1. ABSTRACT

Excitation-contraction (e-c) coupling in muscle cells is a mechanism that allows transduction of exterior-membrane depolarization in Ca\(^{2+}\) release from the Sarcoplasmic Reticulum (SR). The communication between external and internal membranes is possible thanks to the interaction between Dihydropyridine Receptors (DHPRs), voltage-gated Ca\(^{2+}\) channels located in exterior membranes, and Ryanodine Receptors (RyRs), the Ca\(^{2+}\) release channels of the SR. In both skeletal and cardiac muscle cells, the key structural element allowing DHPRs and RyRs to interact with each other is their vicinity. However, the signal that the two molecules use to communicate is different in the two muscle types. In the heart, the inward flux of Ca\(^{2+}\) through DHPRs, that follows depolarization, triggers Ca\(^{2+}\) release from the SR stores. This mechanism is better known as calcium induced calcium release (CICR). Conversely, skeletal fibers do not need extracellular Ca\(^{2+}\) and the signal transduction relies on a mechanical interaction between the two membranes.

The differences between skeletal and cardiac e-c coupling have inspired a series of morphological studies on CRUs to test whether or not there are structural diversities that may explain how and why junctions use different mechanisms in the two muscle types.

2. INTRODUCTION

Muscle fibers are able to finely control cytoplasmic [Ca\(^{2+}\)] to an extremely well organized system of tubules and vesicles that are collectively named Sarcoplasmic Reticulum (SR). The SR is a highly specialized version of the endoplasmic reticulum that closely surrounds myofibrils and that sequesters and releases Ca\(^{2+}\). The signal that activates muscle contraction is the sudden increase in intracellular [Ca\(^{2+}\)] that follows the depolarization of exterior membranes (sarcolemma/T-tubules). This mechanism is named excitation-contraction (e-c) coupling and takes place at calcium release units (CRUs) or junctions, structures in which the SR and exterior membranes are closely associated with one another. CRUs are found in both cardiac and skeletal cells and, while they are structurally quite similar in the two muscle types, they use two different mechanisms to accomplish the same goal: the translation of an electrical signal carried by exterior membranes into Ca\(^{2+}\) release from the SR. In the heart, e-c coupling depends on the inward flux of Ca\(^{2+}\) through DHPRs, which triggers Ca\(^{2+}\) release from the SR stores. This mechanism is better known as calcium induced calcium release or CICR. Conversely, skeletal fibers do not need extracellular Ca\(^{2+}\) and the signal transduction relies on a mechanical interaction between the two membranes.
Figure 1. Different types of Calcium Release Units in muscle cells. CRUs, or junctions, are formed by the close apposition of SR terminal cisternae and exterior membranes. They are called triads, dyads, and peripheral couplings depending on the number and nature of the elements that constitutes them. A) Triads are formed by one T-tubule flanked by two SR cisternae (from adult toadfish swimbladder muscle). B and C) Peripheral couplings and dyads are formed by only two elements: one SR vesicle and respectively the surface membrane or a T-tubule (B, peripheral coupling in a BC3H1 cell; C, dyad in canine heart). The evenly spaced densities pointed by arrows in the junctional gap between the two membranes have been named feet by Clara Franzini-Armstrong and have been identified with the cytoplasmic domain of RyRs, the Ca\(^{2+}\) release channel of the SR. Bar, 0.1 \(\mu\)m (EM courtesy of Clara Franzini-Armstrong).

different names (triads, dyads, and peripheral couplings) depending on the number of elements and the nature of the membranes that constitute them. Triads are formed by three elements, two SR terminal cisternae and one T-tubule (Figure 1 A), while dyads and peripheral couplings are formed by only two elements, one junctional SR and respectively a T-tubule or the surface membrane itself (Figure 1, B and C) (2). Whereas the different kinds of CRUs carry out the same function, it is possible to make a distinction on where and when they can be found. Triads are practically the only kind of junction present in adult skeletal muscle fibers, whereas dyads and peripheral couplings are the predominant type of CRUs in developing muscle and in the heart (6-7). Striated muscles of invertebrates have all three types of junctions, but not necessarily in the same fibers. A different type of CRU has been also described in literature: the extended junctional SR or Corbular SR, found in the interior of cardiac cells, and free of any association with external membranes (8). Corbular SR contains RyRs but not DHPRs and, therefore, will not be further discussed in this review.

3.2. RyRs and DHPRs: the two major players of e-c coupling

One of the first structures described as an integral component of triads are the feet, large electron-dense structures that bridge the narrow gap (about 12nm) separating the SR from the T-tubule/sarcolemma (Figure 1, arrows) (9). Feet were later identified as the cytoplasmic domains of RyRs and RyRs in turn were identified as the Ca\(^{2+}\) release channels of the SR (10). The hydrophobic domain of RyRs (channel region) is inserted in the SR membrane, leaving the large hydrophilic portion (foot region) in the cytoplasm. In electron micrographs (EM) feet appear as evenly spaced densities in both skeletal and cardiac fibers (Figures 1 to 3). Formation of ordered arrays seems to be an intrinsic property of RyRs since arrays are also formed when the protein is expressed in non-muscle cells and under \textit{in vitro} conditions (11). Another extremely important component of CRUs is the dihydropyridine receptors (DHPRs), an L-type Ca\(^{2+}\) channel that plays a central role in triggering SR Ca\(^{2+}\) release (12). DHPRs are specifically localized in areas of exterior membranes that
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Figure 2. Structure of Calcium Release Units in adult skeletal muscle fibers. In adult skeletal muscle, junctions are mostly triads: two SR elements coupled to a central T-tubule. A) A triad from the toadfish swimbladder muscle in thin section EM: the cytoplasmic domains of RyRs, or feet, and calsequestrin are well visible. B) A tri-dimensional reconstruction of a skeletal muscle triad showing the ultrastructural localization of RyRs, DHPRs, Calsequestrin, Triadin, Junctin, and Ca\(^{2+}/Mg^{2+}\) ATPases. Note the localization of DHPRs in the T-tubule membrane: DHPRs are intramembrane proteins that are not visible in thin section EM but can be visualized by freeze fracture replicas of T tubules (see panel C). C) DHPRs in skeletal muscle DHPRs form tetrads, group of four receptors (see enlarged detail), that are linked to subunit of alternate RyRs (see models in B and E). D) In sections parallel to the junctional plane, feet arrays are clearly visible (toadfish swimbladder muscle): feet touch each other close to the corner of the molecule (see enlarged detail). E) Model that summarizes finding of panels C and D: RyRs form two (rarely three) rows and DHPRs form tetrads that are associated with alternate RyRs (RyRs in blue; DHPRs in purple; T-tubule in green). (EM courtesy of Clara Franzini-Armstrong; 3D reconstruction of RyRs courtesy of T. Wagenknecht).

face junctional arrays of feet in both skeletal and cardiac muscle fibers (13, 14). DHPRs are not as visible as RyRs in thin sections because they have smaller hydrophilic domains. However DHPRs can be visualized in freeze-fracture replicas, a technique that allows separation of the two membrane leaflets, exposing intra-membrane domains of proteins. DHPRs appear as large particles clustered in correspondence of CRUs when visualized by freeze-fracture (Figures 2 to 5).

Despite the fact that many other proteins are involved structurally and functionally in e-c coupling (i.e. calsequestrin, triadin, junctin, FKBP12, mitsugumin, junctophilin, etc.), RyRs and DHPRs are still recognized as the two key elements of the mechanism. In both cardiac and skeletal cells the key feature that allows the two proteins to interact with each efficiently is their relative vicinity. DHPRs are always located in areas of exterior membranes that face RyR arrays of the SR, ideally placed to finely and promptly control the activation of Ca\(^{2+}\) release and start muscle contraction (Figure 2, 3, and 5).

4. DIFFERENCES IN DHPR/RYR STRUCTURAL ASSEMBLY BETWEEN SKELETAL AND CARDIAC FIBERS

4.1. Different isoforms of DHPR and RyR are expressed in the two muscle types

We have seen in the previous section how RyRs and DHPRs are localized in CRUs in both skeletal and cardiac muscle. However, a distinction needs to be made: the two muscle types express different isoforms of the two proteins (Figures 2, 3, and 5). In skeletal muscle fibers two different RyR isoforms have been found: type 1 and type 3, or their non-mammalian muscle equivalent α and β. Skeletal muscle contains either RyR1 only, approximately equal amounts of RyR1 and RyR3, or predominantly RyR1 co-expressed with low levels of RyR3 (15). On the other hand,
in the heart practically the only isoform expressed is RyR type 2 [16]. The COOH-terminal region is the most highly conserved and it is predicted to form the intramembrane Ca\textsuperscript{2+} release channel, while the amino-terminal region constitutes the large cytoplasmic domain known as the foot. Three-dimensional reconstruction of RyRs has shown minor but probably significant differences among the three isoforms [17-19]; see section 6 for more details. DHPRs also have different isoforms in cardiac and skeletal fibers, however, the situation for this protein is far more complicated than for RyRs because DHPRs consist of five different subunits (\(\alpha_1\), \(\alpha_2\), \(\beta\), \(\gamma\), and \(\delta\)). The \(\alpha_1\) subunit forms the channel and structurally is very similar to the Na\textsuperscript{+} channel [20]: it is constituted by four domains (I-IV) and contains the segment S4 that has been identified as the voltage sensor of the molecule [21]. Alpha\textsubscript{s-} and alpha\textsubscript{c-} DHPR, the two isoforms expressed in skeletal muscle and heart respectively, share ~66% homology, mostly confined to the transmembrane regions, while higher divergence is found in the cytoplasmic domains and the loops that are thought to interact with RyRs. The other four subunits are regulatory components of the channel and will not be discussed in detail in this review.  

4.2. DHPR and RyR interact differently in skeletal and cardiac muscle  
RyRs are closely associated with each other forming ordered arrays in both skeletal and cardiac muscles. In fact the feet appear as evenly spaced densities in junctions seen in thin section electron microscopy (EM) (Figure 1 to 3). While these images suggest that skeletal and cardiac arrays are identical, this interpretation is not conclusive since views of junctions such as the ones in Figure 1 and Figure 3 B do not identify all the parameters that define an RyR array. Images parallel to the plane of the junction (such as the one in Figure 2 D) have allowed definition of the subtle differences between RyR arrays in skeletal muscle and in body muscles of some invertebrates [22]. Unfortunately no such images are available at this point for cardiac junctions.

While arrays of RyR1 and RyR2 are similar and possibly identical, major divergences exist between DHPRs arrangements. In skeletal muscle, DHPRs are grouped into tetrads, groups of four receptors located at the corner of small squares (see detail in Figure 2 C). Tetrads are superimposed on the feet so that each DHPR is located immediately above one of the RyR subunits and in a specific position relative to it. This spatial tetrad/RyR arrangement generates tetrad arrays that are related to the feet arrays (Figures 2, 4, and 5). Tetrads are specifically and only formed by association of alpha\textsubscript{s}DHPRs and RyR type 1 (see section 5 for more details). Surprisingly, tetrads are not associated with every foot but only with alternate ones [23, 24]. This disposition was found in several different types of fibers regardless of the muscle containing only one (RyR1) or two (RyR1 and RyR3) isoforms, so that uncoupled feet could be either RyR1 or RyR3 (see Figures 2 B and 5 C). The possible reason for this alternate tetrad/RyR1 association will be further discussed in section 6.

In cardiac muscle, immunohistochemistry experiments and morphological studies have shown that DHPRs are also clustered in close correspondence with RyR domains [7, 14]. Indeed, studies in chicken developing peripheral couplings have shown that there is a very close relationship...
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Figure 4. Interaction of RyR1 and RyR3 with skeletal DHPRs. A and B) In dyspedic 1B5 cells (RyR1 –/–), DHPRs are clustered in correspondence of CRUs, but they are not grouped in tetrads as in normal skeletal muscle cells because of lack of RyR type 1 in the SR junctional domains. C and D) DHPR tetrad arrangement is restored by transfection with cDNA encoding for RyR1. Dotting the center of tetrads in the array (C, bottom) results in an ordered pattern that is related to the arrays of feet in the SR. E and F) RyR3 expression does not restore DHPR tetrad arrays suggesting that RyR3 in skeletal muscle cells does not interact directly with DHPRs as RyR1 does. Bar, 0.1 µm (3D reconstruction of RyRs courtesy of T. Wagenknecht).

between the size of arrays of feet in the SR and the area covered by DHPR in the junctional portion of the sarcolemma (25). While DHPRs are clearly clustered closely to RyRs, freeze-fracture studies have shown no evidence of organization of DHPRs into tetrads (7-25). DHPRs are randomly clustered in junctional domains of exterior membranes and no spatial relationship with feet was detected (Figure 3, A and C, and Figure 5, B and D). The lack of tetrads indicates a lack of a direct specific association between α1cDHPR and RyR2 and correlates well with the lack of direct functional interaction between the two (see section 6 for further discussion).

5. DYSGENIC AND DYSPEDIC MICE CONTRIBUTION TO THE UNDERSTANDING OF DHPR/RYR INTERACTION

Molecular biology and gene handling techniques in the last years have revolutionized research approaches in many fields and, among them, also in the study of e-c coupling. Knockouts have become very valuable tools to study protein function and protein/protein interactions. Dysgenic and dyspedic mice, two animal models carrying null mutations for α1sDHPR and RyR1, have been crucial to the study of structural and functional roles of those two proteins in e-c coupling. Muscular dysgenesis is a spontaneous single nucleotide deletion in the gene encoding for the α1s subunit of the DHPR that causes a shift of the translational reading frame (26), while dyspedic mice were created by targeted disruption of the gene encoding for RyR type 1 (27). Initial functional studies have confirmed the central role of the two proteins in e-c coupling. Depolarization of the sarcolemma fails to elicit Ca\(^{2+}\) release from internal stores in both mutants (27, 28) while in both e-c coupling can be restored by transfection with cDNA encoding the missing element (12, 29). From the structural point of view, the two models have allowed a direct demonstration that tetrads and feet are indeed respectively DHPRs and RyRs. In fact the two structures are missing respectively in dysgenic and dyspedic mice and reappear after transfection with cDNA encoding the missing protein (30-32).

Surprisingly, absence of either α1sDHPR or RyR1 does not affect either formation of junctions or targeting of the second protein to junctional domains. In dysgenic muscle, triads containing organized arrays of feet are formed even if DHPRs are missing (33), and in dyspedic mice both triads and peripheral couplings are formed in the absence of RyRs (34). In regards to the targeting of DHPRs to junctions lacking RyRs, there have been some controversial results. Initially in vivo studies seemed to indicate that DHPRs did not cluster in the absence of RyRs (34), whereas more recently DHPRs were found clustered in correspondence to junctions lacking feet in a cell line of muscle origin carrying a null mutation for RyR1 (31). The reason for this discrepancy is still not clear, but it could be due to reduced amounts of α1sDHPR expressed in the in vivo model versus the higher level expressed in 1B5 myotubes.

DHPs and RyRs are not involved in formation of junctions and in targeting of each other to the junctions. However, while RyRs do not need DHPRs to organize...
Figure 5. Structure/function correlation: lack of tetrads in cardiac cells explains the need of Ca\(^{2+}\) in DHPR/RyR communication. A and B) DHPRs clustered respectively in peripheral coupling of BC3H1 cells (A) and in chicken ventricle (B). DHPRs form tetrads in the first, while in the second are randomly disposed in the junctional domain. C and D) both RyR1 (blue, C) and RyR2 (pale green, D) form ordered arrays. However, the specific link that allows associations of tetrads to alternate RyR1s in skeletal muscle cells is missing in the heart. The role and intracellular localization of RyR3 in skeletal muscle fibers is still not completely clear: RyR3 (green, C) may occupy some of the uncoupled positions of the RyR arrays. E and F) Cartoons illustrating the two different mechanisms that allow DHPR and RyR to communicate in e-c coupling: mechanical coupling in skeletal fibers and CICR in cardiac myocytes. Need of external Ca\(^{2+}\) in heart (F) is the result of lack of a direct link between the two molecules, link that probably involves the II-III loop of the DHPR in skeletal fibers (E). Bar, 0.1 \(\mu m\) (3D reconstruction of RyRs courtesy of T. Wagenknecht).

themselves into arrays, DHPRs depend on RyRs for their organizations into tetrads in skeletal muscle CRUs. In fact, skeletal DHPRs clustered at the junctions of dyspedic 1B5 myotubes are not organized into tetrads and resemble DHPR clusters in cardiac myocytes (Figure 4, A and B). The key role of RyR1 in arranging DHPRs in groups of four was directly proven by the restoration of tetrads induced with cDNA encoding for RyR type 1 and interestingly, the alternate RyR/DHPR association was also restored in these cells (Figure 4, C and D) (32). 1B5 myotubes were also used to test functional and structural roles of RyR type 3, the second RyR isofrom expressed in skeletal muscle cells. While RyR1 could restore both K\(^{+}\)-induced depolarization and DHPR tetrads, RyR3 failed to do so (Figure 4, E and F) (32, 35). These results will be further discussed in section 6.

6. PERSPECTIVES: HOW THE STRUCTURE MAY EXPLAIN THE FUNCTION

The structural studies described in the previous sections have shown that, while RyRs and DHPRs are located in close proximity of each other in both cardiac and skeletal muscles, supra-molecular complexes are not assembled in the same way. RyRs are organized in arrays in both muscle types, while DHPRs form tetrad arrays only in skeletal fibers. This finding is extremely important because of its functional implications (Figure 5). In cardiac myocytes the signal that triggers the opening of RyRs is the inward flux of Ca\(^{2+}\) through DHPRs that follows depolarization (CICR) (3), while in skeletal muscle, Ca\(^{2+}\) is not needed for RyR activation as the coupling between the two molecules seems to involve a direct protein-protein interaction (mechanical coupling) (4-5). The functional characteristics of the DHPR and RyR isofroms expressed in the two muscle types are in line with the mechanism they are part of: \(\alpha_{c}\)-DHPR has a higher open probability than \(\alpha_{s}\)-DHPR, and RyR2 is indeed more sensitive to Ca\(^{2+}\) than RyR1. Skeletal muscle has evolved a novel mechanism of e-c coupling that does not rely on Ca\(^{2+}\) influx and the proteins involved in the process have evolved accordingly. The link that allows \(\alpha_{c}\)-DHPRs and RyR1 to form tetrads represents the structural basis for mechanical and Ca\(^{2+}\)-independent e-c coupling of skeletal muscle fibers. Lack of this link in cardiac cells results in need of a diffusible
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Because skeletal e-c coupling does not depend on extracellular Ca\(^{2+}\) the primary role of \(\alpha_\text{sDHPR}\) cannot be to function as a Ca\(^{2+}\) channel. It has been suggested that a positively charged intramembrane segment (S4 in each of the four DHPR domains) is responsible for providing the triggering signal that activates RyRs. In this way the \(\alpha_\text{sDHPR}\) would act as a voltage sensor in the mechanism (37). However, while it is clear that \(\alpha_\text{sDHPR}\) and RyR1 are specifically linked, the nature of the bond is still unknown. In fact, attempts to detect a direct binding between RyRs and DHPRs were unsuccessful, and the possibility of a third protein being interposed among them cannot yet be completely discarded.

Studies performed in knockout mice have allowed identification of DHPR and RyR domains that are critical for the mechanical communication between them. An extremely elegant work by Tanabe et al. (1990)(37) showed that the expression of a \(\alpha_\text{sDHPR}\) chimera, containing only the cytoplasmic loop between the II and III domain from the skeletal isoform, was able to restore skeletal type e-c coupling in cultured dysgenic myotubes. Recently even a shorter domain of the II-III loop (46 amino acids) was found to be sufficient to restore skeletal type e-c coupling (38) and not even drastic alteration of the sequence surrounding those 46 amino acid could abolish its function (39). It has been proposed that also the \(\beta\) subunit of the DHPR plays an important role in e-c coupling. The \(\beta\)-DHPR is localized on the cytoplasmic side of the protein and interacts with the I-II loop of the \(\alpha_\text{sDHPR}\). Studies in \(\beta\)-null mice (\(\beta\)-DHPR -/-) suggest that the carboxy-terminus region of this subunit is important for communicating with RyRs (40). Skeletal/cardiac chimeric RyR receptors expressed in dyspedic myotubes were used to determine which region of RyR1 receives the signal from the DHPR (41) and recent unpublished observations are leading to the conclusion that RyR1 interacts with DHPRs in multiple regions (unpublished data). One more piece was added to the puzzle by the fact that the signaling between the two molecules turned out to be bi-directional (29) and far more complex than expected. An orthograde signal allows triggering of RyR Ca\(^{2+}\) release and a retrograde signal allows DHPRs to function as a channel. Existence of this two-way interaction is strong proof that an intermolecular route indeed functionally links RyRs and DHPRs. However, while it is clear that the orthograde signal is essential to skeletal type e-c coupling, it is not clear whether the retrograde signaling that is necessary for Ca\(^{2+}\) permeation through the DHPR plays any role in e-c coupling events. A correlation between the structural DHPR-RyR link that results in tetrad formation and the functional link that underlies orthograde and retrograde signaling has not yet been established and requires further investigation.

Tetrad is composed of four DHPRs associated with the corners of the four subunits of RyR1, as shown by a combination of thin sectioning and freeze-fracture techniques (Figure 2). Interestingly, three-dimensional reconstruction of RyR1 (skeletal) and RyR2 (cardiac) have shown that one of the major structural differences between the two isoforms is localized in the corner of the cytoplasmic domain (clamp region). The region that bridges domains 5 and 6 of RyR1 is probably missing in RyR2 and differences in this region may definitely contribute to the differences in e-c coupling and DHPR/RyR association (18). DHPR tetrad are not associated with every foot but only and specifically with alternate feet even in muscles that express only RyR type 1. In this configuration only one of every two RyRs has the possibility of interacting directly with DHPRs since the remaining feet are not directly coupled (Figures 2 E, 4 D, and 5 C). The reason for the alternate tetrad/RyR1 structural association is still unclear. It has been speculated that tetrads being bigger than feet, there is no space for tetrad arrays on each RyR (24). However, studies of developing junctions in BC3H1 cells have shown that DHPRs are targeted specifically to alternate RyRs even when the tetrad arrays are incomplete (42). This study ruled out the possibility of simple steric hindrance as the only reason for alternate RyR/tetrad association and suggested that other molecular components of skeletal junctions could be involved in the formation of this pattern. It is also still unclear how uncoupled RyRs are activated. Hypothesis in which alternate RyRs are activated directly by DHPRs whereas the others are activated by CICR have been proposed (4-5). Marx et al. (1998) (43) suggested a different model: according to their results RyR1s are functionally associated in pairs through FKBP12 (coupled gating).

RyR3 is co-expressed with RyR1 in many muscle types and in some cases it constitutes up to 50% of the RyRs. Because RyR3 is never expressed alone in skeletal muscle, it was initially difficult to test how this isoform is activated and what its contribution to CRU structure is under normal conditions. The first evidence has come from an animal model carrying a spontaneous null mutation of RyR1 (cn/cn chicks, crooked neck dwarf phenotype). The muscle fibers in the cn/cn chicks develop poorly and show no e-c coupling in vivo even when RyR3 is expressed (44). Cultured myotubes do show Ca\(^{2+}\) transients, but these are dependent on the presence of external Ca\(^{2+}\). Lack of interaction between RyR3 and skeletal \(\alpha_\text{sDHPR}\) is confirmed by failure of RyR3 to restore skeletal type e-c coupling and DHPR junctional tetrad in dyspedic 1B5 myotubes (32, 35). The relative position of \(\alpha_\text{sDHPRs}\) and RyR3 is similar to that of \(\alpha_\text{cDHPRs}\) and RyR2 in cardiac myocytes, that is the two molecules are near each other but not apparently linked (compare Figures 4 F and 5 D). These results taken together suggest that RyR3 does not participate directly in the interaction with \(\alpha_\text{sDHPRs}\) and support once again the need of tetrads for mechanical coupling.

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**Abbreviations:** CICR, calcium-induced calcium-release; CRU, calcium release unit; DHPR, dihydropyridine receptor; e-c, excitation contraction; EM, electron microscopy/micrographs; RyR, ryanodine receptor; RyR1, RyR2, and RyR3, skeletal isoform of ryanodine receptor, cardiac isoform of ryanodine receptor, and brain isoform of ryanodine receptor; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

**Key words:** Excitation-Contraction Coupling; Skeletal and Cardiac Muscle; Dihydropyridine Receptors, Ryanodine Receptors; Electron Microscopy, Review

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