Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle

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ABSTRACT During excitation-contraction (e-c) coupling of striated muscle, depolarization of the surface membrane is converted into Ca\(^{2+}\) release from internal stores. This process occurs at intracellular junctions characterized by a specialized composition and structural organization of membrane proteins. The coordinated arrangement of the two key junctional components—the dihydropyridine receptor (DHPR) in the surface membrane and the ryanodine receptor (RyR) in the sarcoplasmic reticulum—is essential for their normal, tissue-specific function in e-c coupling. The mechanisms involved in the formation of the junctions and a potential participation of DHPRs and RyRs in this process have been subject of intensive studies over the past 5 years. In this review we discuss recent advances in understanding the organization of these molecules in skeletal and cardiac muscle, as well as their concurrent and independent assembly during development of normal and mutant muscle. From this information we derive a model for the assembly of the junctions and the establishment of the precise structural relationship between DHPRs and RyRs that underlies their interaction in e-c coupling.

Ca\(^{2+}\) released from cytoplasmic stores in response to depolarization of the surface membrane activates the contraction of striated muscle. This process, called excitation-contraction (e-c) coupling, takes place at intracellular junctions between the plasma membrane or its invaginations, the T tubules (1); (ii) a charge movement across the membrane (2) corresponding to the voltage sensing of the dihydropyridine-sensitive L-type calcium channel (DHPR) (3, 4); and (iii) release of Ca\(^{2+}\) from the SR stores through the ryanodine-sensitive Ca\(^{2+}\) release channel (RyR) (5, 6). In both skeletal and cardiac muscle, activation of the SR Ca\(^{2+}\) release channel is under the tight control of the DHPRs, resulting in a rapid and efficient coupling of membrane depolarization and SR Ca\(^{2+}\) release (1, 7). However, the mechanism of e-c coupling differs in the two muscle types. In skeletal muscle, gating of the Ca\(^{2+}\) current through DHPRs is slow and apparently not required for triggering SR Ca\(^{2+}\) release, whereas in cardiac muscle activation of the L-current is fast and necessary for e-c coupling (8). Thus it has been proposed that the main function of the DHPR in skeletal muscle is that of a voltage sensor, which may control the opening of the RyR through direct molecular interactions (2). In cardiac muscle, on the other hand, the Ca\(^{2+}\) current through the DHPR seems to mediate the interaction between DHPRs and RyRs by a process called Ca\(^{2+}\)-induced Ca\(^{2+}\) release (9–11).

FUNCTION AND STRUCTURE OF TRIADS

Function. The signal transduction process occurring at the triad involves: (i) depolarization of the plasma membrane and its invaginations, the T tubules (1); (ii) a charge movement across the membrane (2) corresponding to the voltage sensing of the dihydropyridine-sensitive L-type calcium channel (DHPR) (3, 4); and (iii) release of Ca\(^{2+}\) from the SR stores through the ryanodine-sensitive Ca\(^{2+}\) release channel (RyR) (5, 6). In both skeletal and cardiac muscle, activation of the SR Ca\(^{2+}\) release channel is under the tight control of the DHPRs, resulting in a rapid and efficient coupling of membrane depolarization and SR Ca\(^{2+}\) release (1, 7). However, the mechanism of e-c coupling differs in the two muscle types. In skeletal muscle, gating of the Ca\(^{2+}\) current through DHPRs is slow and apparently not required for triggering SR Ca\(^{2+}\) release, whereas in cardiac muscle activation of the L-current is fast and necessary for e-c coupling (8). Thus it has been proposed that the main function of the DHPR in skeletal muscle is that of a voltage sensor, which may control the opening of the RyR through direct molecular interactions (2). In cardiac muscle, on the other hand, the Ca\(^{2+}\) current through the DHPR seems to mediate the interaction between DHPRs and RyRs by a process called Ca\(^{2+}\)-induced Ca\(^{2+}\) release (9–11).

Structure. The structural organization of DHPRs and RyRs in the junctions of skeletal and cardiac muscle reflects both similarities and differences in the e-c coupling mechanisms of the two muscles (Fig. 1). The most prominent structural feature of all junctions are the “feet,” which are electron-dense structures periodically spanning the narrow junctional gap that separates the membranes of SR and either T tubules or the plasma membrane (12). In rotary shadowed preparations of isolated SR vesicles, an individual foot appears as four equal spheres forming a semicrystalline array in the plane of the membrane (13). The four spheres correspond to the cytoplasmic domains of the homotrameric RyRs (6). High-resolution image reconstruction of the RyR reveals the four-fold symmetry of the channel-forming transmembrane domains and of the cytoplasmic domains (14–16). The latter has a complex system of pores and cavities, which presumably serve to direct the efflux of Ca\(^{2+}\) into the junctional gap. Despite sequence differences, skeletal and cardiac RyRs share the same structural characteristics and a position in the junctional gap, which allows the close interaction with components of the junctional surface membrane (17).

Abbreviations: e-c, excitation-contraction; T, transverse; SR, sarcoplasmic reticulum; DHPR, dihydropyridine receptor; RyR, ryanodine receptor.

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mechanism gated by the Ca\(^{2+}\) released by the coupled channels. However, the steep dependence of SR Ca\(^{2+}\) release on surface membrane voltage in skeletal muscle does not necessarily need such an amplification (25).

In conclusion, the structural and functional properties of the Ca\(^{2+}\) release apparatus in skeletal and cardiac muscle fit nicely into the current hypotheses of cardiac and skeletal e-c coupling. Verification of these hypotheses, however, requires the identification of the molecular interactions of DHPR and RyR that underlie their coordinated spatial organization in the junctions.

**Molecular Bases of DHPR–RyR Interactions.** The bond between surface membranes and SR is strong enough that the junctions survive the strains of muscle contraction as well as the stringent fractionation procedures used in the isolation of triads (26). Even after forced dissociation, junctional T tubule and SR vesicles are capable of reassociating with one another. Yet the nature of the bond between SR and surface membrane is not known. Attempts to show direct binding between isolated DHPRs and RyRs have been unsuccessful (27–29), and the proteins fail to induce formation of junctions between endoplasmic reticulum and plasma membrane when coexpressed in nonmuscle cells (30). However, the cytoplasmic loop between repeats II and III of the α1 subunit of the DHPR is a critical determinant of the specific mode of DHPR–RyR interactions in skeletal and cardiac e-c coupling. When chimeras of the skeletal and cardiac isoform of the α1 subunit were expressed in dygenic myotubes, e-c coupling was restored, and the coupling mode was determined solely by the origin (skeletal or cardiac) of the II–III loop (31, 32). The importance of the II–III loop of the DHPRs is emphasized by the recent observations that peptides from this portion of the molecule affect the properties of the reconstituted channels (33) and influence Ca\(^{2+}\) release from SR vesicles (34). Interestingly, not only the peptide derived from the skeletal DHPR but also that of cardiac origin interacted with the skeletal RyR, while neither had an effect on the cardiac isoform. This was unexpected, considering the results of the above mentioned expression study with skeletal–cardiac chimera. It suggests that the structural difference in the association of feet and DHPR particles (23) and the functional difference in the properties of e-c coupling in skeletal and cardiac muscle are at least partially determined by the RyR isoform. However, the interactions between DHPRs and RyRs in skeletal muscle need not be unidirectional. Recent evidence from a RyR-deficient skeletal muscle cell line indicates that the lack of the RyR reduces the current density but not the charge movement associated with the DHPR. Thus, the DHPR–RyR interactions may also be essential to DHPRs’ function as Ca\(^{2+}\) channels (35).

Since the affinity of DHPRs for RyRs is low, the ability of the two proteins to remain associated with each other in skeletal muscle triads may reflect the concerted action of numerous DHPR/RyR pairs. Alternatively, interactions between the two channels may depend on the presence of an intermediate protein (36). A 95-kDa protein (triadin), showing affinity for both DHPRs and RyRs, has been isolated from the junctional SR (37). Triadin appears simultaneously with DHPRs and RyRs during development and is expressed in skeletal and cardiac muscle (38–41). Thus, triadin is a possible candidate for linking the two major junctional proteins. However, structural predictions from the amino acid sequence suggest that triadin may be mostly positioned on the luminal and not the junctional side of the SR membrane (40). Further analysis of this protein is needed to define triadin’s role in the junction.

**DEVELOPMENT OF TRIADS**

In light of the close proximity of DHPRs and RyRs and of their functional interaction, it has been assumed that these two components also play a major role in the formation and maintenance of the junctions. This idea received support from
Targeting of DHPRs and RyRs. In this section we consider two questions: how do RyRs and DHPRs reach the junction, and how are the two proteins targeted to peripheral versus internal junctions? Fig. 2 illustrates two possible pathways by which RyRs and DHPRs could be transported from their site of synthesis in the rough endoplasmic reticulum to the junctions, either independently or jointly. According to the first hypothesis, RyRs could diffuse from the rough endoplasmic reticulum directly into the SR; in developing muscle, the two membranes are indeed continuous with each other. DHPRs could follow the exocytic route through the Golgi apparatus and be inserted into the surface membrane by constitutive fusion of exocytic vesicles. In this scheme, DHPRs and RyRs would reach the junctions independently of each other. An alternate hypothesis of junction formation has been proposed based on the distribution of DHPRs, RyRs, and a T tubule antigen (TS28) in developing rabbit muscle (42). Colocalized clusters of RyRs and DHPRs were observed either at the fiber periphery or in proximity of TS28-labeled DHPRs. These represent peripheral couplings and triads that are known to coexist in developing myotubes and muscle fibers. However, DHPR/RyR clusters were also noted at some distance from the plasma membrane and from TS28-positive membranes. This has been interpreted to indicate the formation of junctions between Golgi-derived DHPR- and RyR-bearing vesicles before their fusion with the SR and either plasma membrane or T tubules. Consequently, fusion of the preformed junctions with the plasma membrane or T tubules and with the SR had to occur in a highly coordinated manner. Several observations are inconsistent with this hypothesis. First, using similar antibody combinations in myotubes developing in culture, internal DHPR/RyR clusters were never found independent of a general marker for the T-system, but they were expressed simultaneously with or after the formation of T tubules (46, 47). Second, a model in which preformed junctions are inserted into the two membrane systems is inconsistent with observations of junctions without feet in normal and mutated muscle (see below), unless two independent mechanisms of membrane attachment exist—one for the DHPR as RyR vesicles and another for the undifferentiated membrane systems. Third, a quantitative study of junction formation in cardiac muscle (44) supports a mechanism of continuous growth of junctional area occupied by DHPRs and RyRs rather than a mechanism whereby quantal growth of junctions would be expected.

An interesting observation related to the targeting of DHPRs is the successive location of this protein first in junctional domains of the plasma membrane and later during development in junctional T tubules (42, 48, 49). Few skeletal muscles make do without T tubules and simply use peripheral junctions between SR and plasma membrane for e-c coupling. Those muscles that do have T tubules temporarily establish a peripheral Ca\(^{2+}\) release system during early development, which is morphologically, physiologically, and molecularly equivalent to the internal triads (42, 43, 49, 50). However, as soon as a T-system develops, internal junctions between it and the SR are formed and peripheral junctions are eliminated (49). Thus most muscles must have a secondary targeting mechanism, which is activated in parallel to T tubule development and is responsible for the location of DHPR clusters in the T tubules and not in the sarcolemma.

Regardless of whether RyRs and DHPRs find their way to the junctions independently of each other or in associated clusters, the basic questions of how DHPRs and RyRs associate with one another, how the initial SR-surface/T tubule junctions are formed during development, and how junctions are targeted to T tubules are still largely unanswered. Some initial information derived from mutant model systems is discussed below.
**Triad Formation Without DHPRs.** In the absence of compelling evidence for a direct binding of isolated DHPRs and RyRs, investigations have concentrated on the potential contributions of the individual channels to triad formation. Valuable information was obtained from the study of muscular dysgenesis, which is a recessive lethal mutation with a deficiency in skeletal muscle e-c coupling (4, 51). Myotubes from the homozygous embryos lack the DHP-sensitive, L-type Ca\(^{2+}\) currents (4, 52) and the charge movement associated with e-c coupling (4). These properties are restored by transfection with a plasmid carrying the cDNA for the \(\alpha_1\) subunit of skeletal DHPR (53, 54). The primary defect of muscular dysgenesis has been traced to a single point mutation, resulting in a shift in the reading frame and an unstable message for the \(\alpha_1\) subunit (55). The other subunits of DHPRs (\(\alpha_2, \beta, \gamma\) and \(\gamma\)) are expressed (56), but \(\alpha_2\) is not targeted correctly to the T tubules (47), suggesting that the \(\alpha_1\) subunit is required for the normal incorporation of \(\alpha_2\) into junctional membrane domains.

But how does the lack of the DHPR \(\alpha\) subunits affect the formation of junctions with the SR and the normal organization of RyRs within these junctions? T tubules and SR develop normally in dysgenic myotubes in culture (57), but triads with visible feet seemed to be lacking (58, 59). Immunocytochemistry showed failure of RyRs and triadin to cluster (39). The normal coclustered organization of DHPRs, RyRs, and triadin could be restored by de novo expression of the \(\alpha_1\) subunit in segments of dysgenic myotubes rescued by fusion with normal rat fibroblasts. These findings could have been interpreted in support of a requirement of DHPRs in the clustering of RyRs at junctions, had not a small number of dysgenic myotubes been observed that formed normally distributed RyR clusters in the absence of skeletal \(\alpha_1\) subunits. The ability of RyRs to cluster and form junctions in the absence of DHPRs was further confirmed in vivo, by the observation that triads with well-ordered arrays of feet are present in the diaphragm of dysgenic mouse embryos, a muscle that develops fairly well despite the lack of e-c coupling (21). Interestingly, colocalization of triadin and RyR in vitro was restricted to those few myotubes where RyRs were clustered. The distribution pattern of triadin was different from that of the RyRs wherever the latter were diffusely distributed.

These findings suggest an interdependence of RyRs and triadin in the junction. On the other hand, clustering of RyRs in ordered arrays, association of triadin with them, and the formation of junctions between SR and T tubules seem to be independent of the presence of skeletal type DHPRs. This, however, did not entirely rule out the possibility that a different \(\alpha_1\) DHPR isoform, which is not recognized by the skeletal isoform-specific antibody, is responsible for junction formation in dysgenic myotubes. Studies demonstrating transient expression of the cardiac isoform during myogenesis (60, 61) and recordings of DHP-sensitive Ca\(^{2+}\) currents with cardiac characteristics from dysgenic myotubes substantiated these concerns (62). The question could be addressed in a new cell line of dysgenic muscle origin that formed RyR clusters at high frequency but still did not contract (63). These RyR clusters corresponded to T tubule-SR junctions with ordered arrays of feet as seen in electron microscopy, but did not contain \(\alpha_1\) and \(\alpha_2\) subunits of the DHPR. The absence of the \(\alpha_2\) subunit, which associates with all known \(\alpha_1\) isoforms, makes it rather unlikely that another \(\alpha_1\) subunit substituted for the missing skeletal isoform’s role in junction formation but not in e-c coupling. Thus, accumulating evidence indicates that despite the close structural association of DHPRs and RyR in skeletal and cardiac junctions, the DHPR is not needed for clustering of RyRs or for junction formation.

**Formation of Ordered Arrays Is an Inherent Property of RyRs.** The tendency of RyRs to form ordered arrays in junctions and the apparent absence of RyR aggregates outside of junctions in vertebrate skeletal muscle raise the question whether the characteristic disposition of RyRs is dependent on other functional junctional components. In cardiac muscle and in some skeletal muscles of invertebrates, ordered arrays of feet are seen on SR surfaces that are not associated with T tubules or the plasma membrane (64, 65). Immunocytochemistry confirmed that feet in these regions called extended or corbular SR actually represent RyRs (41, 66, 67). Thus the formation of a junction may facilitate the organization of RyRs at these sites but is not needed for the formation of feet arrays. Even more so, it appears that muscle-specific components of the SR are not required at all for the regular organization of RyR. RyRs expressed in Chinese hamster ovary cells become correctly inserted into the endoplasmic reticulum. Their feet face the cytoplasm and form extensive arrays with the same periodicity as in skeletal muscle (30). Finally, even purified RyRs possess the potential for self-assembly into arrays, very much like those in muscle, despite the absence of other proteins (T. Lai, personal communication). Thus the organization of RyRs into ordered arrays can occur independently from interactions with other components of either the SR or the surface membrane and probably represents an intrinsic property of the protein. The array is most likely formed by interactions of the cytoplasmic domains of RyRs (the feet) with each other (18). Some external factors, however, may be needed to specify the location of these arrays in the skeletal muscle junctions and at specific sites in the sarcomeres of cardiac and invertebrate muscles or else to prohibit arrays from forming in large portions of non-junctional SR.

**Triad Formation Without RyRs.** While interactions between RyRs and DHPRs are apparently not required for junction formation between SR and surface membrane, binding of RyRs with an unknown component of the surface membrane could still be involved in the docking of the two membrane compartments. This question was explored in developing muscle fibers from a transgenic mouse with a targeted disruption of the skeletal muscle RyR gene (68). The phenotype of this knock-out mouse is very much like that of the dysgenic mutant. Skeletal muscle fibers initially develop normally, but remain paralyzed due to a defect of e-c coupling. During later embryonic development, this caused retardation of muscle development and the mice died at birth due to respiratory failure. Northern blot analysis verified that the message of the skeletal muscle RyR gene is responsible for depolarization-induced Ca\(^{2+}\) release. However, myotubes from the RyR knock-out mouse still responded weakly to caffeine with a release of Ca\(^{2+}\) from the intracellular stores, indicating the presence of small numbers of RyRs, later shown to be of the neuronal type (69).

How does the lack of skeletal RyR affect the ability of the muscle cell to form junctions between SR and plasma membrane or T tubules, and how does it affect the organization of the DHPRs within the junctional domains? Surprisingly, triads and peripheral couplings formed in the RyR knockout mouse even though the junctions did not contain feet (70). For this phenotype, the RyR mutant was named “dyspedic” mouse. The failure of e-c-coupling in dyspedic mice and the lack of feet in their SR/surface junctions confirms that the disrupted skeletal muscle RyR gene is responsible for depolarization-induced Ca\(^{2+}\) release and that it actually gives rise to the feet. However, feet are apparently not required for the docking of the SR to the T tubule membrane. The width of the junctional gap in dyspedic muscle is irregular and narrower than in normal controls, indicating that in normal junctions, the feet determine the distance between SR and surface membrane and therefore confirming that the feet span the whole distance of the junctional gap.

Whereas RyRs are not required for the formation of the junction, they may be involved in the specific organization of the DHPRs in the junctions. Dyspedic muscle in vivo expresses DHPRs, although at a level much lower than that of normal.
fibers (69). In these muscles, the large intramembrane particles representative of DHPRs were neither found in tetrads, as in normal skeletal muscle, nor randomly clustered in the junctional domain, as in cardiac muscle (70). In contrast, primary myotubes derived from a second dyspedic knockout revealed L-type current densities similar to those of normal myotubes (35) and in a cell line derived from the same mice the DHPR particles were clustered in the junctional domains of the plasma membrane. However, peripheral coupling of these cells still lacked feet and the DHPRs were not organized in tetrads (F. Protasi, P. D. Allen, and C.F.-A., unpublished observations). Thus, DHPRs apparently require the presence of RyR arrays for their own arrangement into tetrads. On the other hand, clustering of DHPRs at the junctions occurs in the absence of RyRs, but may be dependent on expression levels. The random disposition of DHPRs in the junctional domains of cardiac muscle confirms that the DHPRs are capable of aggregating in the junctions despite the lack of a close spatial relationship with the RyRs.

Since the formation of SR-surface membrane junctions involves more than DHPRs and RyRs, we need to look for other specific components of the junctions. In this context it is worth mentioning that during development, the feet appear coordinated with other ultrastructural characteristics of the junction. The electron-dense content of the terminal SR cisternae, presumably calsequestrin, follows closely the differentiation of the feet in the junction. Also, the T tubules frequently show some electron-dense content, most likely components of the extracellular matrix, specifically in the junctional area.

Thus, the mechanism of junction formation may involve transmembrane complexes in the SR and in the surface membrane. Together they make up an extensive junctional complex including extracellular matrix, integral membrane proteins of the surface membrane and the SR, cytoplasmic components between the membrane compartments, and luminal SR proteins. Each of these may contribute in some way to the formation of the functional junction.

**Docking: An Initial Step in Junction Formation.** The capability of junctions to form in the absence of either DHPRs or RyRs, and perhaps also in the absence of both, suggests that during normal development, docking of SR to the surface membrane precedes the clustering of feet and their association with the DHPR particles. This is supported by reports of peripheral couplings entirely or partially without feet in normal developing muscle (45, 71) and of similar SR-surface junctions in a myogenic nonfusing cell line (BC3H1) before expression of DHPRs and RyRs (72). Closer analysis of the BC3H1 cells revealed that cultures with clustered and colocalized DHPRs and RyRs contained junctions that were only partially occupied by feet as well as plasma membrane patches that were only partially occupied by tetrads (44). Further evidence for a gradual accumulation of RyR in newly formed junctions comes from the ultrastructural analysis of developing avian cardiac muscle (23). In these muscle fibers, junctions without feet appeared first, and their frequency decreased in parallel, with the gradual increase in junctions that were either partially or totally occupied by feet. Furthermore, the surface areas of junctional domains occupied by feet and by DHPRs increased precisely in parallel to each other, indicative of a coordinated accrual of the two proteins in the developing junctions.

**A HYPOTHESIS OF TRIAD FORMATION**

Taken together, the above information allows the proposal of a new model for the formation and differentiation of triads and related junctions, with three discrete steps (Fig. 3): (i) the docking of a patch of SR to a corresponding patch of surface membrane; (ii) the accumulation of RyRs in the junction and their organization into arrays; and (iii) the accumulation of DHPRs in the junctional domain of the surface membrane. Skeletal muscle DHPRs associate with RyRs forming tetrads, while cardiac muscle DHPRs become aggregated opposite the RyR arrays but form only loose and irregular associations with the RyRs. The three steps are to be understood as a hierarchy of events, where docking determines the site of RyR aggregation, and the RyR arrays cause the accumulation and tissue-specific organization of the DHPRs. Whereas docking and the incorporation of the channels can be temporarily dissociated, the initial aggregation of RyRs and DHPRs occurs virtually simultaneously. Further gradual accretion of RyRs, closely followed by the DHPRs, occurs as the junction matures.

This sequential mode of triad formation allows predictions about the molecular mechanisms involved in the individual processes. Future investigation will have to identify the specific docking proteins of T tubules/plasma membranes and of the SR, the junctional components that induce aggregation of RyRs at this location, and of course, the molecular bases of the DHPR–RyR interactions that seem to be responsible for the aggregation of DHPRs in the junctional surface membrane and their organization into tetrads in skeletal muscle. The alternating association of feet with tetrads still remains to be a puzzle. The overall size of tetrads is apparently slightly larger than that of the feet; thus steric hindrance may prevent association of a tetrad with every foot. However, steric hindrance alone does not explain the regular alternating association of DHPRs and RyRs. Solving this developmental question may well hold key answers to the problem of the signaling mechanisms in skeletal muscle e-c coupling.
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