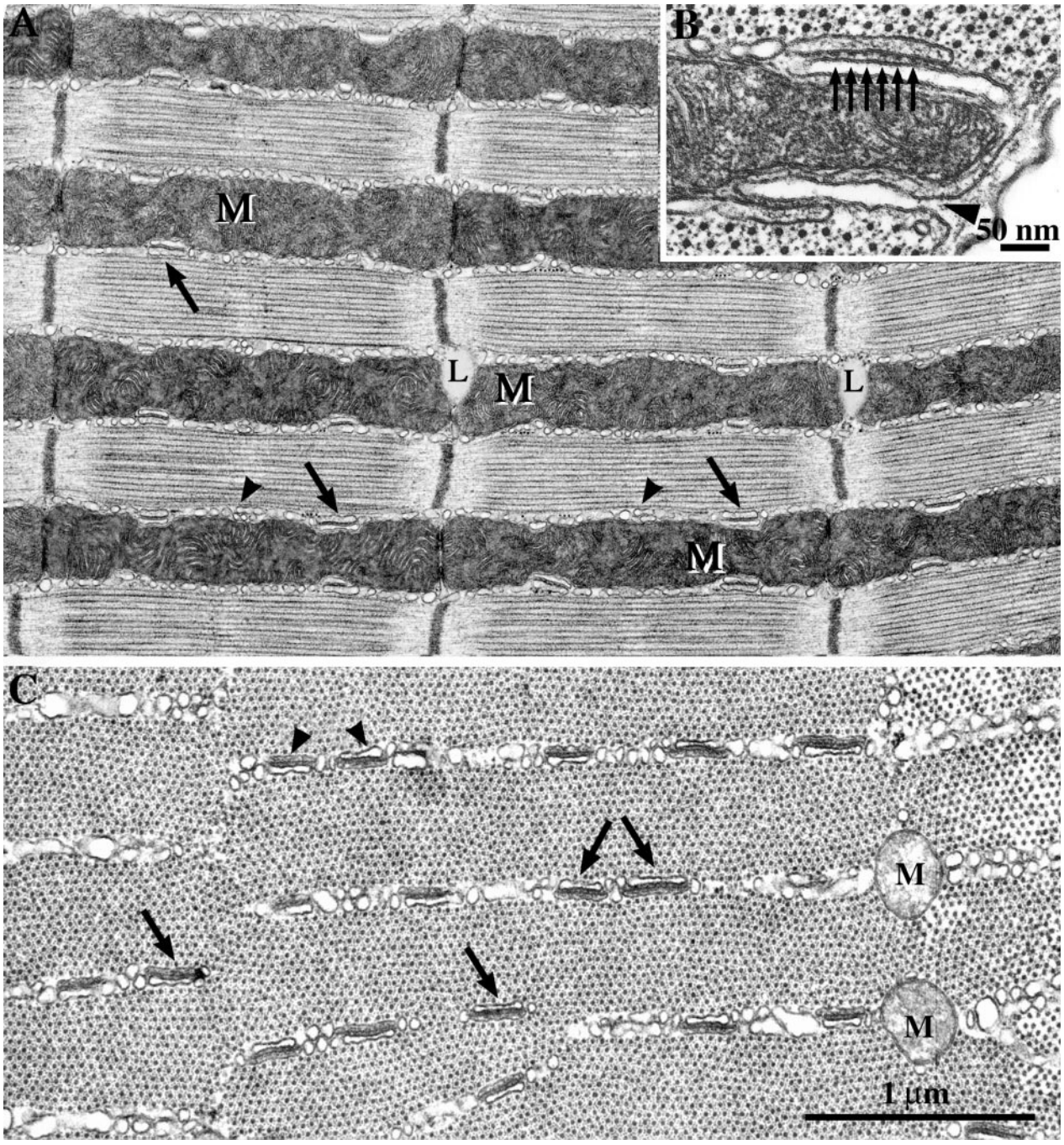

FIGURE 1 Longitudinal and transverse sections of dragonfly flight muscle (*A* and *B*) and transverse section of fly TDT muscle (*C*). (*A*) In the dragonfly flight muscle, myofibrils regularly alternate with large mitochondria (M), and a single or double layer of SR (*arrowhead*) is interposed between them. L = lipid droplets. Two indentations of the mitochondrial surface at every sarcomere provide space for the dyads (*arrows*), composed of jSR (profiles of vesicles with some electron dense content) and jT tubules (*empty profiles*). (*B*) The T tubule membrane is continuous with the plasmalemma, and the lumen with the extracellular space (*arrowhead*). SR and T tubules form dyads. The junctional gap of the dyad is filled by profiles of feet (or ryanodine receptors, *arrows*). The jSR bearing feet does not extend beyond the T tubule surface in the dragonfly muscle. (*C*) In the fly TDT muscle no mitochondria are present over large regions of the fiber. Dyads (*arrows* and *arrowheads*) are usually in doublets because the T tubules extend flat cisternae on two sides and each cisterna forms a dyad with the apposed jSR (compare with Fig. 6). Arrowheads point to two dyads that are on opposite sides of the T tubule in relation to the myofibrils.

feet are also completely resting on a T tubule surface. In scorpion body muscle, however, the foot-covered SR surface extends further than the T tubule profile as first shown for spiders (Sherman, 1973; Sherman and Luff, 1971; Franzini-Armstrong, 1974). In sections grazing the junctional gap, such as shown in Fig. 2, *B* and *C*, the region of the

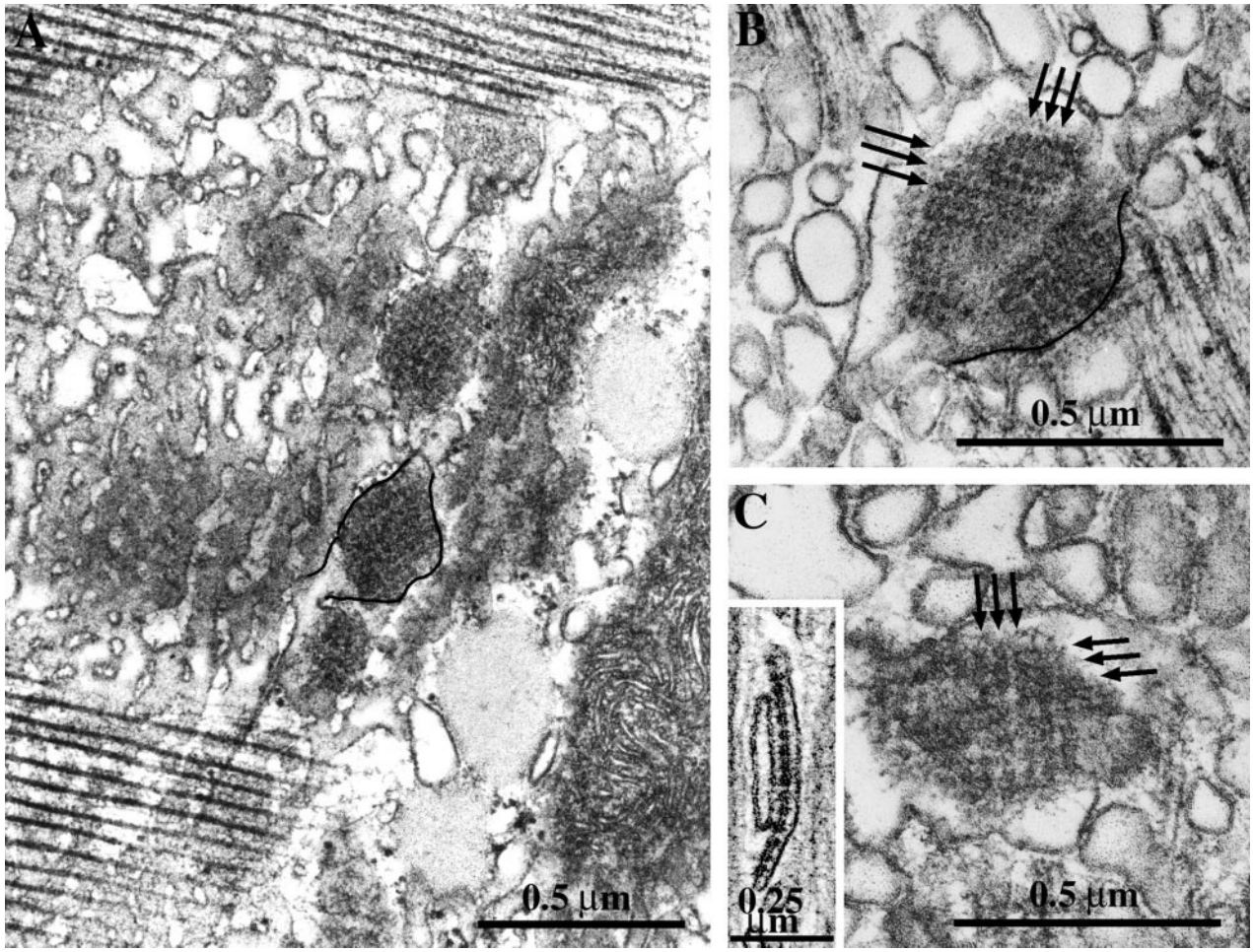

FIGURE 2 Longitudinal sections of dragonfly flight muscle (*A*) and scorpion tail muscle (*B* and *C*), illustrating the disposition of feet as seen in grazing views of the junctional gap of dyads. Feet (*arrows*) are arranged in an extended, coherent, tetragonal array that covers the entire jSR membrane. The two arrays with different orientation in (*B*) belong to two different dyads. In the dragonfly muscle (*A*) the size and shape of junctional domains in T tubule and SR are identical, and the array of feet entirely covers the two junctional membranes. Each junctional patch contains 20–100 feet or more. In the scorpion muscle, the jSR membrane extends farther than the jT membrane. This is seen in (*B*), where the edges of a T tubule have been outlined, and is also more clearly shown in the inset of (*C*), which illustrates a section across a dyad. In the inset, the oval T tubule profile is shorter than the SR profile decorated by feet (see also Sherman and Luff; 1971; Sherman, 1973). The very rarely seen sudden change in orientation of the feet array in (*B*) is probably due to the fact that the figure includes parts of two different dyads. The obvious line without feet at the boundary between the two arrays marks the transition between the two dyads.

junctions where T tubules overlap the array of junctional feet are slightly darker and the edges of one portion of a T tubule have been outlined to emphasize it. It is clear that the array of junctional feet extends beyond the edges of T tubules. The inset in Fig. 2 *C* shows a foot-bearing jSR cisterna that extends beyond the oval-shaped cross-section of T tubule.

An estimate of the content of junctional feet in the junctions from the three arthropods used can be obtained by simply counting the feet in images, such as shown in Fig. 2. Each junctional patch in the three muscles used contains 20–100 feet or more. Based on the approximate center-to-center distance of ~23–28 nm between feet in arthropod dyads (Loesser et al., 1992) the density of feet is 1275–1890/μm^2 of jSR membrane.

Freeze-fracture of T tubules and identification of membrane leaflets

In freeze-fracture replicas from all muscles, T tubule profiles (Fig. 3, from the dragonfly) are readily identified on the basis of their transverse orientation and of the alternate disposition of flat cisternae, forming dyads, and narrow tubules between the dyads. The narrow T tubules do not form junctions, and thus their entire surface is "free." The flat T tubule cisternae within the dyads have one junctional surface, facing the jSR, and one free surface facing myofibrils and/or mitochondria. In dragonfly flight muscles, dyads sit very precisely and consistently in small grooves in the surface of large mitochondria (Fig. 1). T tubule profiles in freeze-fracture images from these muscles are therefore

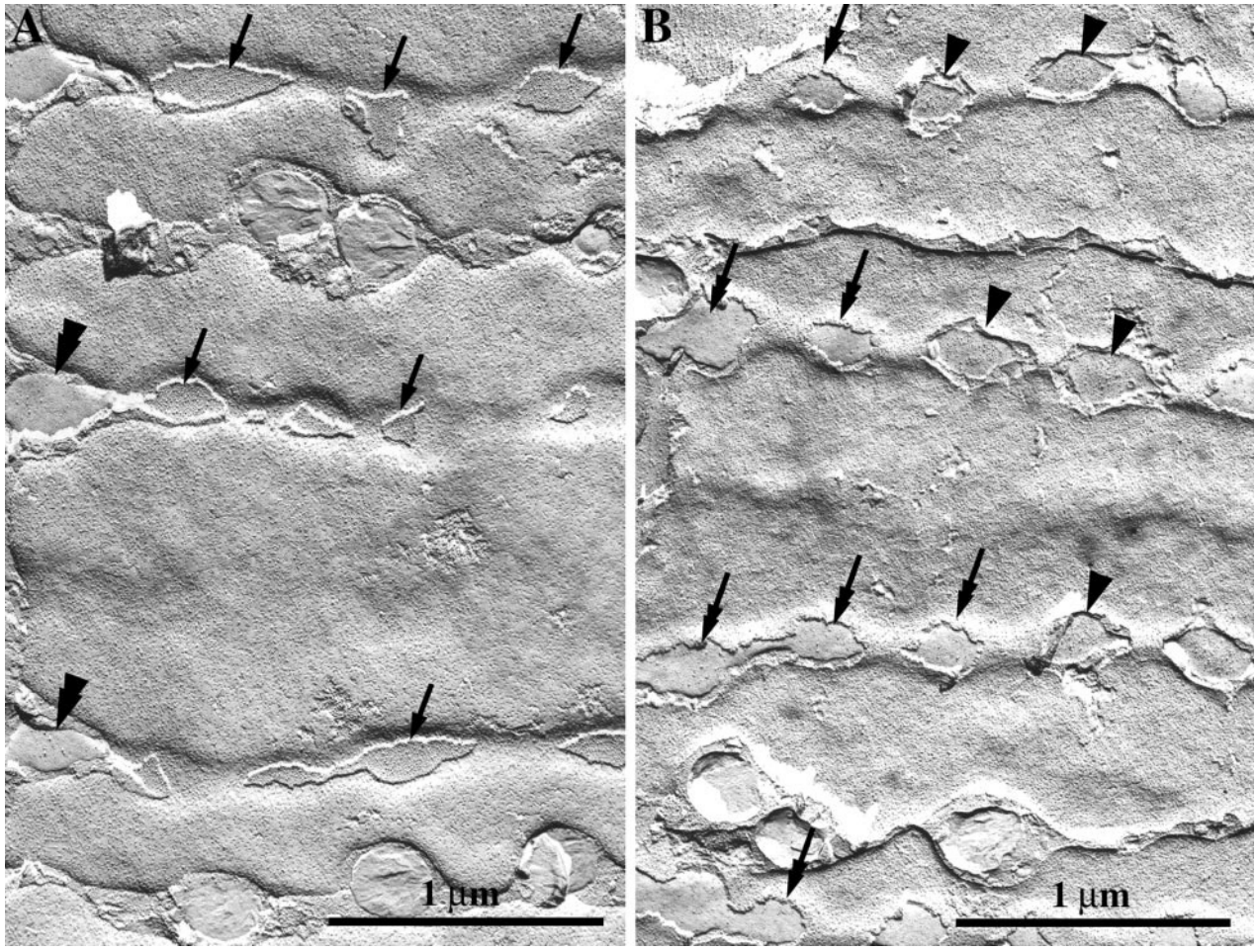

FIGURE 3 Freeze-fracture replica of T-tubules and mitochondria. Both images are entirely occupied by the fractured outer membranes of mitochondria and by T tubules running from left to right. The long axis of the fiber is vertical. (*A*) The viewer is looking at the mitochondrion from the myofibril side. Cytoplasmic leaflets of the free side of T tubules (*arrows*) and luminal leaflet of the junctional side (*double arrowhead*) are seen to lie above the mitochondrial surface. (*B*) The viewer is looking at the mitochondrial outer membrane from the mitochondrial interior. The cytoplasmic leaflet of jT membranes (*arrowheads*) and the luminal leaflets of the free T tubules (*double arrows*) are seen.

always accompanied by extensive views of fractured mitochondrial membranes, which offer excellent reference points for the identification of T tubule membrane leaflets (see also Smith and Aldrich, 1971). In Fig. 3, both images are filled by a view of the mitochondrial outer membrane, except where T tubules are present. In Fig. 3 *A* the profiles of T tubules lie above the grooves in the mitochondrial surface membrane and thus the viewer is looking down on the mitochondrial membrane from the myofibrils' side. In Fig. 3 *B* the T tubule profiles are seen through breaks of the mitochondrial membrane; that is, they are below the mitochondrial surface and thus the viewer is looking at the mitochondrial surface membrane from the inside of the mitochondrion and seeing the T tubules through breaks of the mitochondrial membrane (see below for further details). Note that in these muscles the fracture never splits the SR membrane at dyads.

A detailed interpretation of the paths followed by the fracture planes in proximity of the dyads in dragonfly muscle is given in Fig. 4, which is based on the actual images. Fig. 4 differs considerably from a previous model by Smith and Aldrich (1971), who interpreted some of the membrane views as representing true membrane surfaces. The past interpretation is unlikely to be correct, in view of the fact that wherever membranes are present the fracture plane preferentially follows their interior (Branton, 1966). In Fig. 4, two possible fracture paths are predicted, one following the free (fT, Fig. 4 *A*), the other the junctional (jT, Fig. 4 *B*) surface of T tubules. Four views of fractured T tubules leaflets are expected, two of them showing the cytoplasmic leaflets on the cytoplasmic side of the membrane (fTc and jTc, Fig. 4, *C* and *F*), and the other two showing the luminal leaflets (jTl and fTl, Fig. 4, *D* and *E*). Note that due to the curvature of the T tubule wall, all cytoplasmic leaflets will appear concave and all luminal leaflets convex. In Fig. 4, *C* and *D* the cytoplasmic leaflet of the free T tubule (fTc) and the luminal leaflet of the junctional T tubule (jTl) are seen from the point of view of an observer that is looking at the

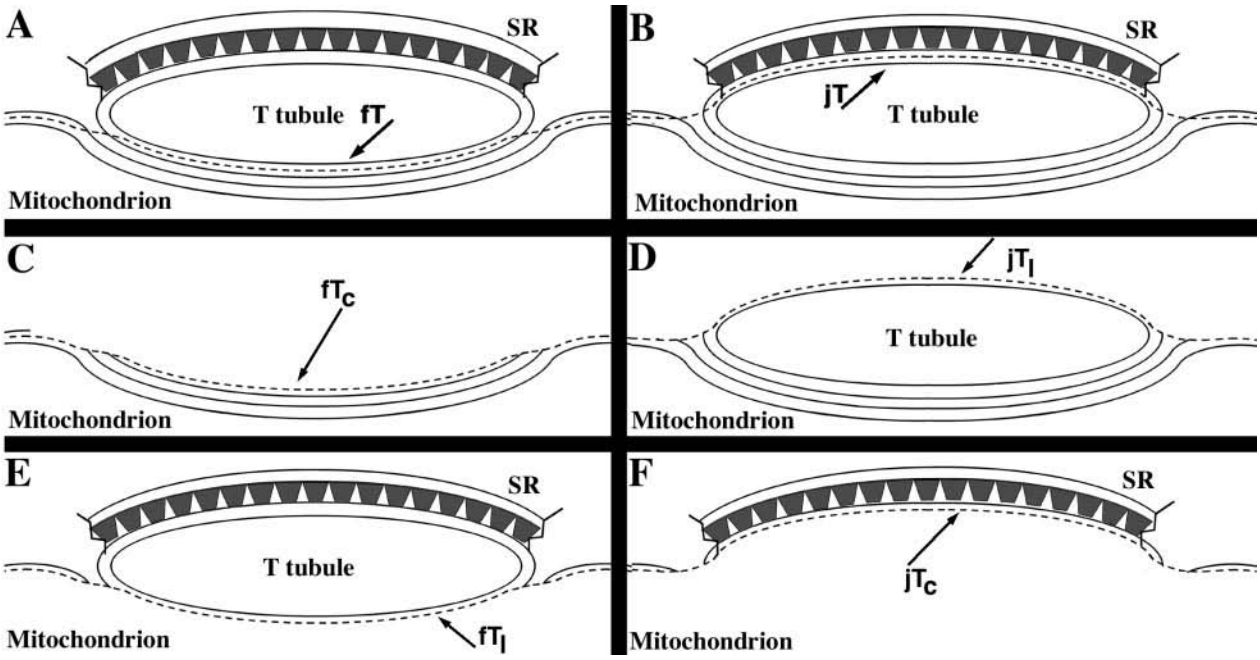

FIGURE 4 Sketch of the fracture planes and resulting images at the sites of dyads in the dragonfly flight muscle. The fracture plane tends to follow the flat areas of the external mitochondrial membrane and to jump into the T tubule membrane at the dyads. Two possible fractures are seen. In one (*A*) the fracture follows the free side of the T tubule, revealing the cytoplasmic (fTc in frame *C*) and luminal (fTl in frame *E*) leaflets. In the other type of fracture (*B*) the jT surface is split, revealing the cytoplasmic (jTc in frame *F*) and luminal (jTl in frame *D*) leaflets. The fracture very seldom splits the SR membrane at the dyads in this muscle. *C* and *D* correspond to the views in Fig. 3 *A*; *E* and *F* correspond to the views in Fig. 3 *B*.

fractured mitochondrial membrane from the cytoplasm. In these images the T tubule profiles appear to lie above the mitochondrial surface as in Fig. 3 *A*. In Fig. 4, *E* and *F*, the luminal leaflet of the free T tubule (fTl) and the cytoplasmic leaflet of the junctional T tubule (jTc) are seen as below the fractured mitochondrial membrane, observed by a viewer located inside the mitochondrion (as in Fig. 3 *B*). Details of the fractured leaflets are described below. Next to the dyads the fracture jumps into the mitochondrial membrane. In the other muscles used, geometrical clues for the distinction of junctional and free T tubule surfaces are less readily available, but the distinction can be readily made on the basis of intramembranous particle distribution (see below).

Freeze-fracture replicas: particle distribution in T tubules

Fig. 5, *A–C* from the dragonfly flight muscles show the cytoplasmic leaflets of free T tubule membranes in the dyad and between dyads (fTc, *arrows*; the wider segments are part of dyads); the cytoplasmic leaflets of junctional T tubule membranes (jTc, *arrowheads*); the luminal leaflets of free T tubule domains (fTl, *double arrows*); and the luminal leaflets of junctional T tubule membranes (jTl, *double arrowheads*).

Cytoplasmic leaflets of free T tubule surfaces, whether within dyads or between them, are decorated by a large number of intramembranous particles of variable sizes. The cytoplasmic leaflets of junctional surfaces constitute distinct domains, with overall fewer particles and an abundance of distinctive large and tall particles of a fairly uniform size. The large particles are clustered toward the center of the junctional domain, occupying a patch of membrane from which other particles are mostly, but not completely, excluded. In the larger inset of Fig. 5 *A*, the transition between the area occupied by large particles and the adjoining areas of the T tubule membrane is a strip with very few particles (*small arrows*). In other junctions the transition is less clear. In the dragonfly flight muscles the entire junctional T tubule profile is in contact with an array of feet (see Fig. 1 *A*), but the area occupied by large particles covers only a central portion of the surface. The number of particles (10–25 per junctional domain) is smaller than the number of feet associated with each junction (see calculations below). Luminal leaflets of free T tubules in the dyads (*double arrows*, Fig. 5 *C*) and between dyads have either very few or no particles. The luminal leaflet of the junctional T tubule (*double arrowheads*, Fig. 5 *C*) also has few particles, but some of the particles have a large diameter and pits may also be present. The latter are better visible in images from another insect shown below.

The apparent diameter of the large particles in the cytoplasmic leaflets of junctional domains, measured in a direction perpendicular to the direction of shadow, is 9.4 ± 1.7

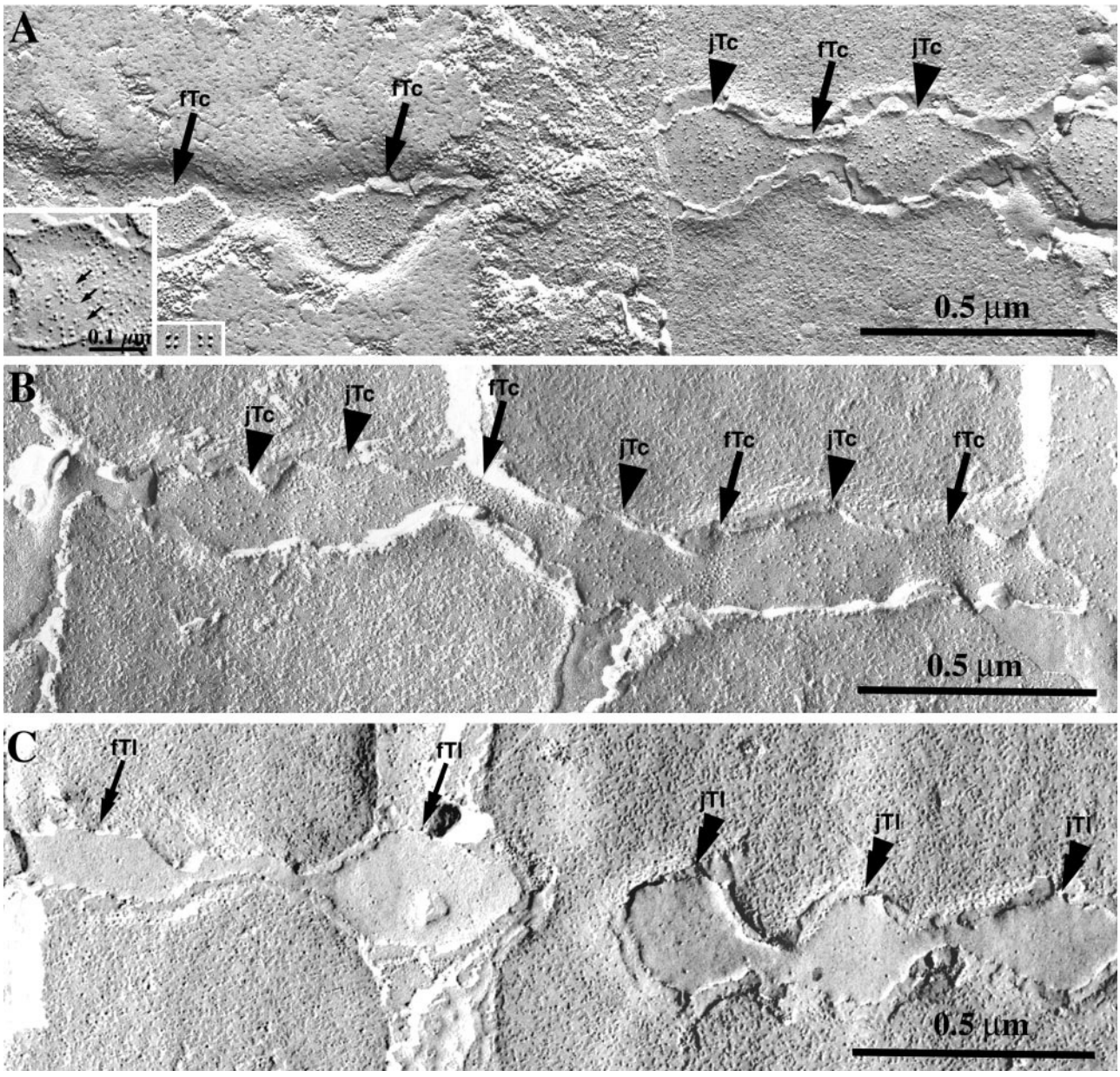

FIGURE 5 Particle distributions in the leaflets of dragonfly T tubules. *A* and *B* show the cytoplasmic leaflets of free T tubules (fTc, *arrows*) and of junctional T tubules (jTc, *arrowheads*); *C* shows the luminal leaflets of free T tubules (fTl, *double arrows*) and of jT tubules (jTl, *double arrowheads*). As shown in Fig. 1 *A* and in the diagram of Fig. 4, large mitochondrial surfaces are immediately adjacent to T tubules, and the two leaflets of mitochondrial outer membranes form the background in Fig. 5 (see Smith and Aldrich, 1971). Cytoplasmic leaflets of free T tubule domains in the dyads and of the short free T segments between the dyads (fTc) are occupied by particles of mostly small and medium size. The cytoplasmic leaflets of jT domains in the dyads (jTc, *arrows*) have a population of large particles, mostly located in groups near the center of the domain. The larger inset at left bottom in (*A*) shows that there is no order in the arrangement of the large particles in the jTc leaflet, and a clear transition (*small arrows*) between the area occupied by large particles and the rest of the T tubule. One group of four particles to the right of the asterisk in the large inset resembles a DHPR tetrad of skeletal muscle, but comparison with two tetrads from a skeletal muscle cell line (*adjacent inset*, from Protasi et al., 1997) shows that the spacing is not sufficiently regular. Closely spaced groups of three or four particles are quite rare in arthropod muscles (see text), indicating no link between surface channels and RyRs. Luminal domains of free and junctional T are mostly smooth, with very few particles (fTl, *double arrows*, and jTl, *double arrowheads*). The cytoplasmic leaflet of the jT domain has pits, located in the central region of the junctional domain, which represent the negative images of the large particles.

nm (mean ± SD, $n = 204$ particles from 28 junctional domains and 2 different freeze-fracture replicas). The average diameter of particles in nonjunctional domains of T tubules is considerably smaller: 5.6 ± 1.3 nm ($n = 100$ particles from 10 areas, 2 freeze-fractures). The difference is significant (Student's *t*-test, $p < 0.0001$).

The disposition of the large particles in the junctional domains is not related to that of the junctional feet (RyRs)

in two respects. First, if the particles were systematically associated with specific domains of the feet, then the particles would tend to be aligned along two orthogonal directions even if very few particles "decorated" each foot. This is not the case, as can be seen glancing along the EM images and rotating them in various directions. Second, if the particles were associated with at least three of the four subunits of feet, then they would form 3- and 4-particle tetrads, with particles disposed at the corners of small squares (see small inset in Fig. 5 *A*, showing tetrads from a skeletal muscle). Counts from 30 junctions shows that 4% of the particles are arranged in small groups of 3 particles resembling an incomplete tetrad. An approximately equal frequency was found in cells derived from dyspedic muscles that lack RyRs (Protasi et al., 2002), indicating that the few "apparent tetrads" are not due to a specific association with feet, but are simply due to random events.

T tubule structure in fly and scorpion body muscles is essentially the same as in dragonfly, with a clear distinction between the junctional and free domains of the T tubules. Fig. 6 *A* shows the general shape of T tubules in the fly TDT muscle, with the distinctive alternation of narrow, free T tubules and flat, junctional cisternae located on either side of the narrow tubule. Arrows and double arrows indicate cytoplasmic and luminal leaflets of the free T tubule (fTc and fTl); arrowheads and double arrowheads indicate cytoplasmic and luminal leaflets of the jT tubule (jTc and jTl).

JTc domains of fly TDT muscle (Fig. 6, *B* and *D*) contain large particles, as in the dragonfly, but with three differences. First, the particles are more frequent and are dispersed over the entire junctional surface; second, the particles partition differently, a relatively large proportion of the particles appearing in the luminal leaflet; third, particles of variable size are almost entirely excluded from the entire junctional domain. This makes the distinction between jT (Fig. 6 *B*, *double arrowhead*) and free T (Fig. 6 *B*, *double arrow*) segments more obvious than in the dragonfly. Small pits are present in the jT domains of the fly, both luminal and cytoplasmic, and no pits are present in the free domains of the T tubule membrane. Particles and pits of jTc and jTl have complementary dispositions: the particles are more frequent in the cytoplasmic leaflet, while the pits are more frequent in the luminal leaflet. The complementary disposition and the correspondence in location of particles and pits indicate that they derive from fractures of the same protein. This is confirmed by similar observations in muscles of vertebrates (Franzini-Armstrong, 1984). The scorpion muscle resembles the fly in that the large particles partition frequently into both luminal (Fig. 6 *D*, *double arrowhead*) and cytoplasmic (Fig. 6 *D*, *double arrow*) leaflets of the jT.

The total density of the protein responsible for the appearance of large particles and complementary pits in the fly muscle was estimated by counting the total number of particles-plus-pits in the junctional domains and referring to the surface area of the domain. The average density of particles is $459 \pm 100/\mu m^2$ (mean $\pm$ SD, $n = 13$ junctions). As in the case of the dragonfly, there is no order in the disposition of the particles and no tetrads are present.

The cytoplasmic leaflet of free T tubule has numerous particles of variable size (*arrows*, Fig. 6, *A* and *D*); the luminal leaflet is devoid of particles (*double arrow*, Fig. 6 *A*). Other membrane profiles in the figures belong to the SR and are dominated by the numerous particles of the Ca^{2+} pump protein.

DISCUSSION

The main finding of this work is that the junctional domains of T tubules in muscles from two insects and a scorpion (arachnida) are occupied by a distinct population of particles of relatively large diameter and prominent height. We propose that these particles represent the L-type Ca^{2+} channels that are involved in e-c coupling of arthropod muscles. This identification is supported by the similarity in size and location with the particles that decorate the junctional domains of surface membrane/T tubules in skeletal and cardiac muscle of vertebrates in vivo and in vitro (Block et al., 1988; Takekura et al., 1994; Sun et al., 1995; Protasi et al., 1996, 1997; Franzini-Armstrong et al., 1991; Franzini-Armstrong and Nunzi, 1983; Franzini-Armstrong and Kish, 1995). In body muscles from arthropods and in vertebrate striated muscles, the particles form a unique population with larger and more uniform diameter than the overall population of membrane particles, and they are located specifically in the T tubule domains that face the SR. The size of the particles is 9.4 nm in dragonfly, 8.5 nm in avian cardiac muscle (Sun et al., 1995), and 8.0 in skeletal muscle of the frog (Franzini-Armstrong, 1984). A similar population of large particles was previously observed in junctional domains of the plasmalemma from muscles of crustacea (Eastwood et al., 1982).

Identification of the unique population of large particles in junctional domains of the plasmalemma and T tubules with L-type channels (DHPRs) is now well accepted for skeletal and cardiac muscle, where it is based on multiple evidence. In skeletal muscle the particles were identified as DHPRs based on coincidental lack of e-c coupling and of the large particle clusters in dysgenic muscle lacking the $\alpha 1$ subunit of DHPRs (Rieger et al., 1987, Tanabe et al., 1988; Romey et al., 1986; Franzini-Armstrong et al., 1991) and on reconstitution of e-c coupling and large particle clusters by transfections with cDNA for the DHPR (Tanabe et al., 1988; Takekura et al., 1994). Cardiac muscle particles were later identified as DHPRs based on similarity in size to the skeletal DHPR particles (Sun et al., 1995; Protasi et al., 1997), in addition to the co-localization of RyR and DHPRs at the light microscope level and the proximity of particle patches to foot-bearing SR in electron micrographs (Carl et al., 1995; Sun et al., 1995; Protasi et al., 1997; Gathercole et

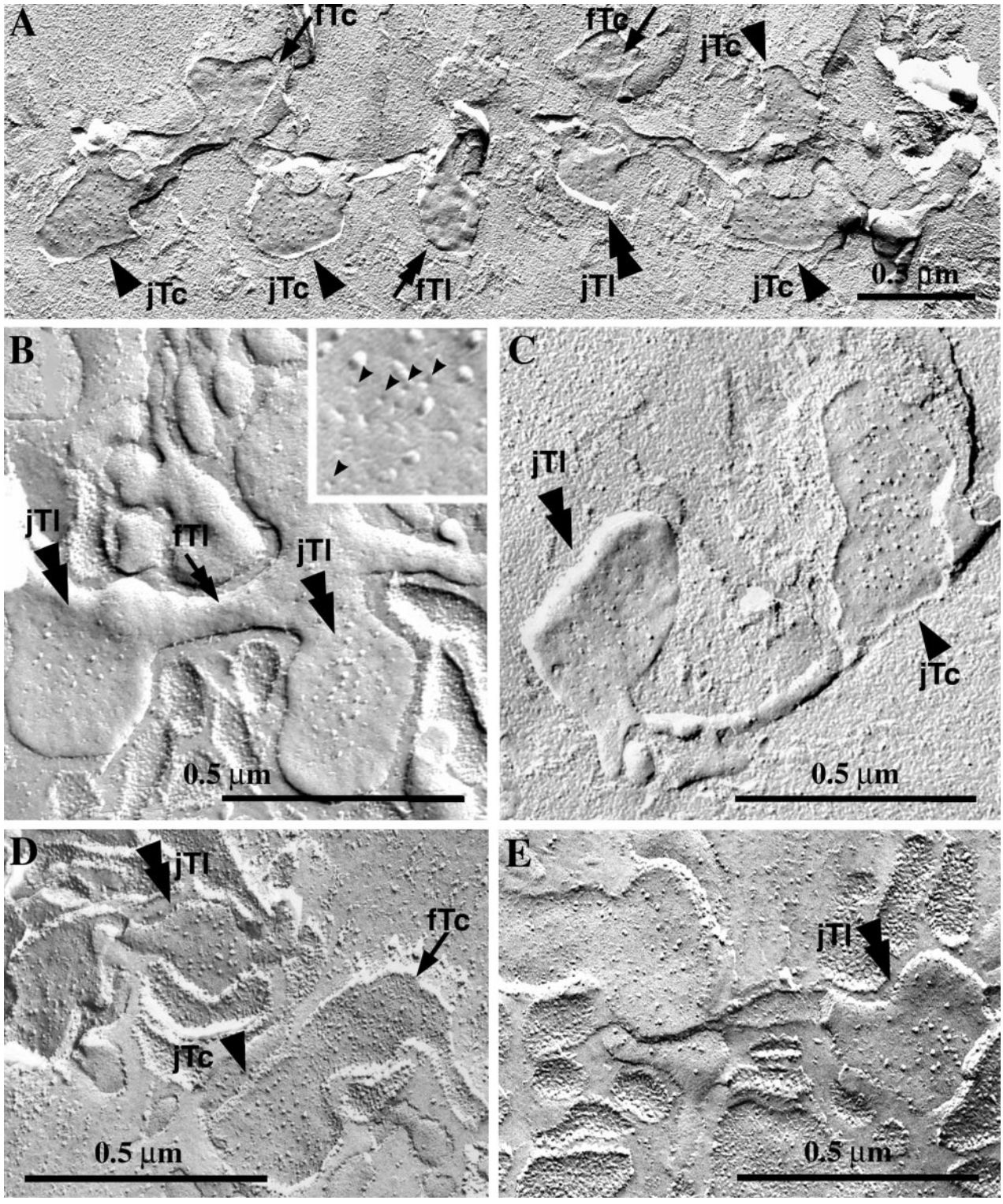

FIGURE 6 (*A*) T tubules of fly TDT muscle run transversely in the cell, and have extended flat cisternae that extend from two sides of the tubule and are components of the dyads. The cytoplasmic leaflets of free T (fTc, *arrows*) and jT (jTc, *arrowheads*) in the dyads and the luminal leaflets of free (fTl, *arrowhead*) and jT (jTl, *double arrowhead*) in the dyads are seen. (*B* and *C*) Both luminal (jTl, *double arrowhead*) and cytoplasmic (jTc, *arrowheads*) leaflets of jT domains have a mixture of large particles and pits. The latter are indicated by small arrowheads in the inset. Particles prevail in the cytoplasmic leaflets and pits are relatively more abundant in the luminal leaflet. Large particles and pits occupy the entire junctional membrane domain and are more abundant than in the dragonfly. Both particles and pits are separated by variable distances. (*D* and *E*) In scorpion muscle large, randomly grouped particles are present in both luminal (jTl, *double arrowhead*) and cytoplasmic (jTc, *arrowheads*) leaflets of jT domains. The cytoplasmic leaflet of free T tubules (fTc, *arrow*) has the usual complement of small variable particles.

al., 2000). Given the distinctive size and positioning of the DHPR particles in muscles of vertebrates, it is appropriate to use these two criteria as the basis for identification of the corresponding Ca^{2+} channels in invertebrate muscle. Our evidence shows that L-type Ca^{2+} channels of arthropod body muscles are located in close proximity to the SR feet. This location constitutes a previously missing crucial link in support of the hypothesis that Ca^{2+}-induced Ca^{2+} release is a possible mechanism of excitation-contraction coupling in these muscles (see below).

The disposition of Ca^{2+} channel-associated particles in arthropod muscles is similar to that in cardiac muscle, and both differ from that of skeletal muscle in one important detail. In cardiac myocytes and in arthropod body muscles Ca^{2+} channels are clustered in junctional domains of surface membrane facing the junctional gap and the arrays of feet (RyRs), but they have a disordered disposition within the clusters (Sun et al., 1995; Protasi et al., 1996). In skeletal muscle, however, DHPRs are also clustered in proximity to RyR, but within the clusters they are arranged in small orthogonal groups (tetrads). The tetrads form arrays complementary to the arrays of feet (Block et al., 1988; Takekura et al., 1994; Franzini-Armstrong and Kish, 1995; Protasi et al., 1996, 1997). The two different DHPR arrangements are interpreted to indicate a close link between DHPR and RyR in skeletal muscle and RyR-DHPR proximity, but either no link or a loose tethering in cardiac muscle. In a dyspedic muscle cell line lacking RyR1, DHPRs are clustered at sites of the SR-surface junction, but they do not form tetrads. Tetrads are restored after transfections with RyR1 cDNA (Protasi et al., 1998, 2000), clearly showing that the RyR1-DHPR link is necessary for the formation of tetrads and indirectly indicating that lack of tetrads is due to lack of such a link.

The structural similarity in Ca^{2+} channel arrangement of arthropod and vertebrate cardiac muscle finds a close correspondence in the channel properties and in the mechanism of e-c coupling of the two types of muscles. Ca^{2+} channels in vertebrate cardiac and arthropod body muscles have fast activation kinetics; Ca^{2+} currents are large, and e-c coupling requires extracellular Ca^{2+} (Zacharova and Zachar, 1967; Fabiato, 1983; Gilly and Scheuer, 1984; Mounier and Goblet, 1987; Tanabe et al., 1991; Bers, 1991; Gyorke and Palade, 1993, 1994). Because Ca^{2+} channels and RyRs are not structurally linked (as shown here), since e-c coupling appears to require Ca^{2+} permeation through the channel, it has been proposed that internal Ca^{2+} release in these two types of muscle is initiated by an indirect activation of the RyR via a Ca^{2+}-induced Ca^{2+} release mechanism. This requires proximity but not close association between Ca^{2+} channels and RyRs, in agreement with our findings for both cardiac and invertebrate muscles. In skeletal muscle, the critical II-III loop of the dihydropyridine receptor is responsible for bidirectional coupling with RyR1: its presence is necessary for activation of the DHPR Ca^{2+} current and for gating of the RyR by the DHPR (Grabner et al., 1999). The II-III loop of L-type Ca^{2+} channels in an insect (*Musca* sp.) does not restore bidirectional coupling, even when inserted into a vertebrate sequence and associated with RyR1 in dysgenic mouse myotubes (Wilkens et al., 2001). Thus the insect e-c coupling apparatus lacks critical components necessary for the direct Ca^{2+} channel-RyR functional coupling.

A few structural details are puzzling in this probably oversimplified view of Ca^{2+} movement events in arthropod muscles. One is the fact that scorpion muscles have such a large inward Ca^{2+} current that it is sufficient to initiate myofibril activation without need of a release from intracellular stores (Gilly and Scheuer, 1984). Yet scorpion muscle has a very well developed system of T tubules, SR, and CRUs with the full complement of RyRs apparently available for internal Ca^{2+} release. A second apparent puzzle is that the clusters of Ca^{2+} channels in the direct flight muscle of the dragonfly are quite small relative to the clusters in the fly TDT and in the scorpion muscles, despite the fact that the dragonfly muscle is a synchronous flight muscle, which requires rapid and repeated activation of Ca^{2+} release. Apparently, a small number of Ca^{2+} channels can, under the appropriate circumstances, rapidly activate a fairly large number of RyRs in this arthropod muscle.

Our results show that the e-c coupling apparatus of vertebrate myocardium and arthropod body muscles share structural characteristics in addition to the previously established functional similarities. In both cases the Ca^{2+} channels involved carry large currents; in both cases Ca^{2+} permeation through the channels is necessary for e-c coupling and in both cases surface membrane Ca^{2+} channel and the SR Ca^{2+} release channels are in close proximity to each other, but do not form a supramolecular complex. Thus the release of calcium from internal stores and an indirect way of communication between channels of the exterior membranes and of the internal stores is of ancient origin. Establishment of the specific DHPR-RyR link (Block et al., 1988) that allows cross-talk between the two molecules (Nakai et al., 1996) is a new evolutionary event that arises in parallel with the vertebrates.

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