TRIADIN IS PRIMARILY CONFINED TO THE JUNCTIONAL SARCOPLASMIC RETICULUM OF MAMMALIAN CARDIAC MYOFIBERS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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ABSTRACT

Triadin is an intrinsic sarcoplasmic reticulum (SR) protein that interacts with calsequestrin (CSQ), the ryanodine receptor (RyR) and junctin and is believed to play a role in excitation-contraction (E-C) coupling (42). Previous immunolocalization studies (44,59) of longitudinal cryosections from rabbit cardiac myofibers have suggested that triadin, like the RyR (85), is densely distributed in junctional SR and corbular SR. However, since interior junctional SR and corbular SR are both localized to the I-band of longitudinally cryosectioned myofibers (9), it is difficult to determine whether the triadin-positive foci correspond to the detection of triadin in junctional SR and/or corbular SR. Since previous immunolocalization studies (115) have shown that it is possible to accurately determine whether junctional SR proteins (e.g. the RyR or junctin) are localized to junctional SR and/or corbular SR, if their subcellular distribution is also examined in the presence of a marker of the sarcolemma (SL) and the transverse (T) tubules of transversely cryosectioned myofibers, it should now be feasible to precisely determine the subcellular distribution of triadin. Thus, the objective of the present study was to reexamine the subcellular distribution of triadin in relation to that of either the RyR, phospholemmal (PLM, a marker of the SL and T-tubules (116)) or junctin, using indirect double immunofluorescence labeling of transversely and longitudinally sectioned canine cardiac myofibers, followed by laser scanning confocal microscopy.

The results presented here show that triadin-positive foci were densely distributed along the SL and T-tubules of canine atrial and ventricular myofibers but not detected in the interior regions of these myofibers where RyR-dense corbular SR is abundantly distributed (85). These results strongly suggest that triadin is densely distributed in junctional SR but is absent from corbular SR in canine cardiac myofibers. Since these results are in contrast to those obtained previously in rabbit cardiac myofibers (59), the subcellular distribution of triadin was also reexamined in transversely and longitudinally sectioned rabbit atrial and ventricular myofibers,
using wheat germ agglutinin as a marker of the SL and T-tubules. The results of these studies, like those obtained in canine cardiac myofibers, strongly suggest that triadin is confined to junctional SR but not detected in corbular SR of rabbit cardiac myofibers.

The finding that triadin, like junctin (115), is confined to junctional SR, while the RyR is densely distributed in both junctional SR and corbular SR, is the second reported difference between the protein composition of junctional SR and that of corbular SR. The possibility that these differences reflect a difference in the mechanism of Ca$$^{2+}$$ release from junctional SR and corbular SR in cardiac myofibers remains to be explored.
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ABBREVIATIONS

a.a = amino acid
ab = antibody
ATPase = adenosine 5'-triphosphate
BSA = bovine serum albumin
$Ca^{2+}$ = calcium
CICR = calcium-induced calcium-release
CSQ = calsequestrin
cSR = corbular sarcoplasmic reticulum
DHPR = dihydropyridine receptor
E-C coupling = excitation contraction coupling
Endo-H = endoglycosidase-H
EXPM = electron probe X-ray microscopy
FITC = Fluorescein isothiocyanate
GST = glutathione-S-transferase
IEM = immunoelectron microscopical
IF = immunofluorescence
ijSR = internal junctional sarcoplasmic reticulum
$IP_3$ = inositol 1,4,5-triphosphate
jSR = junctional sarcoplasmic reticulum
mAb = monoclonal antibody
$\mu$m = micron
mM = millimolar
MV = microsomal vesicle
nm = nanometer

nSR = network sarcoplasmic reticulum

PBS = phosphate buffer saline

PLM = phospholemman

pjSR = peripheral junctional sarcoplasmic reticulum

RyR = ryanodine receptor/calcium release channel

SRD = microsomal vesicles enriched in junctional sarcoplasmic reticulum

SDS/PAGE = sodium dodecyl sulphate/polyacrylamide gel electrophoresis

SL = sarcolemma

SR = sarcoplasmic reticulum

T-tubule = transverse tubule

WGA = wheat germ agglutinin
CHAPTER 1

INTRODUCTION

A. EXCITATION CONTRACTION COUPLING IN CARDIAC AND SKELETAL MUSCLE.

Excitation-contraction (E-C) coupling is the process in which an action potential at the surface membrane of a muscle fiber leads to the contraction of myofilaments within the cell. In cardiac and skeletal muscles Ca\(^{2+}\) plays a key role in the regulation of the contraction process. Following the depolarization of the SL and T-tubules, Ca\(^{2+}\) is released into the myoplasm from its site of storage in the lumen of the sarcoplasmic reticulum (SR). In response to the increase in the intracellular Ca\(^{2+}\) concentration, a contraction regulating complex that consists of the proteins troponin T, I and C and the protein tropomyosin undergoes conformational changes after Ca\(^{2+}\) has been bound by troponin C (8). In turn, this allows the contractile proteins actin and myosin to interact, which then causes the muscle fiber to contract (8). Upon repolarization of the SL the release of Ca\(^{2+}\) from the SR is inhibited and the Ca\(^{2+}\) which was released during depolarization is pumped back into the SR to bring about relaxation (1-3).

In order for Ca\(^{2+}\) to be released from the SR a message elicited during the depolarization of the SL/T-tubule must cross the gap between the SL/T-tubule membranes and the SR membrane. Unlike E-C coupling in skeletal muscle, where a direct mechanical coupling between L-type Ca\(^{2+}\) channels in the SL/T-tubules and Ca\(^{2+}\) channels in the SR is responsible for converting the depolarizing signal into Ca\(^{2+}\) release from the SR (4), E-C coupling in cardiac muscle requires a small amount of extracellular Ca\(^{2+}\) to enter the cell through L-type Ca\(^{2+}\) channels before a large amount of Ca\(^{2+}\) can be released from the SR (5). Once inside the cell the extracellular Ca\(^{2+}\) triggers SR Ca\(^{2+}\) release by binding to sites on the SR Ca\(^{2+}\) channels, causing them to open (5). This process, where the influx of a small amount of extracellular Ca\(^{2+}\)
leads to the release of a large amount of Ca^{2+} from intracellular stores, is called Ca^{2+} induced-Ca^{2+} release (CICR) (6).

Another fundamental difference between cardiac and skeletal muscle is that while the extent of contractions is an all or none phenomenon in skeletal muscle, it is well established that different agents in the circulation, including neurotransmitters, hormones and a variety of pharmacological agents (inotropic agents), enable myocardial cells to change both the rate and extent of their contractions and thus respond to varying demands from the circulation (7). Since the SR in cardiac muscle is primarily responsible for regulating the intracellular Ca^{2+} fluxes that occur during E-C coupling (8,15), it is likely that it might also play a role in regulating cardiac muscle contractions. However, to understand the potential role of the SR in this process, a brief review of the ultrastructure of the SR along with the identification, characterization and subcellular distribution of some of the proteins of which it is composed follows below.

**B. THE STRUCTURE OF THE SARCOPLASMIC RETICULUM IN MAMMALIAN CARDIAC MUSCLE.**

In cardiac myocytes, the Ca^{2+} responsible for causing muscle contraction is stored in a specialized form of smooth endoplasmic reticulum called the sarcoplasmic reticulum or SR (9,10). Ultrastructural studies have revealed that the SR in mammalian ventricular myofibers is composed of three distinct but continuous domains, which are referred to as the network SR (nSR), junctional SR (jSR), and corbular SR (cSR) (fig 1) (9,10). Network SR, which forms an anastomosing network of 25-60 nm sarcotubules that surrounds the myofibrils from Z-line to Z-line along the length of the sarcomere, comprises a majority of the membrane system (118). Junctional SR, the second type of SR, is intimately associated with the sarcolemma and its transversely oriented tubular extensions the T-tubules, which are localized in the plane of the Z-line of the sarcomere. Junctional SR can form peripheral junctional SR (pjSR) by abutting with the sarcolemma at the periphery of the myofiber. It can also form interior junctional SR (ijSR), or
diads/triads in the interior of the I-band close to the Z-line by abutting with T-tubules (118). Ultrastructurally, junctional SR is characterized by the presence of an electron dense material in its lumen (9,10) and regularly spaced electron dense “feet” like processes protruding from its surface (9). These “feet” span the gap between the junctional SR membrane and the SL/T-tubules and appear to make contact with them. The T-tubule/SR junction in cardiac muscle is thought to be significant because of its similarity to the T-tubule/SR junction in skeletal muscle. In skeletal muscle this complex is the site responsible for transmitting the depolarizing signal from the SL to the SR (4,101).

![Diagram of mammalian cardiac muscle showing position of corbular SR (cSR), peripheral junctional SR (pJSR), interior junctional SR (iJSR), and network SR (nSR), in relation to the T-tubules (TT), and sarcolemma (SL). Adapted from ref. 9](image-url)
Corbular SR, the third type of SR, like ijSR, is found in the interior regions of ventricular myofibers but in regions where T-tubules fail to develop (9). Corbular SR is composed of 50-100 nm cisternal expansions of the network SR. Like ijSR, corbular SR is mainly localized in the I-band region of the sarcomere close to the Z-line and like ijSR it also has an electron dense material in its lumen and "feet" like processes protruding from its surface (9,11). Unlike ijSR however, the "feet" like processes which extend from the surface of corbular SR do not make contact with either the SL or the T-tubules and are found at various distances from them (9,11). It should also be noted that some mammalian myocardial cells, such as Purkinje fibers and most atrial myofibers, do not contain T-tubules and therefore lack ijSR. However, these myofibers do contain pjSR and corbular SR, which is densely distributed in their interior regions. (9,11).

C. THE SUBFRACTIONATION OF SARCOPLASMIC RETICULUM MEMBRANE VESICLES.

Although junctional and corbular SR are continuous with the network SR, network SR does not contain an electron dense material in its lumen nor does it have "feet" like structures protruding from its surface. This ultrastructural difference suggests that the three distinct domains of the SR may have different physiological functions and this assumption has been supported by recent biochemical studies. In these studies two unique subfractions of cardiac SR were identified and characterized. One of these was capable of active Ca\(^{2+}\) uptake and was proposed to correspond to network SR (12, 33), while the second was capable of releasing Ca\(^{2+}\) in response to Ca\(^{2+}\) and was proposed to correspond to junctional SR (13). Network SR vesicles were found to be enriched in the SR Ca\(^{2+}\)-ATPase (12) and its regulator phospholamban (12) and are believed to represent the site where Ca\(^{2+}\) uptake into the SR occurs during relaxation. Junctional SR vesicles on the other hand were enriched in proteins believed to be involved in Ca\(^{2+}\) storage and Ca\(^{2+}\) release (13-17), and are thought to correspond to the site in the lumen of the SR where the Ca\(^{2+}\) accumulated by the Ca\(^{2+}\)-ATPase
is sequestered. A membrane subfraction corresponding to corbular SR has not yet been identified, however, the fact that skeletal muscle does not contain corbular SR raises the possibility that corbular SR may have a role which is unique to E-C coupling in cardiac muscle, such as serving as a secondary site of Ca\(^{2+}\) release (to be discussed later).

While a great deal is known about the role of the Ca\(^{2+}\)-ATPase and phospholamban in the process of SR Ca\(^{2+}\) uptake (15), much less is known about the mechanism that regulates the release of Ca\(^{2+}\) from the SR. To better understand the mechanism by which junctional SR releases Ca\(^{2+}\), researchers have purified and characterized several proteins from junctional SR vesicles. These proteins include the ryanodine receptor/Ca\(^{2+}\) release channel (13,14), calsequestrin (15), junctin (16) and triadin (17). A brief review of the structure and function of these proteins and their subcellular distribution in the various domains of the SR follows below.

D. JUNCTIONAL SARCOPLASMIC RETICULUM PROTEINS BELIEVED TO BE INVOLVED IN Ca\(^{2+}\) STORAGE AND RELEASE FROM THE SARCOPLASMIC RETICULUM.

1. The Ryanodine Receptor (RyR)/Ca\(^{2+}\) Release Channel.

Following the discovery of ryanodine, a plant alkaloid that can bind with high affinity to Ca\(^{2+}\) release channels locking them in an open state, radiolabeled ryanodine binding experiments were used to purify the RyR from skeletal (18-20), and cardiac muscle (21). It was observed that the purified RyR, which consisted of a square or quatrefoil shaped particle of ~20 nm on each side with a 2 nm hole in the center (18,19,21,22), was identical in shape to the "feet" structures which project from the surface of the SR (18,21). The reconstitution of the purified receptor into planar lipid bilayers also revealed that the purified protein was capable of forming Ca\(^{2+}\) channels that exhibited activity similar to those described for Ca\(^{2+}\) channels in native SR vesicles (19,23,102). These findings lead to the conclusion that the feet structures that extend from the surface of the SR were actually Ca\(^{2+}\) channels.
Three different RyR genes have been identified in mammals and have been designated RyR-1, RyR-2 and RyR-3 (14, 25). RyR-1 is primarily expressed in fast and slow skeletal muscle (14,25), while RyR-2 is primarily expressed in cardiac muscle (14, 26), however, both RyR-1 and RyR-2 are also expressed at lower levels in brain (24,14). RyR-3 is predominantly expressed in brain (14, 27), but is also expressed at lower levels in other tissues, including smooth, cardiac and skeletal muscle (14,24). Each of the three RyRs is predicted to consist of four identical polypeptide subunits (28,29). Each polypeptide subunit is predicted to have four transmembrane segments located near its C-terminal end, however, as many as 12 transmembrane segments have been suggested for the skeletal (30), and cardiac muscle (26), Ca\textsuperscript{2+} channel isoforms. It is believed that the transmembrane segments in each of the four polypeptide subunits combine to form the pore through which Ca\textsuperscript{2+} is released (22,28,29). The N-termini of each polypeptide subunit, like the extreme C-termini, is predicted to be cytoplasmic. The N-termini in each subunit of the Ca\textsuperscript{2+} channel is also thought to combine to form the foot structure which surrounds the central pore and projects away from the SR membrane towards the SL/T-tubules (22,28,29).

Although the cardiac and skeletal muscle RyR Ca\textsuperscript{2+} channels share similar three dimensional structures and show 66 % identity at the level of their amino acid sequence, (31) the cardiac RyR is more sensitive to Ca\textsuperscript{2+} than its skeletal muscle counterpart (5,15), a finding which is consistent with the Ca\textsuperscript{2+} induced-Ca\textsuperscript{2+} release mechanism of E-C coupling in cardiac muscle (5). It has also been reported that RyR-2, the cardiac Ca\textsuperscript{2+} channel, can also be activated following its phosphorylation by a cAMP activated kinase (32). This suggests that \(\beta\)-adrenergic agonists, which activate cAMP activated kinase, may change the rate and extent of cardiac muscle contraction by modulating the rate at which the RyR releases Ca\textsuperscript{2+} from the SR. The cardiac Ca\textsuperscript{2+} channel may also be regulated by several endogenous effectors. These effectors include Mg\textsuperscript{2+}, which inhibits the RyR by displacing Ca\textsuperscript{2+} from its stimulatory site (31);
adenine nucleotides, which activate the RyR (34) and the cytoplasmic Ca^{2+}-dependent enzyme regulator calmodulin, which may inactivate the Ca^{2+} channel (31,34). However, the functional impact of these different interactions has not yet been determined in vivo.

2. Calsequestrin (CSQ).

It has been suggested that the ability of the SR to store Ca^{2+} at concentrations that approach the mM range is dependent on the Ca^{2+} binding protein CSQ (13,15,35-39). CSQ, which is thought to correspond to the electron dense material observed in the lumen of the SR (40,41), was first isolated more than twenty years ago from skeletal muscle (35,36), and several years later it was also identified and purified from cardiac muscle (37,65). The skeletal and cardiac muscle CSQ proteins, which are the products of different genes, show 65% homology at the level of their amino acid sequence and have been named fast skeletal muscle and cardiac CSQ respectively (38). CSQs are very acidic proteins, having many of their acidic residues located in the carboxy terminal fourth of the molecule and in clusters of two or three amino acids. It is thought that the clusters of acidic amino acids may be involved in Ca^{2+} binding, as sequence analysis of the CSQs reveal that they do not to contain any molecularly recognizable Ca^{2+} binding sites such as E-F hand structures (15,38,64,65).

Because CSQ is a luminal peripheral SR protein (64,65), one important question that arises is how is the protein selectively retained in the lumen of the junctional SR? Recent biochemical studies have identified two proteins, one named junctin (16,42), and the other named triadin (43-45), which may be responsible for retaining CSQ in the lumen of junctional SR. Although junctin and triadin are the products of different genes, the two proteins have several features in common. Both junctin and triadin are single pass transmembrane proteins with similar membrane topologies (16,17,44,47). Additionally, the amino acid sequence of the transmembrane segments of the two proteins are 62 % identical, suggesting that they may belong to the same family.
3. Junctin.

Junctin, a 26 kDa protein, was originally identified as the major CSQ binding protein in junctional SR vesicles isolated from cardiac and skeletal muscle (16,51). The cDNA sequence of junctin predicts a single pass transmembrane protein that consists of 210 amino acids (16). The protein is predicted to have a short cytoplasmic N-terminal region (a.a. 1-22), while the C-terminal region and the remainder of the protein is predicted to reside in the lumen of the SR (16). The luminal segment of the protein also contains long stretches of alternating lysine and glutamic acid residues (KEKE repeats), which are believed to be involved in protein-protein interactions (48-50).

4. Triadin.

a. Skeletal Muscle Triadin

Triadin was originally identified as a component of the triad junction of skeletal muscle and it was reported to bind to both the L-type Ca$^{2+}$ release channel of the SL and T-tubules and the RyR during protein overlay assays and affinity chromatography experiments (52,53). On this basis it was proposed that triadin functions in E-C coupling by forming a physical link between the L-type Ca$^{2+}$ channel and the RyR (54). However, further studies, as we shall see later, have led other investigators to different conclusions regarding the ability of triadin to physically link the L-type Ca$^{2+}$ channel to the RyR.

The cDNA of triadin has been cloned and sequenced from rabbit (17) and human (47) skeletal muscle. The cDNA from rabbit predicts a protein with a molecular mass of ~79 kDa, while the cDNA from human predicts a protein with a molecular mass of ~82 kDa. The two proteins are predicted to be 77% identical at the amino acid level (47). Western blotting with a monoclonal antibody to rabbit skeletal muscle triadin suggests that triadin has an apparent $M_r$ of ~94 kDa on reducing SDS/PAGE (17). In the presence of nonreducing agents such as N-ethylmaleimide, triadin migrates as a series of high molecular mass multimers (17), suggesting
that disulfide bonds formed between triadin molecules and/or other SR proteins may be important in the regulation of Ca$^{2+}$ release from the SR of skeletal muscle (55,56).

Hydrophobicity analysis of the triadin cDNA (17,47), suggests that triadin is a single pass transmembrane protein that has a short cytoplasmic N-terminus that consists of only 47 amino acids. The majority of the protein, including the C-terminus, is thought to reside in the lumen of the SR. The membrane topology proposed for triadin is supported by vesicle protection assays (17,57). Thus, it seems as though triadin's cytoplasmic segment may be too short to bridge the gap between the SR and the T-tubules and as a result, it seems unlikely that triadin interacts with the L-type Ca$^{2+}$ channel (17). However, since the cDNA for triadin predicts that the luminal domain of the protein is highly positively charged, due to an excess of basic residues, it is likely that a non-specific ionic interaction may have occurred between triadin and the L-type Ca$^{2+}$ channel during the protein binding experiments reported by Brandt et al. (52), and Kim et al. (53).

b. Rabbit Cardiac Muscle Triadin

Triadin was initially thought to be a skeletal muscle specific protein because Western blot analysis, using antibodies directed against the C-terminus of rabbit skeletal muscle triadin and Northern blot analysis, using partial cDNA sequences from rabbit skeletal muscle triadin, failed to detect triadin in rabbit cardiac tissue (17). However, the protein was subsequently detected in cardiac tissue (58,59), and three different triadin cDNAs, which are thought to arise by alternative splicing of the same gene that encodes the skeletal muscle triadin isoform (47), have recently been cloned and sequenced from rabbit cardiac muscle (44). The three cardiac isoforms have predicted M$_r$s of ~31 kDa, ~35 kDa and ~75 kDa and are referred to as triadin-1, triadin-2 and triadin-3 respectively. The amino acid sequence of the 32 and 35 kDa isoforms are predicted to be identical to each other and to the skeletal muscle triadin isoform from amino acid 1-264. The large cardiac triadin isoform, unlike the smaller isoforms, is predicted to be identical
to the skeletal muscle triadin isoform throughout most of its length (aa 1-665). However, the cDNAs of the large and small triadin isoforms also predicts that each protein has a distinct amino acid sequence at its C-terminal end (44). Hydrophobicity analysis of the three triadin cDNAs (44), predicts that the three isoforms (fig 2), are single pass transmembrane proteins which adopt a similar membrane topology as their skeletal muscle counterpart and like their skeletal muscle counterpart, the three cardiac triadin isoforms also contain long stretches of basic residues and alternating KEKE repeats within their luminal segment.

The uniqueness of the C-terminus of the three cardiac triadin isoforms and distinctness from that of skeletal muscle triadin explains why earlier attempts to detect triadin in cardiac muscle, using antibodies which were directed against the C-terminus of skeletal muscle triadin, were unsuccessful (17). However, the subsequent Western blotting of rabbit cardiac microsomes, with polyclonal antibodies directed against the conserved luminal domain of triadin (generic triadin), detected three electrophoretic bands that had Mₐₛ that were slightly larger than the Mₐₛ predicted for triadin-1, triadin-2 and triadin-3. The three electrophoretic bands migrated with Mₐₛ of 35, 40 and 92 kDa and are thought to correspond to the triadin-1,2 and 3 respectively (44). It was also observed that there was no major difference in the migration pattern of cardiac triadin in the presence of either β-mercaptoethanol or N-ethylmaleimide, suggesting that disulfide bond formation between triadin molecules and/or other SR proteins may not be required for Ca²⁺ release from the SR of cardiac muscle (44). The significance of there being three isoforms of triadin in rabbit cardiac tissue, verses one in skeletal muscle, remains uncertain. However, the presence of additional triadin isoforms in cardiac tissue may be responsible for the difference in the mechanism by which Ca²⁺ is released from cardiac versus skeletal muscle.
**MODEL SHOWING THE PROPOSED MEMBRANE TOPOLOGY OF THE THREE RABBIT CARDIAC TRIADIN ISOFORMS**

![Diagram showing the membrane topology of the three rabbit cardiac triadin isoforms.](image)

**CYTOPLASM**

**SR MEMBRANE**

**SR LUMEN**

- conserved with skeletal muscle triadin
- unique to 40 kDa isoform
- unique to 92 kDa isoform
- unique to 35 kDa isoform

$S =$ position of cysteine residue

Fig 2. Model showing the membrane topology proposed for the three isoforms of rabbit cardiac triadin. Adapted from ref. 44.

c. **Canine Cardiac Muscle Triadin**

Recent Western blots of canine junctional SR vesicles with antibodies directed against the conserved luminal domain of rabbit triadin (generic triadin), suggested that canine cardiac myofibers express two predominant isoform of triadin (42). In these studies two electrophoretic bands were detected that had predicted $M_s$ of 35 and 40 kDa, the band detected at 92 kDa in rabbit was barely detectable (42). The predicted $M_s$ of these electrophoretic bands, combined with the fact that they were detected with antibodies to rabbit triadin, suggests that they may be homologous to rabbit triadin-1 and triadin-2. However, subsequent attempts to isolate the cDNAs that code for the proteins that give rise to these electrophoretic bands produced results which differed from those reported in the rabbit.
Recently Kobayashi and Jones (98), have cloned and sequenced a single triadin cDNA from canine cardiac myofibers and they have reported that it is highly similar to the triadin-1 cDNA isolated from rabbit cardiac myofibers (44). Like the triadin-1 cDNA from the rabbit, the canine triadin cDNA predicts a protein of ~ 31 kDa. (98). Additionally, the expression of the canine triadin cDNA in Sf21 insect cells showed that the purified protein exhibited an electrophoretic mobility that was indistinguishable from the 35 kDa protein detected in canine junctional SR vesicles and like generic triadin form rabbit cardiac myofibers (44), the recombinant canine triadin protein also bound to CSQ (98).

In addition to isolating the canine triadin-1 cDNA, Kobayashi and Jones (114) have also developed antibodies both to the unique C-terminus of triadin-1 (triadin-1u), and to the conserved luminal domain (triadin-1c), of the protein. Subsequent Western blotting of canine atrial and ventricular SR microsomes using these recently developed antibodies also detected two prominent electrophoretic bands that had predicted M₆s of 35 and 40 kDa (see fig 3) (114). This was rather unexpected however, for although the triadin-c antibody was expected to detect two electrophoretic bands, the triadin-1u antibody was only expected to detect the 35 kDa band that corresponds to triadin-1 (44).

Since the triadin-1u antibody was able to detect the electrophoretic band that correspond to triadin-2, it is possible that the C-termini of triadin-1 and triadin-2 may be identical. However, treatment of the canine cardiac microsomes with Endo H prior to performing Western blots, either with the triadin-1u or the triadin-1c antibodies, resulted in the disappearance of the 40 kDa electrophoretic band and led to an increase in the intensity of the 35 kDa band. These results suggest that canine triadin-1 exists in two forms which have identical amino acid sequences but which differ in the degree to which they are glycosylated.
Fig 3. Immunoblots showing the species specificity of pAb triadin-lu and pAb triadin-lc antibodies. The triadin-lu antibody recognizes two bands that had Ms of 35 and 40 kDa in canine cardiac (lane 1), and canine atrial (lane 11), MVs and in ventricular SRD (lane 12). The triadin-lc antibody also detects two bands with Ms of 35 and 40 kDa in canine cardiac (lane 1), and canine atrial (lane 11), MVs and in ventricular SRD (lane 12). The antibody also detects a single ~60 kDa band in slow (lane 2), and fast (lane 3), skeletal muscle MVs. The abbreviations used are: MV (microsomal vesicles), and SRD (microsomal vesicles enriched in junctional sarcoplasmic reticulum) (Western blot courtesy of Y. Kobayashi and L.R. Jones).

(114). These results also suggest that triadin-1 is the major triadin isoform expressed in canine cardiac myofibers. Kobayashi and Jones (114) have also reported that the 35 and 40 kDa electrophoretic bands detected in cardiac SR vesicles isolated from rabbit, rat, mouse, guinea pig and human cardiac myofibers are also Endo-H sensitive. They have also suggested that triadin-1 is also the predominant isoform expressed in these tissues.

E. INTERACTION BETWEEN THE SARCOPLASMIC RETICULUM EXCITATION CONTRACTION COUPLING PROTEINS.

It was initially believed that the sole function of CSQ was to act as a Ca^{2+} buffer within the junctional SR lumen. However, it is now thought that CSQ may also play an important role in the regulation of Ca^{2+} release from the SR of striated muscle. Biochemical studies have suggested that the amount of Ca^{2+} released from SR vesicles and the rate constant of release
was dependent on the amount of \( \text{Ca}^{2+} \) bound to CSQ (60). Biochemical studies have also suggested that CSQ is capable of undergoing conformational changes following exposure to increasing \( \text{Ca}^{2+} \) concentrations (38,39,51,60). Interestingly, it has also been reported that the \( \text{Ca}^{2+} \) dependent conformational changes induced in CSQ can bring about changes in the conformation of SR \( \text{Ca}^{2+} \) release domain proteins such as the RyR (20). This suggests that conformational changes in CSQ serve as signals which regulate the kinetic properties of the RyR (60). It has also been reported that CSQ must be associated with the SR \( \text{Ca}^{2+} \) release domain if the protein is to release \( \text{Ca}^{2+} \) during depolarization induced \( \text{Ca}^{2+} \) release. This suggests that depolarization induced conformational changes in the RyR are transmitted to CSQ, causing it to release \( \text{Ca}^{2+} \) (62). The \( \text{Ca}^{2+} \) released from CSQ is then thought to increase the intraluminal \( \text{Ca}^{2+} \) concentration of the SR. In turn, this facilitates the binding of \( \text{Ca}^{2+} \) to regulatory sites on the RyR which must be occupied before the \( \text{Ca}^{2+} \) release channel can be opened (62). These and other results (63) suggest that CSQ may be functionally coupled to the RyR, however, because CSQ is a luminal protein (64,65) which does not bind directly to the RyR while in a \( \text{Ca}^{2+} \) bound state (51), it has been suggested that additional proteins may mediate the interaction between CSQ and the RyR.

Electron microscopical studies have suggested that CSQ forms aggregates in the lumen of both skeletal (40), and cardiac muscle junctional SR (41). These studies have suggested that CSQ may be anchored to the \( \text{Ca}^{2+} \) release domain of the SR by filamentous strands which are composed of proteins different from CSQ. It is possible that these filamentous strands may also be responsible for linking CSQ to the RyR (42,43,44). Recently, Guo and Campbell (43), and Guo et. al. (44), reported that a GST fusion protein, containing residues 110-264 from the lysine and glutamic acid (KEKE) rich conserved luminal domain of rabbit triadin, was capable of binding to both the RyR and CSQ in detergent extracts prepared from skeletal muscle homogenates (43), and cardiac microsomes (44). Because of its CSQ binding properties, it was
proposed that triadin may be responsible for anchoring CSQ to the Ca\textsuperscript{2+} release domain of the SR in rabbit skeletal (43), and cardiac muscle (44). Furthermore, triadin's ability to bind to the RyR suggests that triadin may functionally couple CSQ to the RyR (43,44). However, because the fusion protein used to characterize rabbit cardiac triadin was derived from the conserved luminal domain of the triadin molecule, it is unclear whether each of the three triadin isoforms play an equal role in functionally coupling CSQ to the RyR.

Recently, Zhang et. al. (42), have also reported that junctin, through its lysine and glutamic acid rich luminal segment, can also bind to both CSQ and the RyR. They have also reported that junctin, the RyR and CSQ are capable of interacting with the KEKE rich conserved luminal domain of triadin. On this basis, Zhang et. al. (42), have suggested that triadin, junctin, CSQ and the RyR are capable of forming a complex in junctional SR (fig 4). They have also suggested that junctin and triadin, in addition to coupling CSQ to the RyR, may also regulate the release of Ca\textsuperscript{2+} from the SR of striated muscle. However, the physiological significance of these interactions is not as yet fully understood.

F. SARCOLEMMA AND T-TUBULE PROTEINS RELEVANT TO EXCITATION CONTRACTION COUPLING: THE DIHYDROPYRIDINE RECEPTOR/L-TYPE Ca\textsuperscript{2+} RELEASE CHANNEL.

The dihydropyridine receptor/L-type Ca\textsuperscript{2+} release channel (DHPR) is the principal voltage gated Ca\textsuperscript{2+} channel in the SL and T-tubules (66-68). The cardiac isoform of the DHPR is a heterotetramer consisting of subunits, named α-1, α-2, β and δ (69). The α-1 subunit of the DHPR functions as both the DHPR voltage sensor and the channel forming subunit (66-68). The remaining components of the DHPR are thought to modulate the activation and inactivation kinetics of the α-1 subunit, as well as targeting and stabilizing the α-1 subunit in the SL and T-tubule membranes (70,71). The α-1 polypeptide is predicted to contain four repeated domains which are very similar (67). Each of the four repeated domains is predicted to contain six
transmembrane segments which are thought to combine to form the pore of the Ca\(^{2+}\) channel (67). Because the fourth transmembrane segment in each of the four repeated domains contains an ordered pattern of positive charges, it is thought that they may function as voltage sensors (67).

![Diagram showing the arrangement of known E-C coupling proteins]

Fig 4. Model showing the distribution of the proteins believed to be involved in E-C coupling. Adapted from ref 42.

**G. SPARKS AND EXCITATION CONTRACTION COUPLING.**

Since we are now familiar with some of the major proteins thought to be involved in cardiac E-C coupling, we can now begin to explain some of its molecular features. Confocal imaging of isolated ventricular myofibers, which had been loaded with the Ca\(^{2+}\) sensitive probe fluo-3, revealed that the Ca\(^{2+}\) released from the SR during E-C coupling can be visualized as 'Ca\(^{2+}\) sparks' (72-75). Ca\(^{2+}\) sparks are short lived (30-100 ms) localized (2-4 \(\mu\)m), increases in the intracellular Ca\(^{2+}\) concentration proposed to arise from the opening of a few RyRs acting as
a functional unit (75). It has been suggested that the depolarization induced opening of a single DHPR could produce a spark by activating a single RyR functional unit at the Z-line where junctional SR is localized (73,74,77). It was also observed that these depolarization induced Ca\(^{2+}\) sparks had amplitudes and spatio-temporal properties which appeared to be independent of the duration and voltage of the depolarizing signal. Furthermore, they behaved in a stereotypical manner even though the unitary L-type Ca\(^{2+}\) current, their trigger signal, was made to vary (72). It was also observed that the depolarization induced Ca\(^{2+}\) sparks were identical to those occurring spontaneously due to the activation of RyR functional units by the resting cytosolic Ca\(^{2+}\) concentration (72-74, 77). Combined, these results led to the conclusion that Ca\(^{2+}\) sparks reflect the elementary Ca\(^{2+}\) release events underlying cardiac E-C coupling. It was also concluded that modulation of Ca\(^{2+}\) release from the SR was due in part to the number of Ca\(^{2+}\) sparks or functional Ca\(^{2+}\) release units recruited (75). In turn, the number of sparks or functional Ca\(^{2+}\) release units recruited would depend on the strength of the trigger signal, the sensitivity of the RyR to Ca\(^{2+}\) and the transmission of the Ca\(^{2+}\) signal across the coupling space between the SL/T-tubule and junctional SR membranes (75).

Since the influx of a small amount of extracellular Ca\(^{2+}\) can lead to the release of a large amount of Ca\(^{2+}\) from the SR, it is possible that the depolarization induced release of Ca\(^{2+}\) from the SR, which is 10 to 65 times greater than that through the DHPR (78,79), could cause additional Ca\(^{2+}\) efflux from the SR via a positive feedback or regenerative mechanism. However, this does not occur for several reasons. First, it has been proposed that RyRs are fairly insensitive to Ca\(^{2+}\) in the cytosol (72, 73). This proposal was based on the low frequency of occurrence of spontaneous Ca\(^{2+}\) sparks (75) and on the observation that neither spontaneous nor evoked Ca\(^{2+}\) sparks activate further Ca\(^{2+}\) release from neighboring release units, i.e. no regenerating Ca\(^{2+}\) release (72, 73). Combined with the observation that the RyR release units appear to close spontaneously and independently of the duration of single L-type channel flux
and the duration of depolarization (80), Cheng et al. (75), have suggested that under normal conditions Ca\(^{2+}\) sparks do not activate each other and once a spark has been produced by a cluster of RyRs, the spontaneous closure of the channels ensures that they do not behave in an uncontrolled regenerative manner. It has also been observed that RyR clusters are separated by a distance of 30 nm (41), while the foot portion of the RyR fills the 10 nm gap between the SR and the T-tubules (41), suggesting that the distance between the DHPR and the RyR is less than the distance between RyR clusters. It has also been suggested that the diffusion of Ca\(^{2+}\) inside muscle fibers is greatly inhibited due to the presence of buffering proteins (81), the extrusion of Ca\(^{2+}\) from the cell via the Na\(^+\)/Ca\(^{2+}\) exchanger (8) and the uptake of Ca\(^{2+}\) by the SR Ca\(^{2+}\)-ATPase (8). These findings suggest that a DHPR that is particularly close to specific cluster of RyRs will produce larger changes in the intracellular Ca\(^{2+}\) concentration in the region of that RyR cluster and will be in a better position to elicit a spark from that cluster than will the spark form a neighboring cluster (79). Thus, under normal conditions the uncoupling of each SR release unit from its neighboring release unit and the close apposition of SR release units to DHPRs ensures that SR Ca\(^{2+}\) release can be activated without it leading to a positive feedback process, i.e. CICR form neighboring RyR clusters.

During E-C coupling it is thought that Ca\(^{2+}\) entry through the DHPR produces a local increase in the intracellular Ca\(^{2+}\) concentration in the vicinity of a RyR functional unit. Although the Ca\(^{2+}\) that enters the cell through the L-type Ca\(^{2+}\) channel is too low in concentration to produce a spark or activate myofilament contraction, it is enough to directly activate the RyR functional unit by CICR (75). Once activated, the RyRs in the functional unit undergo conformational changes which could then be transferred, possibly via junctin or triadin, to CSQ, causing CSQ to release its bound Ca\(^{2+}\). The resulting increase in the intraluminal Ca\(^{2+}\) concentration of the SR would then be expected to facilitate the binding of Ca\(^{2+}\) to regulatory sites on the RyR (62). Once activated, the RyRs within the functional unit open and allow Ca\(^{2+}\)}
to be released into the myoplasm at concentrations which are high enough to produce a spark. Callewaert (76), has suggested that since most of the Ca²⁺ from a single RyR functional unit will be removed from the myoplasm or will be buffered, the amount of Ca²⁺ reaching neighboring functional units will be insufficient to activate those functional units and as a result, no further amplification of the extracellular trigger Ca²⁺ will occur and the myocyte will not contract. However, if the depolarizing signal is of significant amplitude or duration to activate a large number of L-type Ca²⁺ channels, then a large number of RyR functional units will be recruited and the Ca²⁺ buffering and Ca²⁺ removal systems of the cell will not be able to compensate for the large amount of Ca²⁺ released (76). Therefore, more Ca²⁺ will reach other RyR functional units, leading to their activation and a large enough amplification of the extracellular trigger Ca²⁺ to cause muscle contraction (75,76). The process is stopped by the spontaneous closure of the SR Ca²⁺ release channels and the subsequent extrusion of Ca²⁺ by the Na⁺/Ca²⁺ exchanger and re-uptake of Ca²⁺ by the SR Ca²⁺-ATPase.

H. THE LOCALIZATION OF SARCOPLASMIC RETICULUM PROTEINS AND Ca²⁺ IN THE SARCOPLASMIC RETICULUM OF MAMMALIAN CARDIAC MYOFIBERS.

1. Calsequestrin and Calcium.

Previous biochemical studies (12,13), have suggested that the SR can be divided into two distinct subfractions, the first corresponds to network SR, while the second corresponds to junctional SR. The subfraction that corresponds to network SR was found to be enriched in the SR Ca²⁺-ATPase (12) while the subfraction that corresponds to junctional SR was found to be enriched in several proteins, including CSQ (13). These biochemical studies suggest that the SR Ca²⁺-ATPase should be confined to network SR, while proteins involved in Ca²⁺ storage and Ca²⁺ release, such as CSQ, should be confined to junctional SR insitu. The results of these biochemical studies have been supported by subsequent IEM studies. These studies have shown that the SR Ca²⁺-ATPase (86), and its regulator phospholamban (87), are uniformly
distributed in the network SR, but absent from the junctional SR of cardiac myofibers. These studies, as anticipated, have also demonstrated that CSQ can be detected in the junctional SR, but not in the network SR, of atrial and ventricular myofibers (91). Surprisingly, these studies (91), also revealed that CSQ can be detected in the corbular SR of these myofibers. In fact, Jorgensen et. al. (91), have estimated that ~40 % of the CSQ containing SR in rat ventricular myofibers corresponds to corbular SR. These findings and the results of other immunofluorescence localization studies (89,90), suggest that not only junctional SR but also corbular SR could serve as potential Ca\textsuperscript{2+} storage and release sites because they both contain the Ca\textsuperscript{2+} binding protein CSQ. In support of this proposal, electron probe x-ray microanalysis studies of the subcellular distribution of calcium in resting rat papillary muscle have shown that both the CSQ containing junctional SR (92,93) and corbular SR (92), have a high concentration of calcium, while the network SR, which lacks CSQ, has a very low concentration of calcium.

2. The Ryanodine Receptor.

Assuming that corbular SR is a potential Ca\textsuperscript{2+} release site, then the mechanism by which junctional and corbular SR release Ca\textsuperscript{2+} must differ because corbular SR is too far away from the SL and T-tubules to be directly activated by depolarization of the SL/T-tubule membranes. This suggests that junctional and corbular SR may contain different Ca\textsuperscript{2+} release channels. However, biochemical studies (21), have reported that the purified RyR from cardiac SR is identical in shape to the feet like structures that project form the surface of junctional and corbular SR in cardiac muscle (9). This suggests that the purified cardiac RyR is the Ca\textsuperscript{2+} release channel in both junctional and corbular SR. However, because of the similarity in physical characteristics between the tetrameric RyR (21), and the tetrameric IP\textsubscript{3} receptor (107), and because several studies (108,109), have reported that IP\textsubscript{3} can cause both an increase in the intracellular free Ca\textsuperscript{2+} concentration and the contraction of skinned cardiac myofibers, it is also reasonable to propose that the feet like structures associated with junctional and/or
corbular SR may represent IP\textsubscript{3} receptors. Thus, while the RyR may be the Ca\textsuperscript{2+} release channel in junctional SR, the IP\textsubscript{3} receptor may be the Ca\textsuperscript{2+} release channel in corbular SR or vice versa.

To directly determine whether junctional and/or corbular SR contain the RyR, as has been suggested by biochemical studies, Jorgensen et. al. (85), have determined its subcellular distribution in rat papillary myofibers by IEM labeling and in sheep Purkinje myofibers by immunofluorescence labeling. Jorgensen et. al. (85), have reported that the RyR is present in the internal and peripheral junctional SR and the corbular SR of rat papillary myofibers, but is absent from the network SR. These researchers (85), have also reported that the RyR can be detected at the periphery and in the interior of sheep Purkinje fibers. Because Purkinje fibers lack T-tubules/interior junctional SR, Jorgensen et. al. (85), concluded that the RyR was localized both to peripheral junctional SR and corbular SR (85).

These results suggest that the RyR is not confined only to junctional SR, but is also present in significant amounts in corbular SR, where it codistributes with CSQ and calcium. These results also suggest that both junctional and corbular SR have the potential to release Ca\textsuperscript{2+} via the RyR. However, the fact that corbular SR does not make contact either with the SL or the T-tubules suggests that corbular SR is not triggered to release Ca\textsuperscript{2+} by the depolarization of the SL/T-tubules. Instead, corbular SR may be triggered to release Ca\textsuperscript{2+} by a diffusible agent, possibly the Ca\textsuperscript{2+} that is released from the junctional SR following the depolarization of the SL and T-tubules (5,96).

3. Junctin.

The results of RyR (85), CSQ (89-91), and calcium (92,93), localization studies suggest that they are present in both junctional and corbular SR. It has previously been reported that junctin is the major CSQ binding protein in junctional SR vesicles (16,51). Therefore, it was not surprising that our previous indirect immunofluorescence localization studies (16), suggested that junctin was localized to the I-band region in canine ventricular myofibers, an area where
both junctional and corbular SR are found. However, since both interior junctional SR and corbular SR are present within the I-band region in ventricular myofibers (9), and since it is difficult to differentiate between these two domains in the absence of a marker of either junctional or corbular SR at the light microscopic level of resolution, we were unable to determine whether junctin was indeed present in both junctional and corbular SR. However, subsequent immunolocalization studies, which examined the subcellular distribution of junctin, not only in ventricular myofibers, but also in atrial and Purkinje myofibers, led to other conclusions regarding the subcellular distribution of the protein.

In subsequent studies aimed at localizing junctin, Jorgensen et. al. (95), revealed that labeling for junctin colocalized with labeling for the RyR in the I-band of canine ventricular myofibers (95). However, Jorgensen et. al. (95), also observed that junctin-positive foci were confined to the junctional SR at the periphery of atrial and Purkinje fibers, while RyR-positive foci were detected both along the periphery and in the corbular SR rich I-band region of these myofibers (95). Because the atrial and Purkinje myofibers used in these studies did not contain T-tubules and thus interior junctional SR, the results presented by Jorgensen et. al., (95), strongly suggested that junctin is confined to the junctional SR of canine cardiac myofibers and is not detected in the CSQ and RyR rich corbular SR. In agreement with these results, Thompson et. al., (88), have also shown directly that junctin is densely distributed in junctional SR and not detected in corbular SR of canine cardiac myofibers by immunoelectron microscopical labeling.

Based on junctin’s CSQ and RyR binding properties, its colocalization with CSQ and the RyR in junctional SR and its absence from the corbular SR of cardiac muscle, it has been suggested that junctin may not be essential for coupling CSQ to the RyR in corbular SR (88). Since corbular SR, like junctional SR, contains calcium (92), CSQ (89-91), and the RyR (85), it stands to reason that corbular SR could serve as a potential Ca\(^{2+}\) storage and Ca\(^{2+}\) release site.
however, junctin's absence from corbular SR also suggests that junctional and corbular SR may have different roles in cardiac muscle E-C coupling.

Since immunoelectron microscopical studies have shown that the RyR (85), and CSQ (91), are localized to junctional and corbular SR, it stands to reason that antibodies to these proteins could serve as markers of junctional and corbular SR insitu. Since similar studies have shown that junctin (88), is confined to junctional SR, these results also imply that antibodies to junctin can be used as markers of junctional SR. Recently Jorgensen et. al. (116), have also reported that antibodies to phospholemmman (PLM), channel molecules (99), can be used to mark the SL and T-tubules so that interior junctional SR can be differentiated from corbular SR. Jorgensen et. al., (115), have suggested that it may be possible to use these markers to assess the relative amount of interior junctional SR and corbular SR in ventricular myofibers by immunofluorescence labeling. Jorgensen et. al., (115), have suggested that by showing that RyR-positive foci are localized both along the PLM-positive T-tubules and away from them (fig 5), while junctin-positive foci are localized only along the PLM-positive T-tubules (fig 6), they should be able to differentiate between interior junctional SR and corbular SR if they assume that the junctin and RyR foci that localize along the PLM-positive T-tubules correspond to the detection of the proteins in interior junctional SR, while RyR foci that localize away from the PLM labeled T-tubules correspond to the detection of the RyR in corbular SR. By selecting confocal images of canine ventricular myofibers in which the PLM labeled T-tubules produced a cartwheel like pattern, an indication that the myofibers were transverse, and using Photoshop
Fig 5. Model showing the subcellular distribution of phospholemman (PLM), and the ryanodine receptor (RyR), in atrial myofibers (A), and in ventricular myofibers and atrial myofibers that contain T-tubules (B).

Fig 6. Model showing the subcellular distribution of phospholemman (PLM), and junctin in atrial myofibers (A), and in ventricular myofibers and atrial myofibers that contain T-tubules (B).
software to enlarge the confocal images so that it is possible to differentiate between foci that either do or do not localize along the PLM-positive T-tubules. Jorgensen et. al., (115), found that \(~97\%\) of the junctin-positive foci and \(~70\%\) the RyR positive foci detected in these myofibers were localized along the PLM-positive T-tubules. They also found that \(~3\%\) of the junctin positive foci and \(~30\%\) of the RyR-positive foci detected in these myofibers did not localize along the PLM-positive T-tubules. These results suggest that \(~30\%\) of the RyR-positive foci detected in these myofibers may correspond to the detection of the RyR in corbular SR. These results, which are in good agreement with previous results that suggested that \(~40\%\) of the CSQ labeling detected in rat ventricular myofibers by immunoelectron microscopical localization corresponds to the detection of CSQ in corbular SR (91), suggest that corbular SR represents a substantial proportion of the potential Ca\(^{2+}\) storage and Ca\(^{2+}\) release sites in ventricular myofibers. Furthermore, since researchers are currently unable to biochemically subfractionate junctional and corbular SR, these results also suggest that antibodies to these proteins could serve as markers that may permit researchers to accurately determine the subcellular distribution of any newly discovered E-C coupling proteins.

4. Triadin.

Recently, Carl et. al. (59), have used an antibody whose binding site has been mapped to residues 110-164 within the conserved luminal domain of the triadin molecule (94), (generic triadin), to localize generic triadin in rabbit cardiac myofibers. The results presented by these researchers suggested that generic triadin colocalizes with the RyR at the cell periphery and in the cell interior at the Z-line of longitudinally cryosectioned rabbit ventricular and atrial myofibers. Since the RyR had previously been shown to localize to both junctional and corbular SR (85), these results suggest that generic triadin, like the RyR, is localized to junctional and corbular SR. Recent immunofluorescence localization studies by Guo et. al., (44), also suggest that generic triadin is present in the I-band region of rabbit ventricular myofibers, an area where
junctional and corbular SR are densely distributed. Since biochemical studies (44), have shown that triadin can bind to both CSQ and the RyR, its colocalization with CSQ and the RyR in the junctional and corbular SR of cardiac muscle was an expected result.

Since we have previously seen that junctin is confined to junctional SR, it is possible that additional differences may exist between junctional and corbular SR. Thus, it may be possible that one or more of the three triadin isoforms detected in the rabbit may be confined to either junctional or corbular SR. However, because the antibodies used Carl et. al., (59), and Guo et. al., (44), to localize triadin recognized an epitope common to the three isoforms of triadin expressed in rabbit cardiac tissue, it was not possible to determine whether one or more of the three isoforms is localized to either junctional and/or corbular SR. To determine the subcellular

<table>
<thead>
<tr>
<th>PROTEIN</th>
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<th>CORBULAR SR</th>
<th>NETWORK SR</th>
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<td>HIGH</td>
<td>HIGH</td>
<td>LOW</td>
<td>EPXM 92,93</td>
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IF: IMMUNOFLUORESCENCE
IEM: IMMUNOELECTRON MICROSCOPY
EPXM: ELECTRON PROBE X-RAY MICROANALYSIS
ND: NOT DETERMINED

Table 1. The subcellular distribution of the sarcoplasmic reticulum proteins believed to be involved in Ca\(^{2+}\) release from the SR.
distribution of the three triadin isoforms, it should be possible to develop antibodies to the unique C-terminus of each of the three isoforms and then try to determine whether one or more of these isoforms is confined to junctional and/or corbular SR by immunofluorescence or immunoelectron microscopical labeling. None the less, since rabbit skeletal muscle lacks corbular SR and only expresses a single 94 kDa triadin isoform, it is possible that triadin-3, which is almost identical in sequence to its 94 kDa skeletal counterpart, may be confined to the junctional SR of rabbit cardiac myofibers. Since skeletal muscle also lacks corbular SR and does not express triadin-1 or triadin-2, it is also reasonable to assume that triadin-1 and triadin-2 may both be confined to corbular SR. The subcellular distribution of triadin-1 in canine cardiac myofibers has not yet been determined.

I. DOES CORBULAR SR PARTICIPATE IN Ca²⁺ RELEASE FROM THE SARCOPLASMIC RETICULUM?

The IEM localization of the RyR (85), and CSQ (89-91), and the electron probe x-ray microanalysis studies of the distribution of calcium (92,93), insitu have confirmed earlier biochemical fractionation studies (12,13), which suggested that junctional SR is the Ca²⁺ release site in cardiac muscle. This conclusion is also strengthened by the fact that most available evidence from the study of Ca²⁺ sparks (75,83,84,106), suggests that the site of E-C coupling in ventricular myofibers corresponds to the junctional complex between the junctional SR and the patch of SL or T-tubule membrane to which it is closely apposed. However, the results of RyR, CSQ, calcium and triadin localization studies insitu also suggest that corbular SR constitutes a large proportion of the potential Ca²⁺ storage and release sites in mammalian ventricular myofibers. Additionally, Shacklock et. al., (106), have suggested that sparks could arise from areas more distant from the T-tubules such as corbular SR, which is also localized to the Z-line where T-tubules and junctional SR are densely distributed. This suggestion was based on the observation that the accuracy with which the sites of origin of Ca²⁺ sparks can be
localized is limited by the signal-to-noise ratio of the fluorescent signal, the pixel size and the point spread function of the confocal microscope (106).

While contraction is an all or none phenomenon in skeletal muscle, the rate and degree of contraction in cardiac muscle can be altered (7). In the heart, each muscle fiber contracts during systole. If a greater cardiac output and stronger muscle contractions are required, the option of recruiting more muscle fibers to generate a more forceful contraction is not available, instead more Ca$^{2+}$ must be released from the SR so that there will be a greater inhibition of the contraction regulating complex. Recent studies in Purkinje and atrial myofibers suggests that corbular SR may play a role in regulating the rate and extent to which cardiac myofibers contract by serving as a secondary site of Ca$^{2+}$ release (5,96).

Studies by Fabiato (5), support the idea that corbular SR can be induced to release Ca$^{2+}$ by the Ca$^{2+}$ induced-Ca$^{2+}$ release (CICR), mechanism. Fabiato has shown that skinned canine Purkinje fibers, which lack SL/peripheral junctional SR and T-tubules/interior junctional SR, but contain corbular SR, were capable of SR Ca$^{2+}$ release following a quick elevation of the cytosolic Ca$^{2+}$ concentration. Since corbular SR contains the RyR, CSQ, calcium and triadin, it is reasonable to suspect that corbular SR may be sites of Ca$^{2+}$ release in skinned Purkinje fibers. Studies by Huser et. al. (96), which were based on confocal imaging of isolated cat atrial myocytes that contained peripheral junctional SR and corbular SR, but no interior junctional SR and which were loaded with the Ca$^{2+}$ sensitive probe fluo-3, also support this idea. Huser et. al. (96), showed that electrically induced sparks, presumed to reflect the activation of a small number of RyRs, occurred first at the periphery of these cells where junctional SR is localized and then in the interior regions where corbular SR is localized. It should be noted however, that only peripheral sparks were observed at normal levels of SR Ca$^{2+}$ load. However, when the SR was loaded to simulate an increased inotropic state, an increase in the number of sparks was observed both at the periphery and in the interior of the myofibers. Huser et. al. (96), have
suggested that voltage dependent Ca\textsuperscript{2+} entry through the DHPR triggers Ca\textsuperscript{2+} release from peripheral junctional SR. In turn, the Ca\textsuperscript{2+} released from the junctional SR was proposed to cause Ca\textsuperscript{2+} to be released from corbular SR via a CICR mechanism. Huser et al., (96), suggested that the atrium contains two types of Ca\textsuperscript{2+} stores which differ both in terms of their Ca\textsuperscript{2+} release mechanism and in terms of their kinetics of Ca\textsuperscript{2+} loading (96). These results suggest that while junctional SR can release Ca\textsuperscript{2+} both at normal and elevated SR Ca\textsuperscript{2+} loads, corbular SR may only release Ca\textsuperscript{2+} after the Ca\textsuperscript{2+} load of the SR has been increased following the stimulation of the myocyte by an inotropic agent (fig 7). The Ca\textsuperscript{2+} released from the corbular SR might then function to amplify the amount of Ca\textsuperscript{2+} released from the junctional SR, thus causing a more forceful contraction.

Since \textasciitilde40\% of the CSQ labeling detected in rat ventricular myofibers (91), and \textasciitilde30\% of the RyR positive foci detected in canine ventricular myofibers (115), were estimated to be localized to corbular SR \textit{in situ}, and since it has been reported that corbular SR stores (92) and

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\begin{figure}[h]  
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\includegraphics[width=\textwidth]{fig7.png}
\caption{Fig 7. Model showing the possible role of junctional and corbular SR in potentiated Ca\textsuperscript{2+} release.}
\end{figure}
releases Ca\textsuperscript{2+} under conditions which lead to an increased inotropic state (96), it stands to reason that the corbular SR in ventricular myofibers may also release Ca\textsuperscript{2+} following stimulation of the myofibers by inotropic agents. However, if we are to truly understand the mechanism of E-C coupling, and if we are to understand the role that corbular SR plays in the process, it is important to further characterize the protein composition of junctional and corbular SR. In so doing we may be able to determine what causes the proposed differences in the mechanism affecting Ca\textsuperscript{2+} release from junctional and corbular SR.

J. RATIONALE.

It has been suggested that junctional and corbular SR may have different roles in cardiac E-C coupling (96), and our inability to detect junctin in corbular SR (88,95), supports this suggestion. Since it has not yet been feasible to biochemically subfractionate junctional SR from corbular SR, as has was done for junctional SR and network SR (12,13), it is not yet feasible to biochemically compare the protein composition of junctional and corbular SR. Thus, the \textit{in situ} immunofluorescence and immunoelectron microscopical localization of protein components believed to be involved in E-C coupling is at present the best methods for comparing the protein composition of junctional SR and corbular SR.

Recent double immunofluorescence studies carried out by Carl et. al., (59), and single immunofluorescence labeling studies carried out by Guo et. al., (44), have suggested that triadin is densely distributed in junctional and corbular SR of longitudinally cryosectioned rabbit cardiac myofibers. However, since interior junctional SR and corbular SR are both localized to the I-band of longitudinally cryosectioned cardiac myofibers (9) and because some atrial myofibers and all ventricular myofibers in the rabbit contain T-tubules and thus interior junctional SR (119), it is possible that the results reported by Carl et. al (59) and Guo et. al. (44) may have been misinterpreted. In other words, it is possible that the triadin-positive foci detected in the interior of these myofibers may actually correspond to the detection of the protein in interior
junctinal SR and not corbular SR. This possibility is supported by the fact that junctin was initially thought to be localized to both junctional SR and corbular SR simply because it was densely distributed in the I-band domain of longitudinally cryosectioned canine ventricular myofibers (16). However, when the protein was immunocytochemically localized in transversely cryosectioned myofibers in the presence of a marker of the SL and T-tubules (making it possible to differentiate between interior junctional SR and corbular SR, see fig 6), it was observed that junctin was actually confined to junctional SR. Thus, to accurately determine the subcellular distribution of triadin, it is essential to also localize the protein in transversely cryosectioned myofibers and to compare its localization to that of a marker of the SL and T-tubules. Thus, the objective of this study will be to reassess the subcellular distribution of triadin in canine cardiac myofibers using this more detailed approach to determine if the protein is in fact present in both junctional and corbular SR. If the results obtained differ from those reported in the rabbit (44,59), a similar approach will be used to reassess the subcellular distribution of triadin in rabbit cardiac myofibers.

Fig 8. Diagram showing the position of the triadin-1c and triadin-1u epitopes.

K. APPROACH.

To perform these studies, polyclonal antibodies (see fig 8) specific to the C-terminus (a.a 269-287), of canine cardiac triadin-1 (triadin-1u) were prepared in the laboratory of Dr. L.R.
Jones and made available to us. Since the C-terminus of triadin is not known to interact with any other SR proteins, we anticipate that this epitope should be accessible in both junctional and corbular SR. However, if this site is blocked, polyclonal antibodies (triadin-1c) whose epitope has been mapped to a stretch of amino acids (a.a 165-257) within the conserved region of triadin, were also prepared in the laboratory of Dr. L.R. Jones and made available to us. Although the conserved luminal domain of triadin is thought to be the site through which it interacts with CSQ, the RyR and junctin (42,44), the results from earlier localization studies (44,59) suggest that antibodies to this region of the protein should be able to detect triadin in both junctional and corbular SR. In addition, since it is fairly easy to distinguish between junctional SR and corbular SR in transverse cryosections of atrial and ventricular myofibers, following double immunofluorescence labeling with antibodies to junctional SR proteins such as the RyR and junctin, the subcellular distribution of triadin will also be examined in transversely cut myofibers.

To determine the subcellular distribution of triadin-1, 5-8μm cryosections of canine myocardial tissue containing either atrial or ventricular myofibers were double immunofluorescently labeled, first with antibodies to the unique luminal domain of triadin-1 (triadin-1u) or with antibodies to the conserved luminal domain of triadin-1 (triadin-1c) and then with antibodies to either the RyR, a marker of junctional and corbular SR (59,85), (see fig 5); phospholemman (PLM), a marker of the SL and T-tubules (115), (see figs 5 or 6); or junctin, a marker of junctional SR (88,95), (see fig 6). The double labeled cryosections were then visualized by laser scanning confocal microscopy. If the results obtained from these studies differ from earlier reports (44,59), in the rabbit, a similar approach, utilizing wheat germ agglutinin as the cell surface marker, will be used to reexamine the subcellular distribution of triadin in rabbit cardiac myofibers.
CHAPTER 2

MATERIALS and METHODS

1. Preparation of Affinity Purified Antibodies

   a) αTn-269 Anti-Triadin(triadin-1u): Affinity purified rabbit antibody αTn-269 to canine cardiac triadin-1 was provided by Dr. L.R. Jones (Krahnert Inst. Of Cardiology, Indiana U. School of Medicine, Indianapolis Indiana 46202). αTn-269 was made against an 18-mer peptide (SEEVAGGSKRTLGKKQIQ) starting at amino acid residue 269 of canine cardiac triadin-1. αTn-269 was purified from an 18-mer peptide Tn-269 column. The antiserum specifically identifies the 40 kDa glycosylated triadin-1 isoform and the 35 kDa triadin-1 isoform on Western blots of ventricular and atrial membrane samples.

   b) αdCT-1 Anti-Triadin(triadin-1c): Affinity purified rabbit antibody αdCT-1 to canine cardiac triadin-1 was provided by Dr. L.R. Jones. αdCT-1 triadin was made against full length recombinant canine cardiac triadin-1 expressed in the Baculovirus Expression System and partially purified on phosphocellulose columns. αdCT-1 triadin was then purified from Immobilon-P strips loaded with recombinant canine cardiac triadin-1. The binding site for αdCT-1 has been mapped to a.a residues 165-257, which lies within the conserved luminal segment of triadin. The antiserum specifically identifies the 40 kDa glycosylated triadin-1 isoform and the 35 kDa triadin-1 isoform on Western blots of ventricular and atrial membrane samples. Since the binding site for the antibody has been mapped to the conserved luminal domain of the triadin molecule, the antibody also detects a single band that migrate with a predicted Mr of ~60 kDa on Western blots of canine skeletal muscle microsomal vesicles. The band is thought to correspond to the skeletal muscle isoform of triadin (Kobayashi and Jones personal communication).

   c) GP58 Anti-Triadin: Polyclonal antibody GP-58 to triadin was provided by Dr. K.P. Campbell (Howard Hughes Medical Institute, Dept. of Physiology and Biophysics, Program in Neuroscience, University of Iowa, College of Medicine, Iowa City, Iowa 52242). GP-58 was prepared and characterized
as previously described (44). The DNA fragment corresponding to the conserved region of the luminal domain of triadin (a.a. 69-264) was subcloned into pGEX-2T vector by the EcoRI site and expressed as a GST fusion protein (H-Triadin). Purified H-triadin was used to purify GP58 as described previously (44). After purification, anti-GST antibodies were removed with GST fusion protein-nitrocellulose strips. The affinity purified antibodies to triadin specifically identifies the 35, 40 and 92 kDa cardiac isoforms of triadin on Western blots of rabbit microsomes (44).

d) C3-33 Anti-Ryanodine Receptor: Monoclonal antibody to canine cardiac ryanodine receptor was produced by hybridoma clone C3-33 (103), and was purchased from Affinity Bioreagents, Inc. Golden, Co. This antibody specifically binds the ryanodine receptor present in SR vesicles from canine ventricular muscle.

e) B8 Anti-Phospholemman: Monoclonal antibody mAb8 (IgG2a) to recombinant phospholemman was provided by Dr. L.R. Jones and obtained as previously described (99).

f) C-Terminal Anti-Phospholemman: Affinity purified rabbit antibody to the C-terminus of canine cardiac phospholemman was provided by Dr. L.R. Jones and obtained as previously described (115).

g) 5B3 Anti-Junctin: Monoclonal antibody mAb 5B3 (IgG2b) to recombinant canine junctin was carried out by hybridoma technology according to standard procedures (104). Recombinant canine junctin was expressed and purified from sf21 insect cells and was provided by Dr. L.R. Jones.

h) Wheat Germ Agglutinin: WGA conjugated to biotin was purchased from Vector Laboratories, Burlingame, CA.

2. Dissection and Fixation of Cardiac and Skeletal Muscle

Two mongrel dogs and two rabbits were used in this study. The dogs were anaesthetized with sodium barbital and the hearts and 2cm² strips from the gracilis muscle of the hind legs were excised and rinsed in phosphate buffer saline (PBS, pH 7.4). The rabbits were anaesthetized with fluothane and the heart excised and rinsed in phosphate buffer saline (PBS, pH 7.4).

Myocardial Tissue: Small bundles of myofibers were dissected from the left ventricle and from the left atrial muscle and quickly fixed according to one of the two procedures outlined below.
Skeletal Muscle: Small bundles of canine gracilis muscle were dissected from the dogs and quickly fixed according to procedure 1.

Procedure 1: Methanol Fixation. The unfixed tissue was cryofixed in liquid nitrogen cooled 2-methylbutane. Transverse and longitudinal cryosections (5-8 µm thick) were cut at -20 °C, permeabilized by fixation in methanol (-13 °C) for 5 min. and air dried for 30 min as previously described (46). The methanol fixed sections were either immediately used for immunofluorescence studies or they were stored at 4 °C for up to 4 weeks.

Procedure 2: Nakane Fixation. The unfixed tissue was fixed for 3 h at 4°C in freshly prepared Nakane fixative (105), a periodate-lysine-paraformaldehyde solution containing 2% paraformaldehyde, 0.15M lysine, 10 mM sodium periodate and 0.05 M sodium phosphate (pH 7.4). Tissues were then infused for 30-60 min. with 1.2 M sucrose. The sucrose infused tissue was then frozen in liquid nitrogen. Longitudinal semithin sections (0.5 µm thick) were then cut at -80 °C as previously described (86). The semithin sections were immediately used for immunofluorescence studies.

3. Immunofluorescence Labeling of Canine Atrial, Papillary and Skeletal Muscle Cryosections and Rabbit Atrial and Papillary Muscle Cryosections

Immunofluorescence labeling of 5-8 µm cryosections of methanol fixed canine atrial, papillary and skeletal muscle tissue and rabbit atrial and papillary muscle tissue was carried out as previously described (100). Confocal microscopy was carried out with a confocal fluorescence imaging system (Lasersharp MRC-600, BioRad), using a krypton-argon laser for illumination (97).

a) Single Immunofluorescence Labeling: Methanol fixed cryosections (Procedure 1), were rehydrated for 15 min. at room temperature in 0.1% BSA in phosphate buffer saline (PBS, pH 7.4), and immunofluorescently labeled with one of the following antibodies: (a) affinity purified rabbit antibodies αTn-269 to canine cardiac triadin-1 (25µg/ml in PBS); (b) affinity purified rabbit antibodies αdCT-1 to canine cardiac triadin-1 (50µg/ml in PBS); (c) mouse mAb C3-33 to the canine cardiac ryanodine receptor (25µg/ml in PBS); (d) mouse mAb B8 to canine cardiac phospholemmann (50µg/ml in PBS); (e) affinity
purified rabbit antibodies to canine cardiac phospholemman (50μg/ml in PBS); (f) mouse mAb 5B3 to canine cardiac junctin (0.1mg/ml in PBS); (g) affinity purified guinea pig antibodies GP58 to rabbit skeletal muscle triadin (50μg/ml in PBS); (h) WGA conjugated to biotin (5μg/ml in PBS; Vector Laboratories, Burlingame, CA). The corresponding secondary antibodies were: (a), (b) and (e) affinity purified F(ab)_2 fragment of donkey anti-rabbit IgG conjugated to Texas Red (20μg/ml in PBS; Jackson Immunoresearch Laboratories Inc., Westgrove P.A.), and adsorbed with dog liver powder; (c) affinity purified F(ab)_2 fragment of goat anti-mouse IgG conjugated to FITC (25μg/ml in PBS; Harlan Sera-Laboratories, Crawley Down, Sussex, England); (d) affinity purified F(ab)_2 fragment of goat anti-mouse IgG conjugated to FITC (25μg/ml in PBS; Harlan Sera-Laboratories, Crawley Down, Sussex, England); (f) affinity purified F(ab)_2 fragment of goat anti-mouse IgG conjugated to FITC (25μg/ml in PBS; Harlan Sera-Laboratories, Crawley Down, Sussex, England); (g) affinity purified F(ab)_2 fragment of goat anti-guinea pig IgG conjugated to either FITC or Texas Red (25μg/ml in PBS; Jackson Immunoresearch Laboratories Inc., Westgrove PA.); (h) streptavidin conjugated to Texas Red (10μg/ml in PBS; Vector Laboratories, Burlingame, CA.).

b) Double Immunofluorescence Labeling: Double immunofluorescence labeling of 5-8 μm cryosections, fixed according to Procedure 1, was carried out in sequential steps combining pairs of the seven (a-g), single immunofluorescence labeling procedures described above. Approximately 50-75 slides, each containing 3 tissue sections, were used for each double labeling procedure.

- Double labeling for triadin-1 (using triadin-1u antibodies) and phospholemman combined single labeling procedures (a) and (d).
- Double labeling for triadin-1 (using triadin-1c antibodies) and phospholemman combined single labeling procedures (b) and (d).
- Double labeling for triadin-1 (using triadin-1u antibodies) and the ryanodine receptor combined single labeling procedures (a) and (c).
- Double labeling for triadin-1 (using triadin-1c antibodies) and the ryanodine receptor combined single labeling procedures (b) and (c).
- Double labeling for triadin-1 (using triadin-1u antibodies) and junctin combined single labeling procedures (a) and (f).
- Double labeling for triadin-1 (using triadin-1u antibodies) and junctin combined single labeling procedures (b) and (f).
- Double labeling for the ryanodine receptor and phospholemmman combined single labeling procedures (c) and (e).
- Double labeling for rabbit triadin and WGA combined single labeling procedures (g) and (h).
- Double labeling for rabbit triadin and the ryanodine receptor combined single labeling procedures (g) and (c).

All immunolabeled sections were mounted in slow fade (Molecular Probes, Eugene, OR.) and examined using an MRC 600 Scanning Confocal Fluorescence microscope provided with a Krypton-Argon Laser. Images were processed using Adobe Photo Shop.

c) Double Immunofluorescence Labeling of 0.5 μm Semithin Sections of Nakane Fixed Canine Atrial Muscle

Indirect immunofluorescence labeling of 0.5 μm semithin sections of canine atrial muscle fixed in Nakane fixative (Procedure 2), was carried out as follows: semithin sections from Nakane fixed tissues were rehydrated for 15 min. at room temperature in PBS (pH 7.4), containing 0.1% BSA and 0.5% Triton-X 100 and immunofluorescently labeled as previously described (85), with the exception that all dilution buffers contained 0.1% BSA and 0.5% Triton-X 100. Semithin sections of canine atrial muscle were labeled according to the double labeling procedures outlined above. Briefly, double labeling for triadin-1 (using triadin-1u antibodies) and the ryanodine receptor combined single labeling procedures (a) and (c). Approximately 10 slides, each containing 3 tissue sections, were used.

d) Controls: The specificity of the single labeling procedures were assessed by substituting the primary reagent with PBS (pH 7.4). To eliminate the possibility that staining patterns observed in the
Double labeling procedures were due to cross reactivity of secondary antibodies, comparison between the staining patterns obtained with and without the second primary antibody was carried out for each of the double labeling procedures listed above. Double labeling experiments, in which the order of the primary antibodies were reversed, were also performed.

**e) Adsorption of Affinity Purified Antibodies:** Affinity purified polyclonal rabbit antibodies αTn-269 (triadin-1u) and αdCT-1 (triadin-1c) anti-triadin were incubated at a molar ratio of 4 antigens per antibody binding site with a 20 a.a peptide, corresponding to the unique C-terminus of cardiac triadin-1, conjugated to BSA (αTn-269), or purified triadin-1 protein (αdCT-1), as previously described (85). The supernatant obtained following centrifugation was used as primary reagent in the indirect immunofluorescence labeling procedure.

4. **Length Profiles of Atrial Myofibers**

To perform length profiles, confocal images of methanol fixed 5-8 μm cryosections of canine atrial myofibers were first collected with a bird MRC 600 confocal microscope. The gain and black levels were set so that optimal images would be produced and to ensure that pixel intensities fell between 20 and 220. Confocal images were first collected from single labeled sections to ensure that there was no bleed at the black levels and gain settings used to produce optimum images and to ensure that the double labeling procedure did not alter the sensitivity of either of the two primary antibodies. Confocal images were then simultaneously collected from the same area of sections which were double labeled for triadin-1 (using triadin-1u antibodies) and the RyR. CoMos software was then used to draw continuous lines through the myofibers images from the Texas Red (triadin-1), and FITC (RyR), channels. A graph of the pixel intensities vs. length in microns along the lines was then produced for the lines drawn through myofibers imaged from the Texas Red channel and from the FITC channel. A similar technique was used to compare the pixel intensities of the triadin-1 or RyR images from the single labeled sections to the images from the double labeled sections.
5. **Quantification: Determining the Proportion of Triadin-1, and the Ryanodine Receptor Positive Foci That Correspond to the Detection of the Proteins in Junctional or Corbular SR in Canine Ventricular Myofibers**

To perform these quantifications, confocal images of methanol fixed 5-8 µm cryosections of canine ventricular myofibers were first collected with a BioRad MRC 600 confocal microscope. The gain and black levels were set so that optimal images would be produced and to ensure that pixel intensities fell between 20 and 220. Confocal images were collected from sections which were double labeled either for triadin-1 (using triadin-1α or triadin-1β antibodies) and PLM or the RyR and PLM. To reduce some of the fuzziness of the raw images, CoMos software was used to enhance the edges of the pixels making up the raw images, no additional processing of the images were performed using CoMos software. Edge enhancement makes the image look sharper by accentuating local structural features and de-emphasizing global ones. Photoshop software was then used to add adjustment layers on top of the unprocessed images. Adjustment layers allowed us to adjust the color and tonal qualities of the images to make them look sharper. Because adjustment layers temporarily modify the pixels in the unprocessed image, they can create artifacts by giving the impression that the intensity of the pixels making up the foci are stronger than they actually are or they can allow background labeling to become more foci-like, as a result, adjustment layers were used only to increase the sharpness of the PLM labeled SL and T-tubules. No adjustment layers were made for the triadin or RyR labeled images. Approximately 20 slides, each containing 3 tissue sections, were used for each of the double labeling procedures. Myofibers in which PLM labeling of the T-tubules produced a cart wheel pattern, an indication that the myofibers were transverse, were then selected for analysis (5 myofibers were selected for each double labeling procedure). The unprocessed images and the overlying adjustment layers were then enlarged 4 fold so that it would be easier to differentiate between triadin or RyR foci that either localize along or away from the PLM positive T-tubules. The paint brush tool was then used to mark all visible triadin or RyR foci. Since there was quite a variation in the size of both the triadin and RyR positive foci and since we could not determine whether the larger foci consisted of several smaller foci that were too close together to be resolved into individual
foci, we decided to use a paint brush with a brush size that was equal to the diameter of the smallest visible foci. We observed that the smallest foci were about 5 pixels in diameter. If, while doing the counting, we found that a foci was larger than 5 pixels in diameter, we assumed that that foci was made up of several smaller foci, each having a diameter of ~5 pixels in diameter. For example, if a large foci was ~20 pixels in diameter it often required 4 dots to cover it and would be counted as 4 foci. All foci that localized along the PLM positive T-tubules were marked with an appropriate number of blue dots, while all foci that localized away from the T-tubules were marked with an appropriate number of pink dots. The total number of pink and blue dots required to cover all the triadin or RyR foci in a given myofiber was determined and percentage values calculated.
CHAPTER 3
RESULTS

A. The Subcellular Distribution of Triadin-1 in Transverse and Longitudinally
Cryosectioned Canine Atrial Myofibers

To better understand the potential role of triadin in the release of Ca\textsuperscript{2+} from the SR, it is important that we know its distribution in relation to the ryanodine receptor (RyR). Largely due to the fact that canine cardiac myofibers primarily express one major isoform of triadin (triadin-1) (114), we wanted to determine whether triadin-1, like the RyR, was localized to both junctional and corbular SR in canine atrial and ventricular myofibers.

Unlike ventricular myofibers, most atrial myofibers contain very few or no T-tubules and thus have little or no interior junctional SR (9). However, the interior domains of these myofibers contains an extensive amount of corbular SR and they have significantly more peripheral junctional SR confined to their cell periphery than do ventricular myofibers (11,117,118). Since it fairly easy to distinguish between junctional SR and corbular SR in transverse cryosections of atrial myofibers, at the light microscopical level of resolution, we felt that the subcellular distribution of triadin-1 would be more accurately determined if it were assessed in myofibers of this orientation. Thus, if triadin-1 is expressed in both junctional and corbular SR (fig 9), then triadin-1-positive foci should codistribute with RyR-positive foci both at the cell periphery and in the interior domains of these myofibers. However, if triadin-1 is only expressed in corbular SR, then triadin-1-positive foci should only be detected in interior domains of these myofibers, where they should codistribute with RyR-positive foci. On the other hand, if triadin-1 is only expressed in junctional SR, then triadin-1-positive foci should be confined to the periphery of these myofibers, where they should codistribute with RyR-positive foci and also junctin-positive foci.
Fig 9. Model showing the possible subcellular distribution of triadin-1 and phospholemm (PLM), in transverse cryosections of canine atrial myofibers.

1. Triadin-1 vs. The Ryanodine Receptor in Transversely Cryosectioned Canine Atrial Myofibers.

Confocal imaging of transverse cryosections that were double immunofluorescently labeled with triadin-1u and RyR antibodies showed that intense triadin-1-positive foci (fig 10a, a/a', b, b/b'; red/yellow) were densely distributed at the cell periphery (fig 10a, a/a', b', b/b'; arrowhead) of all of these myofibers. Although the majority of these myofibers were devoid of intense triadin-1-positive foci centrally, a small number of intense foci were detected in the interior domains of the remaining myofibers. These centrally localized foci were either arranged in short rows (fig 10a, a/a'; asterisk) that extended into the cell interior from the cell periphery or they were arranged in multiple rows (fig 10a, a/a'; open arrowhead) that encircled the interior regions of the myofibers close their cell periphery. In addition to these foci, large fluorescent structures (fig 10a, a/a', b', b/b'; short arrow) were also detected in the interior domains of several
myofibers. However, these foci likely correspond to the detection of autofluorescent material because adsorption of the triadin-1u antibodies prior to immunofluorescence labeling had no effect on reducing the intensity of these fluorescent structures, although it lead to a large decrease in the intensity of labeling at the periphery of the myofibers (not shown). In addition, confocal imaging of unlabeled canine atrial myofibers (fig 11) showed that they contained a large number of similar fluorescent structures that, when imaged from the Texas Red channel, were quite prominent. Some of these fluorescent structures were also visible, although to a lesser extent, when the myofibers were imaged from the FITC channel. In addition, we also observed that when the triadin-1u primary antibody was detected with an FITC conjugated secondary antibody (results not shown), the fluorescent structures were no longer visible in the cytoplasm of the triadin-1u labeled myofibers.

In contrast to the distribution of triadin-1, intense RyR-positive foci (fig 10a', a/a', b', b/b'; green/yellow), were densely distributed both at the cell periphery (fig 10a', a/a', b', b/b'; arrowhead) and throughout the cytoplasm (fig 10a', a/a', b', b/b'; long arrows) of all of these myofibers. In merged images it could be seen that the triadin-1-positive foci at the cell periphery and the few foci detected in the interior of some of these myofibers were all localized to areas that were positively labeled for the RyR. Assuming that the RyR-positive foci at the cell periphery represent junctional SR, while those in the interior domains represent corbular SR; these results suggest that triadin-1 is densely distributed in peripheral junctional SR. However, triadin-1 does not appear to be expressed throughout the cytoplasm of canine atrial myofibers, even though the dense distribution of RyR-positive foci clearly indicated that these myofibers contained a large number of corbular SR.

Although each of the intense triadin-1-positive foci in the cytoplasm of these myofibers colocalized with RyR-positive foci, we could not be certain that they correspond to the detection of triadin-1 in corbular SR. Since some mammalian atrial myofibers contain a small amount of
interior junctional SR (9) and because antibodies to the RyR cannot differentiate between interior junctional SR and corbular SR, it is possible that these interior foci could correspond to the detection of triadin-1 in interior junctional SR.

Fig 10. Comparison of the subcellular distribution of triadin-1 (a, a/a', b, b/b': red/yellow), with the RyR (a', a/a', b', b/b': green/yellow), in methanol fixed transverse cryosections of canine atrial myofibers by double immunofluorescence labeling. a/a' and b/b' are the merged images of a, a' and b, b', respectively. b, b' and b/b' are enlarged views of a single myofiber. Triadin-1 labeling colocalizes with labeling for the RyR primarily at the periphery of the myofibers (arrowheads). Very few intense triadin-1-positive foci (asterisk and open arrowheads) were observed in the interior of these myofibers. The interior of the myofibers were labeled primarily for the RyR (long arrows). Autofluorescent foci (short arrows), were also visible in the myofibers imaged from the Texas Red channel. Bar = 10 μm
i) Triadin-1 vs. Phospholemman in Transversely Cryosectioned Canine Atrial Myofibers.

To determine whether the triadin-1-positive foci that appear to be present in the interior of these myofibers were localized to either interior junctional SR or corbular SR, transverse cryosections of canine atrial myofibers were double immunofluorescently labeled with triadin-1 and PLM antibodies.

Confocal imaging showed intense triadin-1-positive foci (fig 12 a, a/a’, b, b/b’ and fig 13 a, a/a’; red/yellow) were indeed localized along the PLM-positive (fig 12 a’, a/a’, b’, b/b’ and fig 13 a’, a/a’: green/yellow) SL at the periphery of all of these myofibers, which confirms our assumption that triadin-1 is densely distributed in peripheral junctional SR. In most myofibers these foci were arranged in a single row around their peripheral regions. Surprisingly, all of the intense triadin-1-positive foci detected in the cytoplasm of these myofibers were also localized to areas that were positively labeled for PLM (with the exception of large globular foci that likely correspond to autofluorescent material). In the presence of the cell surface marker, it became clear that the multiple rows of triadin-1-positive foci that we initially though were present centrally, were actually localized to peripheral junctional SR along tangential/oblique sections through the cell.
sarcolemma (fig 12 b, b', b/b'; arrowheads); while the single rows of triadin-1-positive foci that we observed in interior domains were localized to interior junctional SR along T-tubules (fig 13 a, a', a/a; TT).

Fig 12. Comparison of the subcellular distribution of triadin-1 (a, a' b, b/b; red/yellow), with PLM (a', a' b', b/b; green/yellow), in methanol fixed transverse cryosections of canine atrial myofibers by double immunofluorescence labeling. a/a'; b/b'; are merged images of a, a' and b, b'; respectively. Triadin-1 labeling colocalizes with labeling for PLM primarily along the periphery (arrowheads) of the transverse (a, a', a/a') and oblique (b, b', b/b') myofibers. Note the presence of possible autofluorescent foci (arrows). Bar = 10 μm

Fig 13. Comparison of the subcellular distribution of triadin-1 (a, a/a; red/yellow), with PLM (a', a/a; green/yellow), in methanol fixed transverse cryosections of canine atrial myofibers by double immunofluorescence labeling. a/a' is the merged image of a, a'. Triadin-1 labeling colocalizes with labeling for PLM primarily along the periphery (arrowheads) and along the T-tubules (TT), in the interior of the myofibers. Bar = 10 μm
Assuming that the RyR-positive foci image junctional and corbular SR, while PLM labeling images the SL and T-tubules, the results presented here suggest that the intense triadin-1-positive foci that codistribute with RyR-positive foci at the cell periphery and in interior domains of these myofibers are primarily confined to the SL and T-tubules, where respectively, peripheral and interior junctional SR are localized. In contrast, intense triadin-1-positive foci were not detected in regions of these atrial myofibers where RyR dense corbular SR is abundantly distributed.

2. Triadin-1 vs. The Ryanodine Receptor in Longitudinally Cryosectioned Canine Atrial Myofibers.

Since initial localization studies in the rabbit (44,59) assessed the subcellular distribution of triadin in longitudinally cryosectioned myofibers, we felt our assessment of the subcellular distribution of triadin-1 would not be complete if we failed to localize triadin-1 in longitudinally cryosectioned atrial myofibers. Thus, if our interpretation of the results presented above are correct, intense triadin-1-positive foci should be confined primarily to the cell periphery of longitudinally cryosectioned canine atrial myofibers. In contrast, our results and the results from earlier studies (59,89), predict that intense RyR-positive foci should be detected both at the cell periphery and as uniform transverse bands close to the Z-line in the interior domains of these myofibers.

As anticipated, confocal imaging of double labeled myofibers showed that intense triadin-1 (fig 14a, a/a'; red/yellow) and RyR-positive foci (fig 14a', a/a'; red/yellow) colocalized and were densely distributed along their periphery (fig 14; arrowheads), while their interior domains contained uniform transverse bands of RyR-positive foci (fig 14a', a/a'; long arrow). Surprisingly, we also observed that several of these myofibers also contained transverse bands of triadin-1-positive foci (fig 15a, a/a'; long arrow) in some areas of their cytoplasm. Depending on the region of the tissue section, the number of myofibers showing this banding pattern varied from as few
as one or two, to as many as 30-40% of the total number of myofibers. However, unlike the transverse

Fig 14. Comparison of the subcellular distribution of triadin-1 (a, a': red/yellow), with the RyR (a', a': green/yellow), in methanol fixed longitudinal cryosections of canine atrial myofibers by double immunofluorescence labeling. a/a' is the merged image of a, and a'. Triadin-1 labeling colocalizes with labeling for the RyR primarily at the periphery of the myofibers (arrowheads). The interior of the myofibers were labeled primarily for the RyR (long arrows). ID is intercalated disc. Large globular foci (short arrows), in the interior of myofibers imaged from the Texas Red channel likely correspond to autofluorescent material. Bar = 10 μm

Fig 15. Comparison of the subcellular distribution of triadin-1 (a, a': red/yellow), with the RyR (a', a': green/yellow), in methanol fixed longitudinal cryosections of canine atrial myofibers by double immunofluorescence labeling. a/a' is the merged image of a, and a'. Triadin-1 labeling colocalizes with labeling for the RyR primarily at the periphery of the myofibers (arrowheads). Surprisingly, the interior of these myofibers contain triadin-1 (a, a': long arrows) and RyR-positive foci (a', a': long arrows). However, the triadin-1-positive foci were not visible in all areas of the cell interior (asterisk). Large globular foci (short arrows), in the interior of myofibers imaged from the Texas Red channel likely correspond to autofluorescent material. Bar = 10 μm
bands of RyR-positive foci (fig 15a', a/a'; long arrow), which were similar in intensity to the foci at the periphery of these myofibers (fig 15; arrowheads) and which were evenly distributed throughout their interior domains, the transverse bands of triadin-1-positive foci were very inhomogeneous. The transverse bands were not evenly distributed throughout the interior of these myofibers, being visible in some areas (fig 15a, a/a'; long arrow) but not others (fig 15a, a/a'; asterisk). In addition, they also varied in intensity from region to region within the same myofiber, in some areas being similar in intensity to the foci at the cell periphery, while being much lower in intensity in others. Since we have previously seen that it is possible to misinterpret the results from localization studies if the subcellular distribution of triadin-1 is assessed in relation to only the RyR, and since we did not detect triadin-1 in the corbular SR of transversely cryosectioned myofibers, it is unlikely that the transverse bands of triadin-1-positive foci detected in the interior of these myofibers correspond to the detection of the protein in corbular SR. Instead, these results can be interpreted in several ways. Since we have seen that some canine atrial myofibers contain T-tubules (fig 13), it is possible that the transverse bands of triadin-1-positive foci seen in the interior of these myofibers results from the detection of the protein in interior junctional SR, which like corbular SR, is also localized close to the Z-line (118). Another possibility is that the cross striation is present in an area where the SL has been sectioned tangential/obliquely to the cell surface.

i) Triadin-1 vs. Phospholemman in Longitudinally Cryosectioned Canine Atrial Myofibers.

If the above interpretations are correct, then the triadin-1-positive foci seen in the interior of these longitudinal myofibers, like those seen in the interior of some transverse myofibers, should also localize to areas that are positively labeled for PLM. To test this possibility, longitudinal cryosections of canine atrial myofibers were double immunofluorescently labeled with triadin-1u and PLM antibodies. As anticipated, all of the intense triadin-1-positive foci (fig 16 a, a/a'; red/yellow) detected in interior regions of these myofibers localized to areas that were
positively immunolabeled for PLM (fig 16a', a/a': green/yellow). These domains included either tangential/oblique sections through the surface SL (fig 16; long arrow) and intercalated discs (fig 16; ID) or the occasional T-tubule. Surprisingly, the faint triadin-1 pattern of cross striation (fig 17a, a/a': asterisk), similar to the one seen in the interior of some myofibers that were double labeled for triadin-1 and the RyR (fig 15), was also observed in the interior of some of these myofibers. However, these foci did not localize to areas that were positively immunolabeled for PLM. Since these foci did not appear to localize along either the SL (fig 17; arrowhead) or the T-tubules (fig 17; TT), while similar foci appeared to colocalize with RyR positive foci, these results strongly suggest that they could correspond to the detection of triadin-1 in corbular SR. However, another possibility is that the foci producing the banding pattern, since they were very low in intensity and since they were not uniformly distributed throughout the cytoplasm, are not present in the interior of the myofibers but are actually confined to peripheral junctional SR in out of focus focal planes.

Fig 16. Comparison of the subcellular distribution of triadin-1 (a, a/a': red/yellow), with PLM (a', a/a': green/yellow), in methanol fixed longitudinal cryosections of canine atrial myofibers by double immunofluorescence labeling. a/a' is the merged image of a and a'. Triadin-1 labeling colocalizes with labeling for PLM primarily along the periphery of the myofibers (arrowheads), and along the intercalated discs (ID) in the interior of the myofibers. Intense triadin-1-positive also appear to be densely distributed in the interior of these myofibers (long arrow), however, in the merged image (long arrow), it can be seen that these foci are localized along an oblique section through the surface SL. Note the presence of autofluorescent foci (short arrows). Bar = 10 μm
3. Triadin-1 vs. The Ryanodine Receptor in Semithin Sections of Canine Atrial Myofibers.

The possibility that the faint triadin-1 pattern of cross striation is an artifact is consistent with the fact that the average thickness of the cryosections used were 5-8 μm thick, while the diameter (about 10-20 μm) and orientation of a mammalian cardiac myofiber will vary markedly within the plane of section (9). (see fig 15). Thus, it is possible for a single myofiber to be sectioned at different depths along its length. This implies that when a faint triadin-1 banding pattern is seen in an area of the myofiber, this area of the myofiber may have been sectioned close enough to the SL on its underside to allow the intense foci in the peripheral junctional SR along the SL at that surface to shine through the thickness of the myofiber and give the impression that they are present in the cell interior (see fig 18). Because these foci are actually present in out of focus focal planes, they will be much lower in intensity than the foci in the peripheral junctional SR at the periphery of the myofiber, which are in focus. However, since
antibodies to PLM label the SL on the extracellular side of the membrane (116) while the triadin-1u antibodies label the SR, it is possible that regions of SL on the underside of the myofiber may not be labeled by PLM strongly enough to also shine through and be detected in the cell interior. Thus, while the cell surface marker might be able to image all aspects of the SL, including tangential/oblique sections, in transversely cryosectioned myofibers, it may not be able to image the SL on the underside of these myofibers when they are longitudinally cryosectioned. However, if the myofiber has been sectioned far enough away from the SL on its underside so that foci in out of focus focal planes cannot be detected in the cell interior or if the SL and peripheral junctional SR from both its upper and lower surfaces have been removed, then no banding pattern will not be seen in these areas of the myofiber. However, since the foci in the peripheral junctional SR at the cell periphery in these areas are in focus, intense triadin-1-positive foci will still be detected along the periphery of the myofiber. None the less, these results clearly illustrate the technical difficulties that must be overcome when trying to accurately assess the subcellular distribution of proteins in longitudinal cryosections of atrial myofibers.

Nakane fixed longitudinally cut semithin sections are ~0.5μm thick (~10-20 times thinner than the 5-8μm thick cryosections), and it is likely that they will contain a larger proportion of myofibers that have been sectioned so that the peripheral junctional SR has been removed from both their upper and lower surfaces. Thus, if our interpretations are correct, we can expect to detect triadin-1-positive foci at the periphery but not in the interior of longitudinal semithin sections of canine atrial myofibers. However, since it is expressed in both junctional and corbular SR and because the myofibers in these semithin sections should still contain corbular SR in their interior regions, we should detect RyR-positive foci both at the cell periphery and in the interior of these myofibers. To test this possibility, longitudinally cut semithin sections of canine atrial myofibers were double immunofluorescently labeled with triadin-1u and RyR antibodies.
Confocal imaging of these myofibers showed that triadin-1-positive foci (fig 19a, a'/*; red/yellow) were indeed confined to their periphery, the faint triadin-1 pattern of cross striation seen in 5-8 μm cryosections was not observed in the cytoplasm of these myofibers. In contrast, RyR positive foci (fig 19a, a'/a'; green/yellow) were present at their cell periphery and as transverse bands in their interior regions. In addition the RyR pattern of cross striation seen in these myofibers were indistinguishable from the pattern seen in 5-8 μm cryosections (figs 14 and 15). Furthermore, since these myofibers appear to have been sectioned at the level of their nuclei (fig 19; N), which indicates that the peripheral junctional SR from both their upper and lower surfaces have been removed, and because they did not contain any triadin-1-positive foci centrally, these results strongly suggest that the faint transverse triadin-1 pattern of cross striations seen in 5-8 μm cryosections is due to the detection of triadin-1 in peripheral junctional SR in out of focus focal planes and not due to the detection of the protein in corbular SR. These
results also suggest the small diameter of atrial myofibers, combined with the fact that their orientation varies markedly within the plane of section, may make it difficult, due to shine through, to accurately determine the subcellular distribution of proteins in longitudinal cryosections of these myofibers.

Fig 19. Comparison of the subcellular distribution of triadin-1 (a, a': red/yellow) with the RyR (a', a': green/yellow), in Nakane fixed semithin sections of canine atrial myofibers by double immunofluorescence labeling and confocal microscopy. a/a' is merged image of a and a'. Triadin-1 labeling colocalizes with RyR labeling primarily at the periphery (arrowheads) of the myofibers. Transverse bands of RyR-positive foci were also observed in the interior of the myofibers. The faint triadin pattern of cross striation seen in 5-8 μm thick sections was not visible in the interior of myofibers sectioned in the plane of their nuclei (N). Bar = 10 μm


The results from the double immunofluorescence labeling of transverse and longitudinal cryosections of canine atrial myofibers with triadin-1u antibodies suggested that triadin-1 is primarily confined to junctional SR. There were very few triadin-1-positive foci detected in the interior of these myofibers where abundant RyR dense cormular SR is present. However, most of these foci were either due to autofluorescence, or due to the detection of triadin-1 in junctional SR associated with the T-tubules or with tangential/oblique sections through the SL or intercalated discs. Previous studies (88,95), have also suggested that junctin is primarily confined to the junctional SR of canine cardiac myofibers. This would suggest that the subcellular distribution of triadin-1 should be more like that of junctin than like that of RyR. To
determine if this is the case, transverse and longitudinal cryosections of canine atrial myofibers were double immunofluorescently labeled with triadin-1u and junctin antibodies.

As anticipated triadin-1-positive (fig 20a, a/a', b, b/b'; red/yellow) and junctin-positive foci (fig 20a', a/a', b', b/b'; green/yellow) codistribute and were localized primarily to the periphery (fig 20 arrowheads) of the transverse (fig 20a, a', a/a') and longitudinally (fig 20b, b', b/b') cryosectioned myofibers. In some areas of the longitudinally cryosectioned myofiber the junctin and triadin-1-positive foci produced an intense cross striated banding pattern in what appears to

Fig 20. Comparison of the subcellular distribution of triadin-1 (a, a/a', b, b/b'; red/yellow), with junctin (a', a/a', b', b/b'; green/yellow), in methanol fixed transverse (a, a', a/a'), and longitudinal (b, b', b/b'), cryosections of canine atrial myofibers by double immunofluorescence labeling. a/a', and b/b' are merged images of a, a' and b, b' respectively. Triadin-1 labeling colocalizes with labeling for junctin primarily along the periphery of the myofibers (arrowheads). The interior of the transverse and longitudinal myofibers had very little labeling for either junctin or triadin. Autofluorescent foci (a, a/a'; small arrow), were also visible in the myofibers imaged from the Texas Red channel. Bar = 10 μm
be their cell interior. However, since junctin has only been detected in junctional SR (88,95) it is likely that the banding pattern is not present in the interior of the myofiber but is actually localized to the peripheral junctional SR along the SL at the surface of the myofiber. However, when the SL is removed from the surface of the myofiber (fig 20b, b', b/b'; long arrow), the labeling for both triadin-1 and junctin becomes confined to the periphery of the myofiber. Thus, these results clearly show that the subcellular distribution of triadin-1 appears to be indistinguishable from that of junctin and that triadin-1, like junctin, is primarily confined to junctional SR in canine atrial myofibers.

B. Length Profiles of Canine Atrial Myofibers Double Labeled for Triadin-1 and the RyR

The results from the previous section suggest that triadin-1, in contrast to the RyR, is not expressed in corbular SR. However, it could be suggested that triadin-1 is expressed in corbular SR but the triadin-1u antibodies may not be sensitive enough to detect it. Assuming that the ratio of triadin-1 to RyR in corbular SR is the same as in junctional SR, it is possible that corbular SR, because it is relatively smaller in size than junctional SR and because the triadin-1u antibodies may not be very sensitive, may not contain enough triadin-1 to give rise to foci that are intense enough to be detected above background.

To assess the sensitivity of the triadin-1u and RyR antibodies, length profiles were used to measure the intensity of the triadin-1 (fig 21 a; red) and RyR-positive (fig 21a; green) foci in double labeled transversely cryosectioned atrial myofibers. The length profile for the RyR labeled myofibers (fig 21b; green) showed that the RyR-positive foci at the periphery and in the interior of these myofibers were similar in intensity. This suggests that despite the size difference between junctional and corbular SR, the RyR may be expressed at similar levels in these two domains. These results also predict that if triadin-1 is expressed in both junctional and corbular SR, it should also be expressed at similar levels in these domains. In turn, this predicts that the intensity of the triadin-1-positive foci detected in these areas should also be similar.
although they may only be slightly above background if the triadin-1u antibodies are in fact low in sensitivity. When length profiles of the triadin-1u labeled myofibers (fig 21b: red) were examined, we saw that the triadin-1-positive foci at their cell periphery were substantially higher in intensity than the triadin-1-positive foci in their interior domains. In addition, the triadin-1-positive foci at the periphery of these myofibers were also higher in intensity than the RyR-positive foci. Thus, if the two proteins are in fact present in a 1:1 molar ratio in junctional SR

![Fig 21. Length profile for canine atrial myofibers double immunolabeled for triadin-1 and the RyR. To perform length profiles, confocal images were simultaneously collected from the same area of sections which were double labeled for triadin-1 (a: red), and the RyR (a: green). CoMmos software was then used to draw identical lines through the myofibers in the unprocessed, unmerged images collected from the Texas Red (triadin-1), and FITC (RyR), channels. Graphs of the pixel intensities vs. length in microns along the lines were then produced (b: triadin-1 = red; RyR = green).](image)

(110), then these results suggest that the triadin-1u antibodies may be more sensitive than the antibodies to the RyR. In addition, since the triadin-1-positive foci at the periphery of these
myofibers were so much higher in intensity than the background labeling outside the myofibers and the labeling in their cell interior, these results strongly suggest that our inability to detect triadin-1 in corbular SR is not due to a low sensitivity of the triadin-1u antibodies. Instead, these results show that our inability to detect triadin-1 in corbular SR is likely due to the fact that it is not expressed in this domain of the SR. Furthermore, since the length profiles were performed on unprocessed images, these results also show that the gain and black levels of the Texas Red channel were not adjusted so that the sensitivity of the system would be too low to detect signals emanating from the interior of these myofibers. Results indistinguishable from these were observed when the order of application of the primary antibodies was reversed. Thus, the lower intensity of the RyR-positive foci compared to that of the triadin-1-positive foci at the periphery of these myofibers is not due to the triadin-1u antibodies interfering with the ability of the RyR antibodies to access their epitope.

C. Assessing the Subcellular Distribution of Triadin-1 in Transversely Cryosectioned Canine Atrial Myofibers Using Triadin-1c Antibodies.

The results that we have presented thus far suggest that triadin-1 is densely distributed in junctional SR while it is absent from corbular SR. In addition, our results also suggest that our inability to detect triadin-1 in corbular SR is not due to a low sensitivity of the triadin-1u antibodies. Although biochemical studies have suggested that the epitope for the triadin-1u antibody is not blocked (42,44), the finding that junctional and corbular SR differ in their protein composition (88,94), raises the possibility that the epitope for the triadin-1u antibody may be more accessible in junctional SR than in corbular SR. However, since previous immunofluorescence studies in the rabbit (44, 59), have suggested that antibodies to the conserved luminal domain of triadin can detect triadin in both junctional and corbular SR, it stands to reason that antibodies to the homologous luminal domain of canine triadin-1 should also be able to detect triadin-1 in both junctional and corbular SR, if the protein is in fact expressed in both of these domains.
To determine whether the antibodies to the conserved luminal domain of triadin-1 can detect the protein in both junctional and corbular SR, transverse cryosections of atrial myofibers were double immunofluorescently labeled, first with triadin-1c antibodies and then with antibodies to either the RyR, PLM or junctin. Confocal imaging of these double labeled myofibers showed that the pattern of labeling produced by the triadin-1c antibodies (fig 22a, b, and c; red/yellow) was virtually indistinguishable from the pattern of labeling produced by the junctin antibodies (fig 22c; green/yellow). Thus, the pattern of labeling by the triadin-1c antibodies also indicates that triadin-1 colocalizes with the RyR (fig 22a; green/yellow) and junctin in junctional SR along the PLM (fig 22b; green/yellow) positive SL at the periphery of these myofibers and along the T-tubules (fig 22b; TT) in their interior regions. In contrast, intense triadin-1-positive foci were not detected in the interior of the myofibers where RyR dense corbular SR was abundantly distributed. Although several large and very intense foci were observed in the interior of the atrial myofibers imaged from the Texas Red channel (fig 22; short arrows), these foci probably correspond to autofluorescent material. Additionally, there was some slight labeling of the connective tissue and the endothelial cells by the triadin-1c antibodies (fig 22a; long arrow). However, it is possible that this labeling is due to the presence of a contaminating antibody, because the adsorption of the triadin-1c antibodies with full length triadin-1 prior to immunofluorescence labeling (see fig 28) resulted in a large decrease in the intensity of the triadin-1-positive staining pattern but it had no effect on reducing the intensity with which the connective tissue or endothelial cells were labeled.
D. Triadin-1 vs. The Ryanodine Receptor in Longitudinally Cryosectioned Canine Ventricular Myofibers.

Our results from the previous section suggest that triadin-1 is primarily confined to junctional SR, while the RyR is densely distributed in both junctional and corbular SR in canine atrial myofibers. Since ventricular myofibers contain both interior junctional SR and corbular SR in their interior regions (9), one would expect to see a differential pattern of labeling for triadin-1 and the RyR in cryosections of these myofibers. To assess the subcellular distribution of triadin-1 in canine ventricular myofibers, longitudinal cryosections of canine papillary myofibers were double immunofluorescently labeled with triadin-1 and RyR antibodies. Confocal imaging of these myofibers showed that triadin-1 (fig 23a, red/yellow) and RyR-positive (fig 23a', green/yellow) foci codistributed at the cell periphery (fig 23; large arrowheads) and in transversely oriented rows (fig 23; arrows) in the I-band domain of these myofibers. Although the frequency of labeling in the interior domain of these myofibers was considerably higher for the RyR (fig 23a', small arrowheads) than for triadin-1 (fig 23a, a/a'; small arrowheads), the fact that both...
proteins localized to the I-band, where interior junctional and corbular SR are localized, could easily lead one to conclude that triadin-1, like the RyR, is present in both interior junctional SR and corbular SR. In fact, it was initially concluded that triadin was localized to both junctional and corbular SR of rabbit papillary myofibers based solely on the fact that the protein localized to the I-band domain of these myofibers (44,59). However, since our results clearly show that it is difficult to distinguish between interior junctional SR and corbular SR at the light microscopical level of resolution and because antibodies to the RyR cannot differentiate between interior junctional and corbular SR, it might be premature to conclude, solely on the basis of its colocalization with the RyR in the I-band of ventricular myofibers, that triadin is present in both junctional and corbular SR.

![Image of subcellular distribution of triadin-1 and RyR](image)

**Fig. 23.** Comparison of the subcellular distribution of triadin-1 (a, a' : red/yellow), with the RyR (a'', a''': green/yellow) in methanol fixed longitudinal cryosections of canine ventricular myofibers by double immunofluorescence labeling. a'/a'' is the merged image of a. and a''. Triadin-1 labeling colocalizes with labeling for the RyR primarily along the periphery of the myofibers (large arrowheads) and in transverse bands (arrows), in the interior of the longitudinal myofibers. RyR-positive foci (a''', a'''' ; small arrowheads), were observed in areas of the myofibers that did not contain any triadin-1-positive foci (a, a'' ; small arrowheads). Bar = 10 μm

**E. Imaging Junctional and Corbular SR in Canine Ventricular Myofibers.**

Our previous immunofluorescence and immunoelectron microscopical localization studies (115), which looked at the subcellular distribution of junctin in relation to the RyR and PLM in transversely cryosectioned canine ventricular myofibers, showed that ~97 % of the
Junctional-positive foci detected in interior regions of these myofibers were confined to junctional SR along PLM positive T-tubules. Consistent with it being a marker of junctional and corbular SR, ~70% of the RyR-positive foci detected in these myofibers were localized along the PLM positive T-tubules and were proposed to correspond to the detection of the protein in junctional SR. The remaining ~30% of the RyR positive foci were localized away from the SL and T-tubules, where corbular SR is abundantly distributed. These results, in addition to suggesting that ventricular myofibers contain a significant amount of corbular SR, suggest that if triadin-1 and the RyR are localized in transversely cryosectioned ventricular myofibers and in the presence of the SL and T-tubule marker PLM, we should be able to differentiate between junctional and corbular SR and so assess the subcellular distribution of triadin-1 in ventricular myofibers (see fig 24). Thus, if triadin-1 is primarily confined to junctional SR, triadin-1-positive foci should primarily localized along the SL and T-tubules. Likewise, RyR positive foci corresponding to junctional SR should also be localized along the SL and T-tubules, while RyR positive foci corresponding to corbular SR should be detected away from the SL and T-tubules.

1. Determining the Subcellular Distribution of Triadin-1 and the Ryanodine Receptor in Transversely Cryosectioned Canine Ventricular Myofibers.

To test this possibility, transverse cryosections of canine papillary myofibers were double immunofluorescently labeled with either triadin-1u or triadin-1c antibodies and antibodies to PLM or with RyR antibodies and antibodies to PLM. Confocal imaging of these myofibers showed that the majority of the triadin-1-positive foci (fig 25a, a/a' and b, b/b'; red/yellow), were distributed along the PLM positive SL (fig 25a', a/a' and b', b/b'; green/yellow; arrowheads)and T-tubules(fig 25a', a/a' and b', b/b'; green/yellow; TT). Nevertheless, examination of these myofibers at higher magnification revealed that a small proportion of the triadin-1-positive foci (fig 26a, b; red; small arrowheads) were also localized at some distance away from both the SL and the T-tubules. However, the majority of these foci were substantially smaller in size and much lower in
Fig 24. Diagram of transversely cryosectioned ventricular myofibers showing the possible subcellular distribution of triadin-1 and the RyR in relation to PLM.

intensity than the triadin-1-positive foci that localized along the SL and the T-tubules. Although the foci observed in the interior regions of these myofibers might suggest that triadin-1 is expressed in a small number of corbular SR, their low intensity suggests that they correspond to the detection of the protein in interior junctional SR along T-tubules in out of focus focal planes.

Although a majority of the RyR-positive foci (fig 25c, c/c'; green/yellow) were localized along the PLM positive SL (fig 25c', c/c'; red/yellow; large arrowheads) and T-tubules (fig 25c', c/c'; red/yellow; TT), a large proportion of the RyR-positive foci (fig 25c, c/c'; green) were also observed at a considerable distance from the PLM positive SL and T-tubules, where corbular SR is abundantly distributed. Unlike the unique triadin-1-positive foci just described, these unique RyR-positive foci were similar in size and in intensity to the foci along the SL and T-tubules. In addition, these foci were also uniformly distributed throughout the cytoplasm of these
myofibers and they were clearly visible, both at lower and at higher magnifications (fig 26c; green). Clearly these results, like those observed in the atrium, suggest that triadin-1 is densely distributed in junctional SR but is not detected at significant levels in RyR-positive corbular SR in ventricular myofibers. In fact, the subcellular distribution of triadin-1 appears to be indistinguishable from that of junctin (fig 26d; green/yellow).


To precisely determine the subcellular distribution of triadin-1 and RyR-positive foci in relation to the PLM positive T-tubules, the triadin-1 (fig 27a, b; red/yellow) and RyR-positive (fig 27c; green/yellow) foci that localized along the PLM positive T-tubules (fig 27a,b; green/yellow and 27c; red/yellow) were marked with an appropriate number of blue dots, while the triadin-1(fig 27a’, b’; red) and RyR-positive (fig 27c’: green) foci that localized away from the T-tubules were marked with an appropriate number of pink dots. The proportion of blue and pink dots from several images were enumerated and the results presented in table 2. These results suggest that ~95 of the triadin-1-positive foci in these myofibers were localized along the PLM positive T-tubules, while ~5% of the foci were localizing away from the T-tubules. In contrast it was found that ~66% of the RyR-positive foci localized along the T-tubules, while the remaining 34 % of the foci did not. These results, like those reported by Jorgensen et. al., (115), suggest that ~30 % of the RyR-positive foci detected in canine ventricular myofibers were localized to areas away from the T-tubules where corbular SR is densely distributed. Most importantly, however, these results also show that less than 5 % of the triadin-1-positive foci were similarly localized. Considering that Jorgensen et. al., (115), have suggested that ~97 % of the junctin-positive foci detected in
Fig 25. Comparison of canine ventricular RyR (c, c/c’; gray) and in the intercalated disks myofibrils.
ventricular myofibers were localized along the T-tubules while our results suggest that ~95% of the triadin-1-positive foci detected in these myofibers were also localized along the PLM-positive T-tubules, these results strongly support our conclusion that triadin-1 is densely distributed in junctional SR, while it appears to be absent from most areas where corbular SR is densely distributed.
Fig 27. Determining the proportion of triadin-1 and RyR-positive foci in transverse canine ventricular myofibers by double immunofluorescence labeling. Images were enlarged 4 fold. Triadin-1 (a and b: red/yellow), or RyR (c: green/yellow), positive foci that localize along the PLM (a, b: green/yellow and c; red/yellow) positive T-tubules were then tagged with blue dots. Triadin-1 (a' and b': red), or RyR (c; green), positive foci that localized away from the PLM-positive T-tubules were tagged with pink dots. Blue dots correspond to the detection of the proteins in interior junctional SR, while pink dots correspond to their detection in corbular SR.
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Table 2. A comparison of the number of dots required to cover the triadin-1 and RyR-positive foci that either do or do not codistribute along the PLM-positive T-tubules in canine ventricular myofibers.

F. Specificity of Triadin-1u and Triadin-1c Antibodies.

The specificity of the labeling observed in canine ventricular myofibers with triadin-1u (fig 29a) or triadin-1c (fig 28a) antibodies was demonstrated by adsorbing the affinity purified antibodies with, respectively, C-terminal triadin-1 peptide (fig 29b) or with purified full length triadin-1 (fig 28b) prior to immunofluorescence labeling. The results show that the intensity of labeling by both antibodies was greatly reduced following adsorption. However, the adsorption of the triadin-1c antibodies lead to a slight increase in the intensity with which the connective tissue was labeled. Thus, it is possible that the triadin-1c antibodies may contain a contaminating antibody that labels the connective tissue. Prior to adsorbing the antibodies, the
connective tissue labeling might not be visible because it is masked by the strong intensity of the triadin-1-positive foci. However, after the triadin-1c antibodies are adsorbed, these intense foci disappear, thus allowing the labeling by the contaminating antibody to become visible. Consistent with the results from Western blots (fig 3), specific immunofluorescence labeling by triadin-1u antibodies (fig 30a) was not detected on cryosections of canine skeletal muscle. On the other hand, the triadin-1c (fig 30b) antibodies produced a chicken wire pattern of labeling on cryosections of canine skeletal muscle that was similar to the one produced by an antibody specific to rabbit skeletal muscle triadin (not shown).

Fig 28. The adsorption of triadin-1c antibodies with purified triadin-1 before immunofluorescence labeling (b) shows that the intensity of the triadin-positive labeling in unfixed canine ventricular myofibers is greatly reduced compared to labeling by the antibody before adsorption (a). Adsorption appears to have caused an increase in the intensity with which the connective tissue is labeled, suggesting that the triadin-1c antibody might contain a contaminating antibody.
Fig 29. The adsorption of triadin-1u antibodies (b), with C-terminal triadin-1 peptide before immunofluorescence labeling shows that the intensity of the triadin-1 labeling in unfixed canine ventricular myofibers is greatly reduced compared to labeling by the antibody before adsorption (a).

Fig 30. Confocal images of canine skeletal muscle single labeled with (a) triadin-1u and (b) triadin-1c antibodies. While labeling by the triadin-1u antibody was slightly above background, a chicken wire like pattern could be observed in myofibers immunolabeled with the triadin-1c antibody. Arrowheads indicate the periphery of the muscle fibers. Bar = 10 μm
G. The Subcellular Distribution of Triadin in Transverse and Longitudinally Cryosectioned Rabbit Atrial Myofibers.

The results from earlier immunofluorescence studies by Carl et al., (59), suggested that triadin and the RyR colocalizes at the cell periphery and at the level of the Z-line in rabbit atrial and ventricular myofibers. This implies that triadin is localized to both junctional and corbular SR in these myofibers. In agreement with these results, single immunofluorescence studies carried out by Guo et al., (44), also suggested that triadin is localized to the I-band region where junctional and corbular SR are densely distributed. The results from the double immunofluorescence labeling of transverse and longitudinal cryosections of canine atrial and ventricular myofibers, with antibodies to two distinct domains of triadin-1, also suggest that triadin-1 is mainly confined to junctional SR along the SL, intercalated disc and T-tubules of these myofibers. However, in contrast to earlier results, the results from our studies suggest that triadin-1 is not expressed at significant levels in the RyR-dense corbular SR of these atrial and ventricular myofibers. In addition, our results also suggest the small diameter of atrial myofibers, combined with the fact that their orientation varies markedly within the plane of section, may make it difficult to accurately determine the subcellular distribution of proteins in longitudinal cryosections of these myofibers. In addition, the fact that antibodies to the RyR cannot differentiate between interior junctional SR and corbular SR clearly indicates that there are technical difficulties associated with attempting to determine the subcellular distribution of triadin in relation to only the RyR in longitudinal cryosections of atrial and especially ventricular myofibers.

Although Carl et al., (59), demonstrated that atrial myofibers double labeled for the RyR and the DHPR had DHPR-positive foci confined to the cell periphery, while RyR-positive foci were found both at the cell periphery and in their cell interior near the level of the Z-line, they did not show directly that triadin positive foci could also be detected near the Z-line of atrial myofibers which lacked DHPR-positive foci and presumably T-tubules. This is important
because some rabbit atrial myofibers contain T-tubules (119), and since we have seen that it is difficult to differentiate between interior junctional SR and corbular SR, in the absence of a marker of the SL and T-tubules, it is possible that some of the triadin-positive foci seen in the interior domains of these myofibers may be due to the detection of the protein in interior junctional SR, which like corbular SR is also present at the level of the Z-line. Thus, it is possible that the results reported by Carl et. al. (59) and Guo et. al. (44), may have been misinterpreted because these researchers failed to localize triadin in the presence of a marker of the SL and the T-tubules and because they also failed to localize the protein in transversely cut cryosections.

To determine if triadin is in fact present in both the junctional and corbular SR of rabbit cardiac muscle, 5-8μm transverse and longitudinal cryosections of rabbit atrial and ventricular myofibers were double immunofluorescently labeled, first with antibodies to the conserved luminal domain of triadin (44) and then with antibodies to either the RyR, a marker of junctional and corbular SR (59,85), or with wheat germ agglutinin (WGA), a lectin which binds to N-acetylglucosamine on the cell surface thus providing a continuous marker of the cell sarcolemma and T-tubules (113). The double labeled cryosections were then visualized by laser scanning confocal microscopy.

1. Triadin vs. The Ryanodine Receptor.

Confocal imaging of double labeled transverse (fig 31a, a', a/a', b, b', b/b') and longitudinal (fig 31c, c', c/c') cryosections of rabbit atrial myofibers showed that intense triadin-positive foci (fig 31a, a/a', b, b/b', c, c/c'; red/yellow) were densely distributed along the SL of these myofibers, where they colocalized with RyR-positive foci (fig 31a', a/a', b', b/b', c', c/c'; green/yellow). In contrast to earlier reports and in contrast to the dense distribution of RyR-positive foci in interior regions of these myofibers, the majority of these myofibers appeared to be devoid of centrally localized triadin-positive foci. However, a few of the transverse and longitudinally cryosectioned myofibers (not shown), did have a few intense triadin-positive foci in their interior regions. In addition, several of the longitudinally cryosectioned myofibers also
contained a faint triadin-positive banding pattern that was similar to the pattern observed in some canine atrial myofibers (see fig 15).

![Image of subcellular distribution of triadin and RyR](image)

**Fig 31.** Comparison of the subcellular distribution of triadin (a, a', b, b', c, c'; red/yellow), with the RyR (a', a'/a', b', b'b', c', c'; green/yellow), in methanol fixed transverse (a, a'/a', b, b', b'/b'), and longitudinal (c, c', c'/c'), cryosections of rabbit atrial myofibers by double immunofluorescence labeling. a/a', b/b', and c/c', are merged images of a, a'; b, b'; and c, c', respectively. Triadin labeling colocalizes with labeling for the RyR primarily at the periphery of the myofibers (large arrowheads and small arrows). The interior of the myofibers were labeled primarily for the RyR (b', b'b', c', c'; long arrow). Bar = 10 μm

2. **Triadin vs. Wheat Germ Agglutinin.**

Confocal imaging of transverse (fig 32a, a', a'/a', b, b', b'/b') and longitudinal (fig 32c, c', c'/c', d, d', d'd') rabbit atrial myofibers showed that a majority of the triadin-positive foci (fig 32a, a/a', b, b'b', c, c'; d, d'd'; green/yellow) were distributed along the WGA (fig 32a', a'/a', b', b'b', c', c'; d, d'd'; red/yellow) labeled surface SL. Furthermore, most of the occasional intense triadin-
positive foci detected in what appeared to be the interior domains of these myofibers were actually confined to regions that were also positively labeled by the cell surface marker. These regions included the occasional T-tubule (fig 32b, b/b'; TT), the intercalated disc (fig 32c, c'; c/c'; ID) or tangential/oblique cryosections through the surface SL (fig 32d, d', d/d'; long arrow). In contrast, the faint banding pattern observed in some of these myofibers failed to colocalize with labeling by the cell surface marker, which led us to conclude that they, like the faint banding pattern seen in some longitudinally cut canine atrial myofibers, were artifacts. Thus, these results suggest that triadin is densely distributed in junctional SR, while it is absent from the corbular SR in these myofibers.

H. The Subcellular Distribution of Triadin in Transverse and Longitudinal Cryosections of Rabbit Ventricular Myofibers.

1. Triadin or The Ryanodine Receptor vs. Wheat Germ Agglutinin.

If triadin, as suggested from the previous results, is localized to only junctional SR, one would expect to observe a differential staining pattern for triadin and the RyR in cryosections of ventricular myofibers. In agreement with these results, confocal imaging of transverse rabbit ventricular myofibers (fig 33) revealed that intense labeling for triadin (fig 33a, a/a'/green/yellow) and the RyR (fig 33b, b/b'; green/yellow) colocalized with labeling by WGA (fig 33a', a/a', b/b'; red/yellow) both at the periphery (fig 33a,a',a'/a, b, b', b/b'; large arrowheads) and along the T-tubules (fig 33a,a',a'/a, b, b', b/b'; TT) in the interior of these myofibers. Although very few triadin-positive foci were localized at a distance from the SL and T-tubules, a large proportion of the RyR-positive foci were observed at a discernible distance from the both the SL and the T-tubules, where corbular SR is densely distributed. In addition, confocal imaging of transverse myofibers that were double immunofluorescently labeled with antibodies to triadin (fig 33c', c/c'; red/yellow) and the RyR (fig 33c, c/c'; green/yellow) showed directly that there was a clear difference in the subcellular distribution of these two proteins. Although the two proteins were densely distributed and colocalized both at the periphery and in the interior of these myofibers, the interior of the myofibers contained considerably more RyR-positive foci (fig 33c, c/c'; small
arrowheads) and a large proportion of these foci did not colocalize with triadin-positive foci (fig 33 c', c/c'; small arrowheads). Thus, these results suggest that triadin is densely distributed in the junctional SR of both atrial and ventricular myofibers, while it is either absent from corbular SR or it is expressed in this domain at levels too low to detect.

Fig 33. Comparison of the subcellular distribution of triadin (a, a'; green/yellow), with WGA (a', a'a': red/yellow); the RyR (b, b/b': green/yellow), with WGA (b', b/b': red/yellow); and the RyR (c, c/c'; green/yellow), with triadin (c', c/c': red/yellow), in methanol fixed transverse cryosections of rabbit ventricular myofibers by double immunofluorescence labeling. a/a': b/b', and c/c', are merged images of a, a'; b, b', and c, c', respectively. Large arrowheads indicate the periphery of the myofibers. TT indicates the position of T-tubules. Long arrows indicate the position of T-tubule like structures. Labeling for the RyR was also observed in areas of the myofibers (b, b/b', c, c/c'; small arrowheads), which did not contain any labeling for WGA (b', b/b': small arrowheads) or triadin (c', c/c': small arrowheads). Bar = 10 μm
2. Triadin vs. The Ryanodine Receptor in Longitudinal Cryosections of Rabbit Ventricular Myofibers.

Confocal imaging of longitudinal cryosections of rabbit papillary myofibers showed that triadin-positive foci (fig 34a', a/a': red/yellow) and RyR-positive foci (fig 34a, a/a': green/yellow), colocalized in a cross striated banding pattern in the interior of these myofibers. These results, like those reported by Carl et. al. (59), and Guo et. al. (44), suggest that triadin and the RyR colocalize in the I-band region of these myofibers, where junctional and corbular SR are densely distributed. Based on these results alone, it can easily be seen how Carl et. al. (59), and Guo et. al. (44), could have incorrectly conclude that triadin is present in both the junctional and corbular SR of ventricular myofibers.

Fig 34. Comparison of the subcellular distribution of triadin (a', a/a': red/yellow), with the RyR (a, a/a': green/yellow), in longitudinal cryosections of methanol fixed rabbit ventricular myofibers by double immunofluorescence labeling. a/a', is a merged image of a and a'. Triadin positive foci colocalize with RyR positive foci primarily along the cell periphery (large arrowheads), and in transverse bands in the interior of the myofibers. Labeling for the RyR was also observed in areas of the myofibers (small arrows, which did not contain any labeling for triadin (small arrows). Bar = 10 μm
CHAPTER 4

DISCUSSION

A. The Subcellular Distribution of Triadin-1 in Canine Cardiac Myofibers

To better understand the potential role of triadin in the release of Ca\textsuperscript{2+} from the SR, it is important to know its distribution in relation to the RyR, calsequestrin and junctin. In this study the subcellular distribution of triadin-1 was determined in transverse and longitudinal cryosections of canine atrial and ventricular myofibers by double immunofluorescence labeling and laser scanning confocal microscopy.

The results of the immunolocalization studies presented here suggest that triadin-1 colocalizes with the RyR and junctin primarily in the junctional SR of canine atrial myofibers\textsuperscript{1}. There were very few intense triadin-1-positive foci detected in the interior of these myofibers where RyR dense corbular SR is abundantly distributed and although these triadin-1-positive foci colocalized with RyR-positive foci, we subsequently observed that a majority of these foci were distributed in regions of these myofibers that were positively immunolabeled for the cell surface marker PLM. These regions included either oblique sections through the SL and intercalated discs or the T-tubules, and suggests that the foci were largely confined to junctional SR. In addition, the faint triadin-1 pattern of cross striations which did not colocalize with labeling for PLM in some 5-8 μm longitudinal cryosections were not visible in the interior of 0.5μm semithin sections of atrial myofibers. On the other hand, the RyR pattern of cross striations seen in these semithin sections were virtually indistinguishable from the pattern of labeling seen in 5-8μm longitudinal cryosections. These results suggest that the faint triadin-1 pattern of cross striation seen in some myofibers is an artifact that arises from the detection of triadin-1 in peripheral junctional SR in out of focus focal planes. These results also show that it

\textsuperscript{1}Subsequent immunoelectron microscopical studies have confirmed that triadin-1 is densely distributed in junctional SR but is sparsely distributed in corbular SR (Thompson et. al., manuscript in prep).
is difficult to accurately determine the subcellular distribution triadin-1 if it is assessed in only longitudinally cryosectioned myofibers. However, our results also suggest that the subcellular distribution of triadin-1 can be accurately determined if it is assessed in the presence of a marker of the cell surface and the T-tubules and in transversely cut cryosections.

Furthermore, the quantitative assessments of the number of triadin-1 and RyR-positive foci in transverse ventricular myofibers also support these conclusions. Although we were unable to differentiate between interior junctional SR and corbular SR in longitudinal cryosections of ventricular myofibers, confocal imaging of transversely cryosectioned myofibers showed that it was feasible to use double immunofluorescence labeling to distinguish between these domains of the SR, as well as to assess their relative proportions in situ. The results of our quantification studies suggest that ~95% of the triadin-1 positive foci in these myofibers fell along the T-tubules and are proposed to correspond to the detection of the protein in junctional SR. In contrast, 66% of the ryanodine receptor positive foci localized along the T-tubules, while 34% of the foci were unique and are proposed to correspond to junctional and corbular SR respectively. Since earlier studies using a similar approach (115), found that ~97 % of the junctin positive foci and ~67% of the RyR positive foci fell along the T-tubules, these results clearly show that triadin-1, like junctin, is primarily confined to junctional SR in both canine atrial and ventricular myofibers. In addition, the finding that ~5% of the triadin-1-positive foci detected in these myofibers were not localized along the T-tubules, while ~3 % of junctin-positive foci were similarly localized, strongly supports our conclusion that the unique triadin-1-positive foci detected in these myofibers are localized along T-tubules in out of focus focal planes.

The results presented here suggest that triadin-1 is very densely distributed in junctional SR but is not expressed in corbular SR of canine cardiac myofibers. Alternatively, it could be suggested that triadin-1 is expressed in corbular SR and at levels comparable to that of junctional SR, but the triadin-1u antibodies used in this study may not be sensitive enough to
detect the protein in corbular SR. Thus, while the triadin-1u antibodies may be sensitive enough to detect triadin-1 in the larger junctional SR structures, they may not be sensitive enough to detect triadin-1 in the smaller corbular SR structures. However, this seems unlikely because line scans of double labeled atrial myofibers revealed that the triadin-1-positive foci at the periphery of these myofibers were actually higher in intensity than the RyR-positive foci at their cell periphery and in their interior regions. Thus, if the two proteins are present in junctional SR in a 1:1 molar ratio (110), then these results suggest that the sensitivity of the triadin-1 antibodies may actually be higher than the sensitivity of the RyR antibodies. Thus, it seems that our inability to detect triadin-1 in corbular SR at levels comparable to that of junctional SR is not due to a too low sensitivity of the immunolabeling procedure for triadin-1, but rather these results serve as a strong indication that triadin-1 is absent from corbular SR.

We should note however, that our inability to detect substantial levels of triadin-1 in corbular SR could stem from the fact that the epitope for the triadin-1u antibodies is a luminal one and it is possible that the epitope may be blocked in corbular SR but not junctional SR. Thus, the protein environment within the two SR domains may make triadin-1 more accessible when it is found in junctional SR than when it is found in corbular SR. However, while the epitope for the triadin-1c antibodies may be blocked, since it is the same site to which CSQ, the RyR and junctin (42,44), are thought to interact, neither of these proteins is known to interact with the C-terminus of triadin-1. This suggests that the triadin-1u epitope should not be blocked since it is not known to interact with other SR proteins. Additionally, since antibodies to the RyR (59,85) and the luminal protein CSQ (89-91), can detect these proteins in both junctional and corbular SR, it stands to reason that antigens in the two SR domains should be equally accessible, despite any differences that may exist in the ultrastructure of junctional and corbular SR. Although we are certain that our inability to detect triadin-1 in corbular SR is not due to the inability of our antibodies to detect the protein, we are currently attempting to develop antibodies
to the N-terminus of triadin-1. Since this epitope is predicted to be cytoplasmic (44), it should be equally accessible, whether present in junctional or corbular SR.

B. The Subcellular Distribution of Triadin in Rabbit Cardiac Myofibers

Since Guo et al. (44) and Carl et al. (59), concluded that triadin was localized to both junctional and corbular SR by assessing its subcellular distribution in relation to only that of the RyR and in only longitudinally cryosectioned myofibers, we felt that their results may have been misinterpreted due to the technical difficulties associated with localizing proteins in longitudinally cryosectioned myofibers and in relation to only the RyR. Thus, we reassessed the subcellular distribution of triadin in relation to the RyR and WGA in transverse and longitudinally cryosectioned rabbit atrial and ventricular myofibers.

The results presented here support the conclusion that triadin and RyR-positive foci colocalize and are densely distributed both at the periphery and along the T-tubules in the interior of rabbit atrial and ventricular myofibers. However, in contrast to earlier reports (59), intense triadin-positive foci were not detected in the interior regions of these myofibers where RyR dense corbular SR was abundantly distributed. Although a faint triadin-positive pattern of cross striation, which did not localize along the WGA-positive SL or T-tubules, was visible in the interior of some longitudinally cryosectioned atrial myofibers, the fact that all of the intense triadin-positive foci detected in the interior of transverse cryosections of these myofibers localize along the SL or the T-tubules, suggested that the banding pattern was an artifact similar to the one observed in longitudinal cryosections of canine atrial myofibers. These results suggest that triadin is densely distributed in junctional SR of canine and rabbit cardiac myofibers, while it is not expressed at detectable levels in the interior regions of these myofibers where corbular SR is abundantly distributed.

C. The Role of Triadin in Canine and Rabbit Cardiac Myofibers

It is generally agreed that junctional SR is the principal Ca\(^{2+}\) storage/release site in cardiac muscle relevant to E-C coupling and that the RyR, calsequestrin, triadin and junctin
interact with each other to form a complex where calsequestrin stores Ca\textsuperscript{2+}, the RyR releases Ca\textsuperscript{2+} while triadin and junctin represent physical and/or signaling links between calsequestrin and the RyR (42). However, previous \textit{in situ} immunolocalization studies have identified not only junctional SR but also corbular SR as potential Ca\textsuperscript{2+} storage/release sites by demonstrating that calsequestrin (89-91), and the RyR (59,85) are very densely distributed in both of these domains of cardiac SR.

Our finding that corbular SR represents ~34\% of the total RyR containing SR in ventricular myofibers, combined with the fact that corbular SR, like junctional SR, is localized to the Z-line where 'sparks' originate during E-C coupling, suggests that corbular SR represents a significant proportion of the Ca\textsuperscript{2+} storage/release sites that could provide a substantial proportion of the total Ca\textsuperscript{2+} released from the SR during E-C coupling. In support of this possibility, recent evidence has suggested that corbular SR can be induced to release Ca\textsuperscript{2+} (5,96). In the most recent of these two studies, it was reported that electrically induced Ca\textsuperscript{2+} transients or 'sparks' (which reflect the opening of a small number of RyRs acting as a functional unit), occurred first at the cell periphery of cat atrial myofibers, where junctional SR is localized, and later in the interior regions of these myofibers, where corbular SR is densely distributed (96). However, it was reported that only peripheral 'sparks' were observed at normal levels of SR Ca\textsuperscript{2+} load, while the interior 'sparks' occurred only after the SR was loaded to simulate an increased inotropic state (96).

These results suggested that under conditions that simulate an increased inotropic state, the Ca\textsuperscript{2+} released from junctional SR could cause Ca\textsuperscript{2+} to be released from corbular SR via a CICR mechanism. However, the exact mechanism by which Ca\textsuperscript{2+} is released from corbular SR is not known. Nor is it known how the release of Ca\textsuperscript{2+} from corbular SR is regulated to prevent positive feedback. However, one possibility is that the Ca\textsuperscript{2+} released from corbular, like the Ca\textsuperscript{2+} released from junctional SR (75), is released in a small microenvironment so that it is unable to activate further Ca\textsuperscript{2+} release from RyRs in nearby corbular SR. In addition, the fact
that the RyR is fairly insensitive to Ca$^{2+}$ in the cytosol (72,73) may also prevent positive feedback. None the less, the differences in the Ca$^{2+}$ release mechanism and kinetics of Ca$^{2+}$ loading proposed to exist between junctional and corbular SR (96) may be directly related to the fact that triadin and junctin are confined to junctional SR. Thus, although it has been suggested that triadin, like junctin (42), is responsible for anchoring CSQ to the Ca$^{2+}$ release domain of the SR and functionally coupling it to the RyR (42,44), the data presented here suggests that triadin, like junctin, may have a role which is specific to E-C coupling in the junctional SR of rabbit and canine cardiac myofibers.

If correct, our results point to the possibility that corbular SR may contain proteins which are different from triadin and junctin but which are also responsible for anchoring CSQ to the Ca$^{2+}$ release domain of corbular SR and functionally coupling it to the RyR. However, since evidence has suggested that small calsequestrin binding proteins (~ 30 kDa), may play a key role in regulating the release of Ca$^{2+}$ from the SR (60, 111, 112), it is possible that these unknown proteins may be similar in size to triadin and junctin. However, further studies will be required to identify and characterize these proteins and determine what role they may play in the mechanisms of Ca$^{2+}$ release from corbular SR. However, this task should be made easier since our findings that triadin, like junctin, is a specific marker of junctional SR, should enable researchers to differentiate between subfractions from cardiac SR that correspond to either junctional or corbular SR. In turn, this will enable the detailed characterization of the protein composition of junctional and corbular SR and provide additional insight into the mechanism by which Ca$^{2+}$ is released from these domains.
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