Triadin, a Linker for Calsequestrin and the Ryanodine Receptor

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Introduction

Protein components of the triad junction play essential roles in muscle excitation-contraction coupling (EC coupling). Considerable research has been performed on the identification and characterization of proteins that regulate calcium storage and release from the sarcoplasmic reticulum (McPherson and Campbell, 1993; Franzini-Armstrong and Jorgensen, 1994). Key proteins characterized include the dihydropyridine receptor; the voltage sensor and L-type calcium channel in t-tubules; the ryanodine receptor/Ca^{2+}-release channel in the terminal cisternae of the sarcoplasmic reticulum; and calsequestrin, a moderate-affinity, high-capacity calcium-binding protein located in the lumen of the junctional sarcoplasmic reticulum. Study of these proteins has been instrumental to our understanding of the molecular mechanisms of EC coupling. Recent research from our laboratory has focused on triadin, an abundant transmembrane protein in the junctional sarcoplasmic reticulum. Here, we briefly review recent results on the structure of triadin and its interactions with other protein components of the junctional complex in skeletal and cardiac muscle.

Identification of Triadin

Using purified skeletal muscle triads, we generated a library of monoclonal antibodies against different proteins of the junctional sarcoplasmic reticulum (Campbell et al., 1987). Several monoclonal antibodies recognize a protein of 94 kD (now called triadin) on reducing SDS-PAGE (Fig. 1 *A*). Triadin is highly enriched in the junctional membrane preparations including heavy sarcoplasmic reticulum, triads, and junctional face membranes, but it is not detectable in longitudinal sarcoplasmic reticulum (SR) or T-tubules. Triadin migrates just slightly faster than the Ca^{2+}/ATPase, the mobility of which prevents the identification on Coomassie blue-stained gels. However, triadin is clearly detected in the junctional face membrane preparation, which is deplete of most of the Ca^{2+}/ATPase (Knudson et al., 1993*a*). The migration of this protein and its localization in the junctional membrane fractions closely resembles a 95-kD protein characterized by Brandt et al. (1990) and Caswell et al.

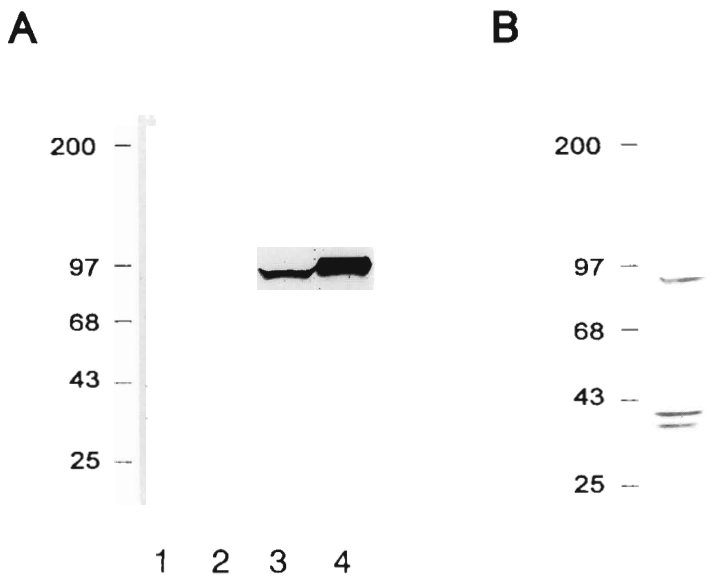

Figure 1. Identification of triadin in skeletal and cardiac muscle sarcoplasmic reticulum. (*A*) Immunoblot of light sarcoplasmic reticulum (*lane 1*), T-system (*lane 2*), triads (*lane 3*), and junctional face membrane (*lane 4*) stained with monoclonal antibodies XIIH11-2 and IIG12 against skeletal muscle triadin. (*B*) Identification of cardiac triadin isoforms in rabbit cardiac muscle microsomes by use of polyclonal antibody GP57.

(1991). This protein was named *triadin* (Brandt et al., 1990) because of specific association with isolated triads.

Localization of Triadin by Immunofluorescent Staining

The localization of triadin in skeletal muscle was carried out by immunofluorescence staining (Knudson et al., 1993*a*; Flucher et al., 1993). Labeling of transverse cryosections with anti-triadin antibodies showed a polygonal pattern throughout the cytoplasm of the myofibers. The staining pattern observed in longitudinal cryosections appeared as transversely oriented rows of discrete foci. The distribution of the rows corresponded to the interface between the A-band and the I-band. This pattern corresponds to the location of triads in skeletal muscle. To determine more precisely whether triadin is confined to the terminal cisternae, its subcellular distribution was determined by immunocolloidal gold labeling and compared with the ryanodine receptor/calcium release channel. Triadin was distributed predominantly over the terminal cisternae, and the labeling was generally indistinguishable from the ryanodine receptor. This nearly identical pattern of staining suggests these two proteins colocalize at the junctional face membrane and may interact with each other. Triadin was also examined in dysgenic mice (Flucher et al., 1993). Dysgenic myotubes with a deficiency in the α_1 subunit of the dihydropyridine receptor show

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SKM  MTEITAEGNASTTTTVIDSKNGSVPKSPGKVLKRTVTEDLVTTFSSPAAWLLVIALIITWSAVAVVMFDLVDYKN
CT3  MTEITAEGNASTTTTVIDSKNGSVPKSPGKVLKRTVTEDLVTTFSSPAAWLLVIALIITWSAVAVVMFDLVDYKN
CT2  MTEITAEGNASTTTTVIDSKNGSVPKSPGKVLKRTVTEDLVTTFSSPAAWLLVIALIITWSAVAVVMFDLVDYKN
CT1  MTEITAEGNASTTTTVIDSKNGSVPKSPGKVLKRTVTEDLVTTFSSPAAWLLVIALIITWSAVAVVMFDLVDYKN

SKM  FSASSIAKMGSDPLKLVHDAVEETTDWIYGFFSLLSDIISSDGDEEDDEGDEDTAKGEIEEPPLKRKDIHKEKIE
CT3  FSASSIAKMGSDPLKLVHDAVEETTDWIYGFFSLLSDIISSDGDEEDDEGDEDTAKGEIEEPPLKRKDIHKEKIE
CT2  FSASSIAKMGSDPLKLVHDAVEETTDWIYGFFSLLSDIISSDGDEEDDEGDEDTAKGEIEEPPLKRKDIHKEKIE
CT1  FSASSIAKMGSDPLKLVHDAVEETTDWIYGFFSLLSDIISSDGDEEDDEGDEDTAKGEIEEPPLKRKDIHKEKIE

SKM  KQEKPERKIPTKVVHKEKEKEKEKVKEKEKPEKKATHKEKLEKKEKPETKTVTKEEKKARTKEKIEEKTKKEVKG
CT3  KQEKPERKIPTKVVHKEKEKEKEKVKEKEKPEKKATHKEKLEKKEKPETKTVTKEEKKARTKEKIEEKTKKEVKG
CT2  KQEKPERKIPTKVVHKEKEKEKEKVKEKEKPEKKATHKEKLEKKEKPETKTVTKEEKKARTKEKIEEKTKKEVKG
CT1  KQEKPERKIPTKVVHKEKEKEKEKVKEKEKPEKKATHKEKLEKKEKPETKTVTKEEKKARTKEKIEEKTKKEVKG

SKM  VKQEKVKQTVAKAKEVQKTPKPKEKESKETAAVSKQEQKDQYAFCRYMIDIFVHGDLKPGQSPAIPPPSPTEQAS
CT3  VKQEKVKQTVAKAKEVQKTPKPKEKESKETAAVSKQEQKDQYAFCRYMIDIFVHGDLKPGQSPAIPPPSPTEQAS
CT2  VKQEKVKQTVAKAKEVQKTPKPKEKESKETAAVSKQEQK**ECIFLSAATPQGIPNRQQLNDIHHCFLKTKKGGNGQ**
CT1  VKQEKVKQTVAKAKEVQKTPKPKEKESKETAAVSKQEQK**GKQSEEAAGCFKRTLGKKQMQ**----------

SKM  RPTPALPTPEEKEGEKKKAEKKVTTETKKKAEKEDAKKKSEKETDIDMKKKEPGKSPDTKPGTVKVTTQAATKKD
CT3  RPTPALPTPEEKEGEKKKAEKKVTTETKKK-EKEDAKKKSEKETDIDMKKKEPGKSPDTKPGTVKVTTQAATKKD
CT2  **HAFCLKGC**----------
CT1  ----------

SKM  EKKEDSKKAKKPAEEQPKGKKQEKKEKHEEPAKSTKKEHAAPSEKQAKAKIERKEEVSAASTKKAVPAKKEEKTT
CT3  EKKEDSKKAKKPAEEQPKGKKQEKKEKHEEPAKSTKKEHAAPSEKQAKAKIERKEEVSAASTKKAVPAKKEEKTT

SKM  KTVEQETRKEKPGKISSVLKDKELTKEKEVKVPASLKEKGSETKKDEKTSKPEPQIKKEEKPGKEVKPKPPQPQI
CT3  KTVEQETRKEKPGKISSVLKDKELTKEKEVKVPASLKEKGSETKKDEKTSKPEPQIKKEEKPGKEVKPKPPQPQI

SKM  KKEEKPEQDIMKPEKTALHGKPEEKVLKQVKAVTTEKHVKPKPAKKAEHQEKEPPSIKTDKPKSTSKGMPEVTES
CT3  KKEEKPEQDIMKPEKTALHGKPEEKVLKQVKAVTTEKHVKPKPAKKAEHQEKEPPSIKTDKPKSTSKGMPEVTES

SKM  GKKKIEKSEKEIKVPARRESHQLQNVTKAEKPARGSKEGFEDVPA**SKKAKEEAEEVSSTKKQKSPISFFQCVYLD**
CT3  GKKKIEKSEKEIKVPARRESHQLQNVTKAEKPARGSKEGFEDVPA**LATVGIWGMNQWMEDLSVTLPSK**-------

SKM  **GYNGYGFQFPVTPAQYPGESSGKPNSPGPKQ**
CT3  ----------
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Figure 2. Protein sequences of skeletal and cardiac muscle triadin isoforms. Amino acid sequences of skeletal muscle triadin (SKM) and cardiac muscle triadin (CT1, CT2, and CT3) are aligned. The residues that are different among these isoforms are indicated with bold characters.

reduced expression of the ryanodine receptor and triadin. However, both proteins are still capable of forming clusters and attaining mature cross-striated distribution.

Biochemical Characterization of Triadin

Triadin has been thoroughly characterized by biochemical methods (Caswell et al., 1991; Knudson et al., 1993*a*). Endo H treatment shifts the mobility of triadin by approximately 2 kD; this suggests that it is a glycoprotein. In addition, ConA directly binds to triadin. Furthermore, this protein labeled by ^{125}I-TID, a compound that binds to the hydrophobic domains of proteins. The membrane topology of triadin was also examined by the vesicle-protection assay. The result suggests that only a small portion of triadin is cytoplasmic, while the bulk of this protein is located in the lumen of the sarcoplasmic reticulum.

Another major characteristic of skeletal muscle triadin is that it may form disulfide-linked oligomers (Caswell et al., 1991; Knudson et al., 1993*a*). This protein

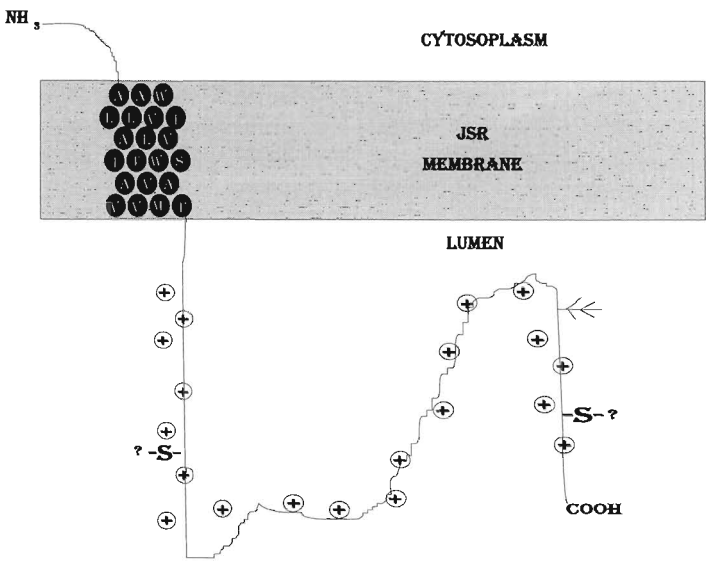

Figure 3. Proposed model for the membrane topology of skeletal muscle triadin. Amino acids 48–68 of triadin are represented in the membrane domain of the protein. The approximate locations of the 2 cysteines that are located at positions 270 and 671 and are likely disulfide-linked are represented by an *S* and a *question mark*. The basic nature of the luminal domain of triadin is depicted by the positive charge symbols (+). Possible glycosylation site is depicted by the *glycosylation symbol*.

requires strong sulfhydryl reducing agents to migrate at 94 kD on SDS-PAGE. In the presence of non-reducing reagents such as *N*-ethylmaleide and absence of reducing agents, triadin migrates as a series of high-molecular-mass multimers. The functional implications of this biochemical characteristic have been investigated, and it was suggested that these disulfide groups may be important in the regulation of calcium release by the sarcoplasmic reticulum (Liu et al., 1994).

Primary Structure of Triadin

The full-length cDNA encoding the rabbit skeletal muscle triadin was obtained by library screening using anti-triadin monoclonal antibodies (Knudson et al., 1993*b*), and the primary structure of the protein was deduced from the cDNA sequence (Fig. 2). This protein is composed of 706 amino acid residues. On the basis of hydrophobicity analysis, triadin contains a single transmembrane domain that separates it into cytoplasmic and luminal segments (Fig. 3). Only the NH_2-terminal 47 amino acids are cytoplasmic; the rest of the protein including the C-terminus is located in the lumen of the sarcoplasmic reticulum. This membrane topology is consistent with the results of the vesicle-protection assay (Knudson et al., 1993*b*). The amino acid content of the luminal domain of triadin is remarkable for a very high abundance of charged residues. The relative abundance of the basic residues over acidic residues results in a predicted isoelectric point of 10.18. The positively

charged luminal domain may interact with the negatively charged calsequestrin in the lumen of the junctional sarcoplasmic reticulum (Knudson et al., 1993*b*).

Triadin in Cardiac Muscle Sarcoplasmic Reticulum

Results from Western blot analysis with a combination of monoclonal antibodies and Northern blot analysis using partial DNA sequence of skeletal muscle triadin suggested that triadin is specific for skeletal muscle (Knudson et al., 1993*b*). However, several recent reports suggested that triadin is also present in cardiac muscle (Brandt et al., 1993; Peng et al., 1994; Sun et al., 1995; Carl et al., 1995). Peng et al. (1994) were able to detect several messages of triadin in cardiac muscle using Northern blot analysis. Recently, we generated polyclonal antibodies against the luminal domain of triadin-glutathione *S*-transferase (GST) fusion proteins. All the polyclonal antibodies recognize proteins in cardiac muscle microsomes as well as skeletal muscle (Guo et al., 1996). In fact, three isoforms of triadin were detected with these antibodies. One migrates at a molecular mass of about 92 kD, slightly smaller than the skeletal muscle counterpart. The two other isoforms correspond to the doublet of about 35 kD (Fig. 1 *B*). It is of interest that none of the monoclonal antibodies that were used to isolate the skeletal muscle triadin cDNA recognized these proteins (Guo et al., 1996).

Using RT-PCR and library screening, three cDNAs encoding cardiac triadin isoforms were obtained (Fig. 2). The deduced amino acid sequences show that these proteins are identical in their NH_2-terminal sequences, whereas the COOH-terminal sequences are distinct from each other and from that of skeletal muscle triadin. In fact, the NH_2-terminal sequence identity is also shown at the nucleotide level. The isoforms of triadin in skeletal and cardiac muscle are probably encoded by the same gene by alternative splicing. Recently, we have localized the human triadin gene to chromosome 6 (data not shown).

Immunofluorescence staining of rabbit cardiac muscle shows that cardiac triadin is primarily confined to the I-band region of cardiac myocytes where the ryanodine receptor and calsequestrin-containing junctional and corbular sarcoplasmic reticulum are localized (Guo et al., 1996; Jorgensen et al., 1984, 1993). Immunoelectron microscopical studies will be required to determine whether one or more of the three isoforms of cardiac triadin is confined to either corbular or junctional sarcoplasmic reticulum.

Molecular Interactions of Triadin

Molecular interactions of triadin in skeletal muscle were first examined by a protein overlay assay and by affinity chromatography. Both the solubilized ryanodine receptor and the α_1 subunit of the dihydropyridine receptor were found to bind triadin (Brandt et al., 1990; Kim et al., 1990). It was therefore thought that triadin was a coupling protein spanning the triad junction that mediates the signal transduction between the two calcium channels. However, membrane analysis of triadin has led to some controversy regarding the ability of triadin to interact with the dihydropyridine receptor (Knudson et al., 1993*b*). Only 47 amino acids of triadin are located in

the cytosol. This segment may be too small to cross the triad cleft to interact directly with the dihydropyridine receptor that is located in the T-tubule membrane. Instead, since the bulk of this protein is intraluminal, triadin probably exerts its function in the lumen of the sarcoplasmic reticulum.

The molecular interactions of skeletal muscle triadin were also examined by Guo and Campbell (1995). Domains of skeletal muscle triadin that are localized in the cytoplasm or the lumen of the sarcoplasmic reticulum were expressed as GST–fusion proteins and immobilized on glutathione Sepharose to form an affinity column. Detergent-solubilized homogenates were prepared from rabbit skeletal muscle and applied to the columns. Two proteins of ~63 kD and ~550 kD bound to the Sepharose that conjugated with the luminal domain of triadin (L-triadin Sepharose). These two proteins were identified as the ryanodine receptor and calsequestrin. The ryanodine receptor and calsequestrin are minor components in the solubilized whole-muscle homogenates; however, they are the major proteins from the homogenates that bind to the L-triadin Sepharose. This demonstrates the specificity of the interactions.

The association of the ryanodine receptor with triadin was demonstrated further in [^{3}H]ryanodine receptor–binding experiments. The luminal portion fusion protein bound the labeled ryanodine receptor in a dose-dependent manner. Using the same beads assay, we examined the possible interaction of triadin Sepharose with the [^{3}H]PN-200-110 labeled dihydropyridine receptor. However, neither the cytoplasmic nor the luminal portion of triadin was able to bind to the [^{3}H]PN-200-110 labeled receptor in the muscle homogenate. These results demonstrate a specific interaction between triadin and the ryanodine receptor that occurs in the lumen of the sarcoplasmic reticulum; and we have obtained no evidence for an interaction of triadin with the dihydropyridine receptor.

Calsequestrin binds Ca^{2+} with moderate affinity and high capacity (MacLennan et al., 1983), and the binding of Ca^{2+} leads to dramatic conformational change of this luminal protein (Mitchell et al., 1988; He et al., 1993). We therefore examined the effect of Ca^{2+} on the interaction between calsequestrin and triadin. In the presence of $CaCl_2$, L-triadin Sepharose was able to bind calsequestrin. However, when EDTA was present, the interaction was inhibited. Thus, calsequestrin binds to the luminal domain of triadin in a Ca^{2+}-dependent manner.

Calsequestrin is a soluble protein that remains associated with the luminal side of the junctional membrane of the sarcoplasmic reticulum through its interaction with a previously unidentified membrane protein (Franzini-Armstrong et al., 1987). To test whether triadin is the anchoring protein for calsequestrin, we examined whether the triadin-luminal-domain-GST fusion protein was capable of inhibiting the reassociation of calsequestrin with the junctional face membrane. When calsequestrin was incubated with the junctional face membrane, calsequestrin reassociated with the junctional face membrane. However, when increasing amounts of triadin-GST fusion protein were added to the incubation buffer, less calsequestrin reassociated to the junctional face membrane. This result suggests that one function of triadin is to anchor calsequestrin to the luminal side of the junctional sarcoplasmic reticulum in proximity to the "SR feet" (ryanodine receptor), where calsequestrin stores calcium that is available for release (Franzini-Armstrong, 1970; Franzini-Armstrong et al., 1987).

Since different isoforms of triadin also exist in cardiac muscle, we examined

whether the cardiac triadin binds to the ryanodine receptor and calsequestrin in cardiac muscle. Our results indicate that the segment of the luminal domain of triadin, which is homologous among all the isoforms in the two tissues, is able to interact with both the ryanodine receptor and calsequestrin.

Calsequestrin is a soluble protein that remains associated with the luminal side of the junctional membrane of the sarcoplasmic reticulum through its interaction with a previously unidentified membrane protein (Franzini-Armstrong et al., 1987). Calsequestrin can be extracted from the junctional face membrane by treatment with EDTA or high concentrations of NaCl (Ikemoto et al., 1991; Costello et al., 1986); this indicates that the interaction between calsequestrin and its membrane-anchoring protein can be inhibited by EDTA and is disruptable by high ionic strength. Notably, the association between calsequestrin and triadin-GST fusion protein is also inhibited by EDTA. Futhermore, high salt concentration can elute calsequestrin from L-triadin Sepharose. These binding properties resemble those of the interaction of calsequestrin with its anchoring protein in the junctional face membrane. In addition, like calsequestrin, triadin is an abundant protein that is specifically localized in the junctional region, with the bulk of this protein in the lumen of the sarcoplasmic reticulum (Knudson et al., 1993*a*, *b*). Together, these characteristics strongly suggest that triadin is the physiological anchoring protein for calsequestrin at the junctional face of sarcoplasmic reticulum. This hypothesis was further confirmed in the calsequestrin/junctional face membrane reassociation experiment.

It is known that the intraluminal Ca^{2+}-binding protein, calsequestrin, and the ryanodine receptor/Ca^{2+}-release channel are functionally coupled (Ikemoto et al., 1991; Ikemoto et al., 1989; Gilchrist et al., 1992; Kawasaki et al., 1994; Donoso et al., 1995). The ryanodine receptor, when activated by Ca^{2+}-release agents, such as caffeine, induces dissociation of calcium from calsequestrin (Ikemoto et al., 1991). Conversely, changes in luminal Ca^{2+} concentration lead to conformational changes in calsequestrin, and this information can be transmitted to the ryanodine receptor, thus affecting the activation of the ryanodine receptor/Ca^{2+}-release channel (Ikemoto et al., 1989; Gilchrist et al., 1992; Kawasaki et al., 1994; Donoso et al., 1995). However, despite the biophysical evidence for functional coupling between the ryanodine receptor and calsequestrin, there is no biochemical evidence for direct interaction between the two proteins. It is thought that the interaction is mediated by a third protein. Our data and previous studies suggest that triadin binds to the ryanodine receptor (Caswell et al., 1991; Liu et al., 1994) and the luminal domain of triadin also interacts with calsequestrin. Together, these results suggest that triadin anchors calsequestrin to the junctional face membrane and thus may be involved in the functional coupling between calsequestrin and the ryanodine receptor/Ca^{2+}-release channel in the lumen of the sarcoplasmic reticulum. A model illustrating the molecular interactions of triadin in the lumen of the sarcoplasmic reticulum is presented (Fig. 4).

These results have provided biochemical evidence for a structural and/or functional role of triadin in the lumen of the junctional sarcoplasmic reticulum. However, many questions still await further investigations. For example, what are the cDNA sequences for the different isoforms of triadin in skeletal muscle? What is the function of the different isoforms of triadin? How does triadin mediate the functional coupling between the ryanodine receptor and calsequestrin? Answers to

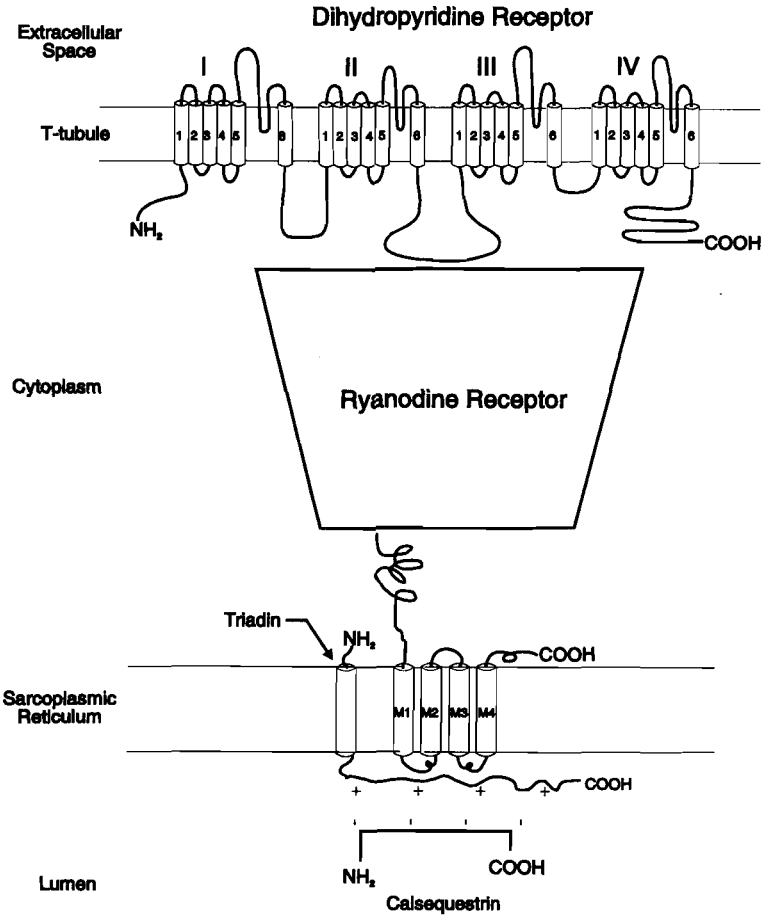

Figure 4. Proposed model for the interactions of triadin with calsequestrin and the ryanodine receptor/calcium release channel in triads.

these questions will help us understand the triad structure and EC coupling at a new level.

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