This is to certify that the

dissertation entitled

THE INTERACTION OF CALMODULIN WITH THE SARCOPLASMIC RETICULUM CALCIUM CHANNEL PROTEIN FROM NORMAL AND MALIGNANT HYPERTHERMIA SUSCEPTIBLE SKELETAL MUSCLE

presented by

HSIU-CHING YANG

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science & Human Nutrition

Jack Strasburg
Major professor

Date 8/13/96

THE INTERACTION OF CALMODULIN WITH THE SARCOPLASMIC RETICULUM CALCIUM CHANNEL PROTEIN FROM NORMAL AND MALIGNANT HYPERTHERMIA SUSCEPTIBLE SKELETAL MUSCLE

By

Hsiu-ching Yang

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1996

ABSTRACT

THE INTERACTION OF CALMODULIN WITH THE SARCOPLASMIC RETICULUM CALCIUM CHANNEL PROTEIN FROM NORMAL AND MALIGNANT HYPERTHERMIA SUSCEPTIBLE SKELETAL MUSCLE

By

Hsiu-ching Yang

The Ca^{2+}-release channel (ryanodine receptor) of the skeletal muscle sarcoplasmic reticulum (SR) is modulated by various physiological and pharmacological ligands. Calmodulin (CaM), a ubiquitous Ca^{2+}-binding protein, has been demonstrated to play a role in regulating SR Ca^{2+} channel activity depending on myoplasmic calcium concentration. However, there have been no direct binding data on the interaction of CaM with the channel protein. The major biochemical defect in malignant hyperthermia (MH), an inherited disorder of skeletal muscle, is associated with a point mutation (Arg615Cys) of the Ca^{2+}- channel protein which is likely responsible for the abnormal Ca^{2+} release from SR in porcine MH susceptible (MHS) skeletal muscle. The altered Ca^{2+} channel activity in MHS SR may results, in part, from abnormal CaM regulation.

The first objective of this project was to define the equilibria of CaM binding to the Ca^{2+}-release channel in porcine skeletal muscle using fluorescence anisotropy. Our results demonstrated that there are five CaM-binding sites per channel subunit with the affinities depending on Ca^{2+} and Mg^{2+} concentrations. The binding of CaM to SR Ca^{2+} -channel was modulated by caffeine, an activator of the Ca^{2+}-channel activity.

The second objective was to test the hypothesis that the altered Ca^{2+}-channel activity

in porcine MHS SR results, in part, from abnormal CaM regulation of the Ca^{2+}-channel. This was examined by determining the binding equilibrium and stoichiometry of MHS and normal SR Ca^{2+}-channel with CaM under defined metal ion concentrations. The stoichiometry of CaM to the channel protein in MHS SR was significantly altered compared to normal SR in the presence of EGTA indicating the possibility of abnormal CaM regulation of Ca^{2+}-channel in MHS SR.

The third objective was to identify CaM-binding sites in Ca^{2+}-channel from rabbit skeletal muscle SR in order to further understand structure-function relationship of Ca^{2+}-channel activity. Two CaM-binding sites, amino acid residues 1333-1508 and 2400-2515, in the central regions of Ca^{2+}-channel were identified by limited proteolysis combined with photoaffinity labeling and immunoblotting analysis.

These results provide sufficient evidence that CaM plays an important role in regulating skeletal muscle SR Ca^{2+}-channel by binding to specific domains with different affinities depending on other channel modulators concentrations.

To my parents

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor, Dr. Gale M. Strasburg, who help me grow not only as a scientist but also in character. It is absolutely crucial to have his guidance, advice and support throughout the course of graduate study and preparation of the dissertation. I gratefully acknowledge the members of my guidance committee: Dr. Alfred Haug, William Helferich, John Linz, and Robert Merkel, who critically read my manuscripts and offered valuable suggestions. I am indebted to Dr. Linz for generously giving up his time and equipments to assist in teaching me molecular biology experiments. I also am indebted to Dr. Helferich for his generosity to let me use equipments in his lab. I am gratefully to my collaborators: Dr. James Michelson at Minnesota University, who provided us MHS SR vesicles; Dr. Susan Hamilton at Baylor College of Medicine who provided us rabbit SR vesicles and gave me a wonderful opportunity to finish my last study in her lab; Dr. Yili Wu, from Dr. Hamilton's lab, who provided us calpain digestion results. Thanks to the past and present members of Dr. Strasburg lab: Mark Reedy, Christ Burke, Dr. Li-ju Wang, Arti Arora, Dr. Todd Byrem and Dr. Yu-chen Chang; and coworkers at Dr. Hamilton's lab for their friendship and support. Special thanks to Dr. Todd Byrem for reviewing and giving valuable suggestions for part of my dissertation. I also would like to express my deepest

gratitude to my family and friends for their support and encouragement. Finally, I forever am indebted to my parents, Feng-O Yang and Ai-Min Yang for their love, support, and encouragement throughout this graduate study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION AND OBJECTIVES

Contraction of skeletal muscle is triggered by the release of Ca^{2+} from the sarcoplasmic reticulum (SR); this process is coupled to the depolarization and repolarization of the transverse tubular (T-tubule) membrane. Communication of voltage changes between T-tubule and SR occurs at the triad junction where "foot" proteins, also known as ryanodine receptor or Ca^{2+}-release channel protein, span the gap between the two membrane structures.

Purified ryanodine receptor protein has four identical subunits of relative molecular mass of 565,000. The channel properties and subcellular distribution of the ryanodine receptor suggest its involvement in the SR Ca^{2+} release that occurs in skeletal muscle excitation-contraction (E-C) coupling. Regulation of the SR Ca^{2+}-release channel includes numerous physiological and pharmacological molecules such as channel activators: μM Ca^{2+}, adenine nucleotides, caffeine, nM ryanodine; and channel inhibitors: Mg^{2+}, mM Ca^{2+}, μM ryanodine, calmodulin (at μM $[Ca^{2+}]$) which can modify channel gating properties and thereby alter the sensitivity of the channel in response to the change of T-tubule membrane potential (Meissner et al., 1986).

One of these channel activity modulators is calmodulin (CaM), an acidic protein

composed of four homologous Ca^{2+}-binding domains, mediates Ca^{2+} stimulation of numerous cellular processes. Initial studies on the role of CaM in Ca^{2+} regulation show that Ca^{2+} release from heavy SR vesicles is partially inhibited by CaM in the presence of Ca^{2+} via a direct interaction with the Ca^{2+} channel. Recently, it was reported that CaM can also activate Ca^{2+} release from SR when myoplasmic [Ca^{2+}] is < 0.1 μM (Tripathy et al., 199). The Ca^{2+} -channel modulators may also be involved in altering the binding of CaM to the channel protein. In the absence of detailed CaM and channel protein binding data, it is difficult to assess the overall role of CaM in regulation of Ca^{2+}-channel activity. Therefore, the first objective of this study was to examine the effects of some channel modulators such as Ca^{2+}, Mg^{2+}, KCl, and caffeine, on CaM/Ca^{2+} -channel binding interaction to define further the physiological role of CaM in channel regulation.

Malignant hyperthermia (MH) is an inherited myopathy in which skeletal muscle contracture with attendant hypermetabolism and elevation in body temperature are triggered by inhalation of anesthetics and skeletal muscle relaxants. In swine homozygous for the defect, MH can also be triggered by stress; thus, the disease in the affected animals is also referred to as porcine stress syndrome (PSS). Major economic losses in the swine industry result from the development of pale, soft, exudative (PSE) pork that arise from postmortem manifestation of the disease in MH susceptible (MHS) pigs. Because Ca^{2+} is the main regulator of muscle contraction and metabolism, the defect in MH was believed to lie in Ca^{2+} regulation. There is now considerable evidence that the primary biochemical defect in MH is associated with an abnormal SR Ca^{2+}-release mechanism (Mickelson et al., 1988). A single point mutation (C1843 to T1843) in the porcine skeletal muscle rynodine receptor gene (*ryr*

1) has been identified (Fujii et al., 1991). This mutation, together with phenotypic indicators demonstrate that this single amino acid alteration is the causal abnormality for MH. However, direct evidence concerning the functional role of the mutated Ca^{2+}-channel protein and the possible role of altered regulation by channel modulators in altering Ca^{2+} release properties of the channel protein in MH is absent. To address this question, the second objective of this study was to test the hypothesis that altered Ca^{2+}-channel activity present in SR from MHS swine results in part, from altered CaM binding to and regulation of the Ca^{2+}-channel.

Cloning, sequencing and functional expression of cDNAs encoding the Ca^{2+}-channel have provided some clues regarding structural-functional relationships within the normal and MHS Ca^{2+}-channel protein. The localization of binding sites for various modulators including CaM on Ca^{2+}-channel has been attempted by prediction methods using the primary and secondary structure algorithms (Takeshima et al., 1989; Zorzato et al., 1990). CaM binding domain candidates have been identified by several groups using the ligand overlay method on rabbit skeletal muscle channel protein fragments expressed as fusion proteins. These results are questioned by the fact that the fusion protein containing the channel protein fragment may not refold like that of the intact protein (Menegazzi et al., 1994; Chen et al.,1994). Furthermore, the fact that up to nine CaM-binding sites have been identified by the two laboratories using this method, and the fact that there is little agreement on identity of sites, together suggest identification of sites must be done in the native protein. The roles of specific CaM-binding sites as activators or inhibitors is important in our understanding of the mechanism of Ca^{2+} regulation in health and disease.

In summary, the objectives of my research are as follows:

1. To determine CaM/channel protein equilibrium dissociation constant (K_d) and binding capacity (B_{max}) under defined conditions in normal skeletal muscle.
2. To test the hypothesis that altered Ca^{2+} channel activity present in SR from MHS swine results in part, from altered CaM binding to and regulation of the Ca^{2+}-channel.
3. To localize CaM binding domains in native skeletal muscle SR Ca^{2+}-release channel.

The dissertation addresses these three objectives and is organized in a series of chapters. The first two sections include the Introduction and Literature Review for the entire dissertation. Each study was organized as a manuscript with its specific Introduction, Experimental Procedure, Results and Discussion. The last sections were the Overall Conclusions and Bibliography for the entire dissertation.

CHAPTER 2

LITERATURE REVIEW

2.1 Sarcoplasmic Reticulum in the Skeletal Muscle

2.1.1 Sarcoplasmic reticulum and excitation-contraction coupling

Muscle contraction and relaxation are regulated by the myoplasmic free calcium concentration, which in turn depends on appropriate communication between two membrane systems, the sarcolemma/transverse-tubule (T-tubule) membranes and the sarcoplasmic reticulum (SR) membranes in myofibrils. The actual role of Ca^{2+} in the excitation-contraction (E-C) coupling mechanism in skeletal muscle was not completely understood until discovery of the Ca^{2+}-dependent regulatory proteins in the late 1960s (Ebashi and Endo, 1968).

In the late 1940s, it was known that depolarization of the muscle cell membrane induced an influx of some substances necessary to induce contraction and these substances were recognized to be released from an internal source so that a lesser period of time elapsed between excitation and contraction (For review see Entman, 1986). Several important experiments in the 1950s implicated the involvement of the SR in muscle contraction. Marsh

(1951, 1952) discovered that homogenized muscle, which was normally shrunken by ATP, could be induced to swell by addition of ATP plus a muscle extract. This swelling corresponded to the muscle relaxation process and the relaxing effect induced by the muscle extract was subsequently demonstrated with muscle fibers (Bendall, 1952,1953; Fujita, 1954). The relaxing factor ('Marsh' factor) was isolated and identified as the SR. Huxley (1957) and Huxley and Taylor (1958) demonstrated that microelectrode application of depolarizing potassium solutions at the region of the T-tubular system resulted in only localized contraction of the adjacent hemisarcomeres. Podolsky and Constantin (1964) ionophoretically injected Ca^{2+} at the triad region which also resulted in hemisarcomere contraction, suggesting that calcium was the important link between excitation and contraction.

The role of calcium in mediating actin-myosin interaction during muscle contraction was first demonstrated by Ebashi and Ebashi (1964) who demonstrated that a protein component complexed to tropomyosin imparted calcium sensitivity to reconstituted actin-myosin. In the period between 1968 and 1970, this factor was demonstrated to be troponin (Ebashi et al., 1968); the mechanism by which troponin and tropomyosin regulate actin-myosin interaction has been studied in considerable depth since then. The concentration of Ca^{2+} required to elicit muscle contraction was consistent with the affinity of troponin for Ca^{2+}. This strongly implicated calcium-induced conformational changes in troponin as the major modulator of actin-myosin interaction and contractility.

2.1.2 Structure of the sarcoplasmic reticulum

Skeletal muscle in higher animals consists of bundles of long fibers or cells. Each muscle fiber has numerous myofibrils which are composed of linear arrays of repeating sarcomeres that run the length of the fiber. The cylindrical sarcomeres form the structural units of muscle contraction. Each sarcomere is surrounded by a sarcotubular membrane system, a sleeve-like network consisting of invaginations of the sarcolemma (plasmalemma of muscle cells) called T-tubules, and the SR which is nestled between the T-tubules (Fig. 2.1). This SR/T-tubule membrane system is directly responsible for regulating the Ca^{2+} concentration in the immediate vicinity of the muscle filaments (for reviews see Franzini-Armstrong, 1980; Martonosi, 1984).

In skeletal muscle, the total SR complement is broadly divided into two morphologic categories: junctional SR which is closely apposed and attached to either the sarcolemma or its derivative, the T-tubule; and nonjunctional SR which does not make a physical connection with other membrane systems. The junctional SR region that is tightly apposed to the T-tubule forms large dilated sacs commonly termed the terminal cisternae. Since most skeletal muscles display a triple structure, i.e., terminal cisterna-T-tubule-terminal cisterna, Porter and Palade (1957) referred to this anatomical structure as a triad, a term which still persists. The gap between the junctional SR and T-tubule is about 15 nm. The junctional gaps are bridged by regularly spaced, densely staining structures termed foot structures or junctional feet. In mammalian skeletal muscle, the SR/T-tubule junctions invariably occur at each A-I band region. Freeze-fractured preparations of intact skeletal muscle have revealed the presence of

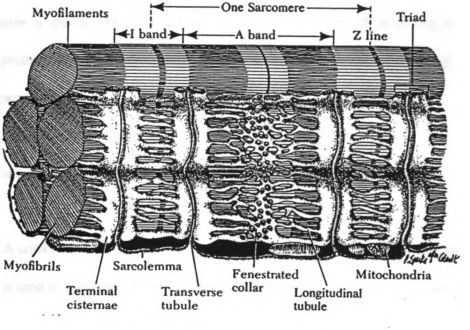


Figure 2.1 Diagrammatic representation of the sarcoplasmic reticulum and T-tubules, and their relation to the myofibrils of mammalian skeletal muscle (From Judge et al., 1989).

30 nm "dimples" or indentations usually as a single row in membranes of the terminal cisternae (Rayns et al., 1975; Beringer, 1976). There is a definite correlation between number of indentations in terminal cisternae and increasing speed of muscle contraction. Evidence suggests that charge movement and Ca^{2+} fluxes are related to density of indentations (Dulhunty and Valois, 1982; Dulhunty and Valois, 1983; Dulhunty et al., 1983). Compared to the degree of specialization in junctional SR, the nonjunctional SR is considerably simpler. The longitudinal tubules connect medially with two terminal cisternae, forming contiguous SR compartments.

2.1.3 Components of sarcoplasmic reticulum

A combination of differential centrifugation and isopycnic zonal ultracentrifugation methods is used to separate SR vesicles into light and heavy SR fractions. Light SR vesicles are obtained from the 30-32.5% zone of sucrose concentration, whereas heavy SR vesicles are obtained from the 38.5-42% zone of sucrose gradients. Freeze-fracture replicas of the light SR vesicles show an asymmetric distribution of intramembranous particles with the same orientation and distribution as the longitudinal SR *in vivo* (Campbell et al., 1980). Heavy SR vesicles appear as rounded vesicles of uniform size filled with electron dense material, similar to that seen in the terminal cisternae of the SR (Campbell et al., 1980). Biochemical characterization of light and heavy SR vesicles demonstrated that heavy SR contains greater than six times the calcium content of light vesicles, and the rate of passive Ca^{2+} efflux from the heavy vesicles is double that of light vesicles. The biochemical and morphological data

strongly support the view that the light vesicles are derived from the longitudinal SR and that the heavy vesicles are derived from the terminal cisternae (Campbell et al., 1980).

The SR comprises about one-third phospholipid and neutral lipids and two-thirds protein (Meissner and Fleischer, 1971). There are several major proteins in the SR and enzymatic activities that may or may not be related directly to the Ca^{2+} regulatory mechanisms of the membrane. Sodium dodecyl sulfate gel electrophoresis shows that the light SR contains predominantly (80-90%) Ca-ATPase or Ca-pump protein and 5% of a 53 kDa protein (MacLennan and Wong, 1971). The latter is a high affinity Ca^{2+}-binding protein within the lumen of the SR (MacLennan and Wong, 1971). The heavy SR fraction contains 50% Ca-ATPase, 25% calsequestrin, 5% 53 kDa protein, 3% each of 30 and 34 kDa proteins, and 2% Ca^{2+} release channel protein (Campbell and MacLennan, 1981).

The Ca^{2+} and Mg^{2+}-dependent ATPase enzyme, a single polypeptide of 110 kDa, carries out the enzymatic function of Ca^{2+} transport in skeletal muscle SR (de Meis and Vianna 1979). Immunofluorescence and immunoferritin labeling techniques were used to show that the Ca^{2+}-ATPase pump is localized throughout the longitudinal SR and nonjunctional regions of the terminal cisternae, but is absent from the junctional region of the terminal cisternae, the region apposed to the T-tubule (de Meis and Vianna 1979).

Two glycoproteins of 53 kDa and 160 kDa are transmembrane proteins, intrinsic to the SR membrane. Calsequestrin is a major SR protein, accounting for about 7% of the total membrane protein. It binds nearly 1000 nmol of Ca^{2+} per mg protein with a dissociation constant of about 1 mM (MacLennan et al., 1983). Calsequestrin is luminally located, mostly in the terminal cisternae and it has been postulated that it acts to sequester Ca^{2+} in the interior

of the SR (MacLennan et al., 1983)

Approximately 20% of the terminal cisternae consists of junctional face membrane which contains the foot structures spanning the gap between the apposed SR and T-tubules. The feet have an unusually large size (2,000 kDa per foot protein) and characteristic shape which allowed their direct identification with the large spanning proteins (Kawamoto et al., 1988; Kawamoto et al., 1986) or ryanodine receptors (Block et al., 1988; Inui et al., 1987a,b; Kawamoto et al., 1988) that constitute the channel responsible for release of calcium from the SR (for review see McPherson and Campbell, 1993). Thus, the terms "junctional foot protein", "ryanodine receptor", and SR "Ca^{2+}-release channel protein" are synonymous.

2.1.4 Physiological role of the sarcoplasmic reticulum in skeletal muscle

The SR has a central role in regulating Ca^{2+} homeostasis in the muscle cell. This function may be divided into three activities: Ca^{2+} uptake, Ca^{2+} storage, and Ca^{2+} release during a contraction and relaxation cycle.

1) Calcium uptake

The SR reduces the calcium concentration within the myofibrillar space to values sufficiently low ($<10^{-7}$ M) to allow and to maintain relaxation of muscle (see Weber and Sanadi, 1966). The Ca^{2+}-pump protein is responsible for translocating Ca^{2+} against the concentration gradient from the myoplasm to the lumen of the SR. The energy is provided by ATP hydrolysis; two Ca^{2+} ions are translocated per molecule of ATP hydrolyzed (MacLennan and Holland, 1975; Tada et al., 1978; Ikemoto, 1982).

The Ca^{2+} pump protein has been extensively studied in terms of structure, enzyme kinetics, and thermodynamics by measuring Ca^{2+} uptake, by isolating SR vesicles and by characterizing kinetic events in the purified protein (review in Fleischer and Inui, 1989).

2) Calcium Storage

The SR is the sole source of calcium responsible for muscle contraction during activation of E-C coupling in skeletal muscle. Ca^{2+} taken up by the Ca^{2+} pump protein is stored within the SR during relaxation until an electro chemical signal from the T-tubule causes its release from the SR junctional terminal cisternae for contraction (Somlyo et al., 1981). The Ca^{2+} storage function of SR is mainly attributed to calsequestrin which is localized in the luminal space of the terminal cisternae of skeletal muscle SR (Meissner et al., 1973). Calsequestrin from fast-twitch skeletal muscle has molecular mass of 65,000 estimated from primary structure (Campbell et al., 1983). The most important property of the calsequestrin is its high capacity for binding Ca^{2+} with moderate affinity. Calsequestrin from skeletal muscle can bind 40-50 Ca^{2+} ions per molecule with a dissociation constant of ~1 mM in isotonic salt (Maurer et al., 1985; Meissner, 1973). By binding Ca^{2+}, calsequestrin performs the important function of keeping the free Ca^{2+} concentration within the SR lumen low, since mM free Ca^{2+} concentrations inhibit the calcium pump. The moderate affinity of calsequestrin for Ca^{2+} (K_{Ca}=mM) is also important for rapid dissociation of Ca^{2+} for its release from the SR lumen upon E-C coupling (MacLennan and Wong., 1971).

3) Calcium Release

Release of Ca^{2+} from the SR via the Ca^{2+}-release channel protein is under the control of the membrane potential across the T-tubule. In striated muscle, rapid release of Ca^{2+} from SR is initiated by a surface membrane action potential that is communicated to the SR from the T-tubule via the "junctional feet". The latter structures are now known as Ca^{2+} release channels (for review see Meissner, 1994).

Based on previous reports, a general model for E-C coupling in striated muscle has emerged as follows: depolarization of the T-tubule membrane by a nerve impulse triggers a charge movement at the T-tubule/SR junctional structure, which results in the release of calcium from the SR membrane into the myoplasm. The myoplasmic Ca^{2+} concentration increases from resting levels of $< 10^{-7}$ M to $\sim 10^{-5}$ M. Calcium binding to troponin changes the structure of the thin filaments and facilitates the interaction of myosin with actin, with the subsequent activation of the cyclic cleavage of ATP and the development of contractile tension. The general applicability of this model suggests several important properties of the SR which bear directly on the control characteristics of E-C coupling. In this process, three events must take place: detection by a voltage sensor of changes in potential across the T-tubule membrane; transmission of the depolarization signal to the SR; and release of calcium from the SR. All elements necessary for these events are contained within an E-C unit or triad.

Morphology studies suggested that a specific transmission of the depolarization signal to the SR was necessary for E-C coupling (Franzini-Armstrong, 1970). Block et al. (1988) suggested that there is a direct interaction between the Ca^{2+} channel-foot proteins in the SR membrane and a protein component of the T-tubule membrane referred to as the

dihydropyridine receptor (DHPR) which serves as a voltage sensor in muscle. Biochemical evidence demonstrates that Ca^{2+} channel and DHPR are present in the triad membrane preparation in a complex, thus supporting a model of direct physical linkage of DHPR and Ca^{2+} channel in E-C coupling (Fig. 2.2) (Marty et al., 1994b). How signal transmission occurs at the T-SR junction is one of the major unsolved problems of muscle biology. Two major E-C coupling mechanisms in muscle have been proposed.

1) Molecular coupling model. Based on early studies, it was proposed that in response to an action potential, a movement of membrane-bound electrical charge, termed a gating charge, from the cytosolic surface to the extracellular surface of the T-tubule membrane would initiate release of accumulated Ca^{2+} into the myoplasm by the Ca^{2+}-release channels in the junctional region of SR (Fig. 2.3) (Chandler et al., 1976; Schneider, 1981; Schneider and Chandler, 1973). This mechanical interaction hypothesis for E-C coupling may involve modulation by additional interacting, coupling, or linking proteins. (Block et al., 1988; Fleischer and Inui, 1989; Kim et al., 1990; Caswell et al., 1991; Marty et al., 1994).

2) Diffusible chemical-transmitter hypothesis. The alternative to direct mechanical regulation of the SR Ca^{2+}-release channel by the T-tubule voltage sensor is that a diffusible chemical transmitter is responsible for signal transmission from T-tubule to SR. The calcium ion has long been considered as a candidate for a diffusible transmitter. There is morphological evidence to suggest that, at least in some skeletal muscles, only a subpopulation of Ca^{2+} release channels are mechanically linked to T-tubule sensors (Franzini-Armstrong and Jorgensen, 1994). Ca^{2+} ions released by these channels could serve to amplify further SR Ca^{2+} release by opening the remaining DHPR-unlinked channels (Franzini-

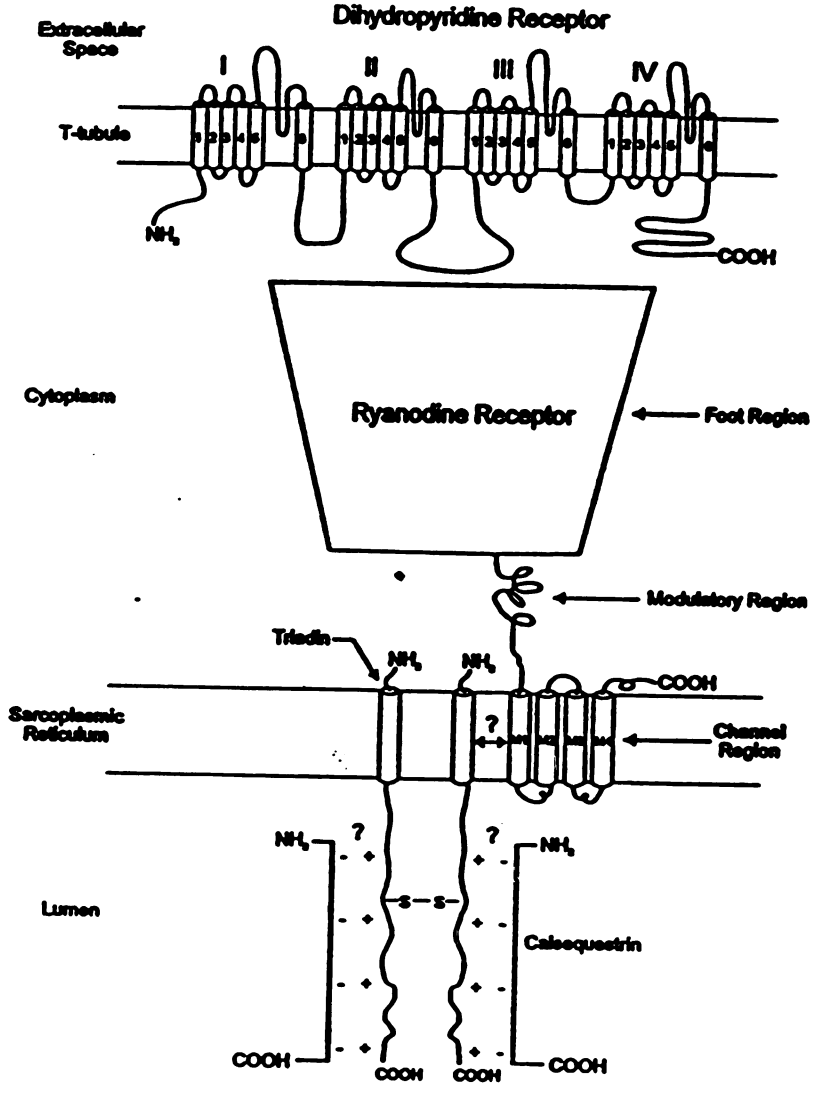


Figure 2.2 Model of the skeletal muscle triad junction (From McPherson and Campbell, 1993).

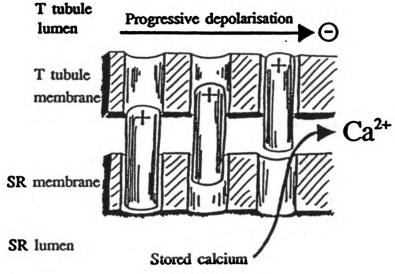


Figure 2.3 Molecular coupling model for the release of Ca^{2+} from SR. Voltage sensors in the T-tubular membrane move outwards in response to depolarization. With sufficient depolarization the calcium channels in the SR membrane are open and calcium escapes into the cytoplasm and diffuses to the myofibrils (From Jones and Round, 1990).

Armstrong and Jorgensen, 1994). This would be consistent with the presence of Ca^{2+}-induced Ca^{2+} release (CICR) in skeletal muscle (Endo et al., 1970; Ford and Podolsky, 1970). Inositol, 1,4,5-triphosphate (IP_3) has been considered as a possible candidate as the hypothetical chemical transmitter from the T-tubule to the SR based on its role as a secondary messenger for activation of Ca^{2+} release in smooth muscle and most non-muscle cells (Berridge, 1993). Two early studies showing that IP_3 promotes the release of Ca^{2+} from skinned skeletal muscle fibers (Vergara et al., 1985) and SR vesicles (Volpe et al., 1985) suggest that IP_3 may also play a central role in the mechanism of skeletal muscle E-C coupling. However, IP_3 appears to be generated in concentrations too low and on time scale which is too slow to account for the rapid release of Ca^{2+} in skeletal muscle (Walker et al., 1987).

Sufficient evidence for the necessary involvement of a diffusible transmitter from T-tubule to SR has not been provided and some indication against necessary participation has been obtained. The direct molecular interaction model would thus seem to be the current hypothesis of choice for the mechanism of T-tubule to SR signal transmission (Schneider, 1994).

2.2 The Ca^{2+} Release Channel Protein/Ryanodine Receptor of Skeletal Muscle Sarcoplasmic Reticulum

2.2.1 Purification and characterization of Ca^{2+}-release channel protein/ryanodine receptor

Ryanodine, a neutral alkaloid, which was isolated from the stems of the plant *Ryania speciosa* Vahl, is a muscle-paralyzing agent (Jenden and Fairhurst, 1969). Ryanodine induces a progressive contracture in skeletal muscle and loss of contractile tension in cardiac muscle by affecting the Ca^{2+} release mechanism in SR (see Jenden and Fairhurst, 1969, for review). Fairhurst and Jenden (1962) and Jones et al. (1979) were the first to show, in skeletal muscle and cardiac muscle, respectively, that ryanodine stimulated accumulation of Ca^{2+} by the SR. Sutko et al.(1985) proposed that ryanodine specifically blocks the Ca^{2+}-channel of SR at high concentration. Further studies demonstrated that the effect of ryanodine bound to the Ca^{2+}-release channel is concentration-dependent; at concentrations in the range of 0.01-10 μM ryanodine, Ca^{2+} release is stimulated, whereas, at concentrations in the range of 10-300 μM ryanodine, release is inhibited (Gilchrist et al., 1992; Hasselbach and Migala, 1987; Lattanzio et al., 1987; Meissner, 1986a; Nelson, 1987). This led to the widespread use of ryanodine as a probe of SR function in both cardiac and skeletal muscle and provided the rationale for using radiolabeled ryanodine to identify the presence of the Ca^{2+}-release channel protein in junctional SR. Pessah et al. (1985 and 1986) demonstrated that [^{3}H]ryanodine binds with high affinity in Ca^{2+}-dependent manner in heavy SR preparations from rabbit skeletal muscle and

provided direct evidence for Ca^{2+}-ryanodine receptor complexes involved in the release of Ca^{2+} for contraction during E-C coupling. Using [^{3}H]ryanodine binding as an assay for channel protein activity, Lai et al. (1988, 1989) purified the Ca^{2+}-release channel and characterized the protein as a large, 30S homotetramer of negatively charged and allosterically coupled polypeptides, each of M_r>400,000. Purification of the Ca^{2+}-release channel has also been accomplished by immunoaffinity chromatography using an anti-ryanodine receptor monoclonal antibody (Imagawa et al., 1987) and by sequential column chromatography on heparin-agarose and hydroxylapatite in the presence of CHAPS (Inui et al., 1987a). Electron microscopy of the purified ryanodine receptor revealed a four-leaf clover structure which is comparable in size and shape to the feet structures identified by Franzini-Armstrong (1970) in cisternae of SR, suggesting that the ryanodine receptor is equivalent to the feet structures (Inui et al., 1987a; Lai et al., 1988; Kawamoto et al., 1986).

The purified ryanodine receptor has been reconstituted into planar lipid bilayers where it exhibits Ca^{2+} conductance properties and pharmacological modulation consistent with that of the native Ca^{2+}-release channel from SR vesicles (Lai et al., 1988; Lai et al., 1989; Imagawa et al., 1987; Smith et al., 1988; Hymel et al., 1988). The Ca^{2+} release channel has a characteristically large unitary conductance and low divalent-over-monovalent ionic selectivity, and is gated into the open state by cellular ligands (Smith et al., 1985, 1986a, 1986b).

2.2.2 Structure and Function of Ryanodine Receptor (RyR) Ca^{2+}-Channel

Comparisons between freeze-fracture images of the junctional SR and rotary-shadowed images of isolated triads and of the isolated foot protein reveal that the RyR/Ca^{2+} channel has two domains: one is the large hydrophilic foot structure which spans the junctional gap; the other is a hydrophobic domain buried in the membrane which presumably forms the actual Ca^{2+}-release channel (McPherson and Campbell, 1993). Hydropathy plots are consistent with these morphological studies and suggest that the carboxy-terminal pore region, which is thought to consist of as few as four (Takeshima et al., 1989) or as many as ten to twelve (Zorzato et al., 1990) putative transmembrane segments.

Analysis of the purified ryanodine receptor using freeze-drying and rotary shadowing methods show a uniform population of large molecules, with four apparently identical subunits, symmetrically disposed around a central depression. The entire large quatrefoil structure is 26-28 nm side-to-side and 33-36 nm along the diagonal (Block, 1988). Averaged images of negatively stained and frozen hydrated specimens have provided a more detailed view of the structure of the rabbit skeletal muscle Ca^{2+}-release channel protein (Wagenknecht et al., 1989; Radermacher et al. 1992)). An overall dimension of 27x27x14 nm was observed for the skeletal Ca^{2+}-channel with an unusual ion-conduction structure composed of a central (membrane-spanning) channel that branches into four radial channels in the cytoplasmic (foot) region of the complex (Fig 2.4). The pathway for calcium efflux is suggested by a central pore from which four radial channels extend to each of four openings contiguous with the myoplasm (Wegenknecht, 1989) (Fig 2.4).

Surface topology analysis combined with protease sensitivity has been used to provide independent information identifying amino acid sequences with high likelihood for appearing

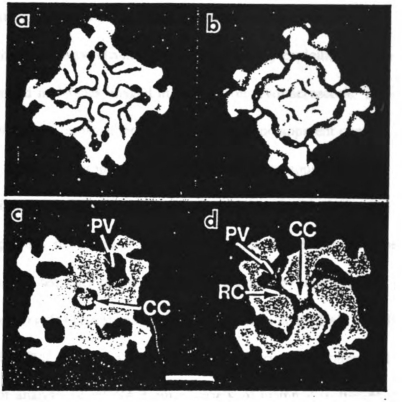


Figure 2.4 Three-dimensional reconstruction of the calcium release channel obtained from negatively stained specimens. Labeled are the central cavity (CC), the radial canal (RC), and the peripheral vestibules (PV). (a,b) Top and bottom faces;(c,d) reconstruction sliced open to reveal internal structure. (From Radermacher et al., 1992).

on the surface of the Ca^{2+}-channel molecule and for predicting sequences with low surface probability which may be buried within the hydrophobic core of the structure (Marks, et al., 1990). Recently, Serysheva et al. (1995) determined the low resolution three-dimensional structure of the Ca^{2+}-release channel in its closed state by exploiting the random orientations of ice-embedded molecules imaged in an electron cryomicroscope. Their results reveal a structure in which the transmembrane region exhibits no apparent opening on the SR lumen side and the extended cytoplasmic region has a hollow appearance and consists, in each monomer, of a clamp-shaped and a handle-shaped domain (Fig 2.5).

The primary structures of three mammalian RyR isoforms have been determined by cDNA cloning and sequencing. The isoforms are encoded by three different genes coding for skeletal muscle (*ryr1*) (Takeshima et al., 1989; Zorzato et al., 1990), cardiac muscle (*ryr2*) (Nakai et al., 1990; Otsu et al., 1990), and brain (*ryr3*) (Hakamata et al., 1992) RyR. Northern blot analysis of mRNA from a variety of mammalian tissues indicates that the skeletal isoform appears to be restricted to fast- and slow-twitch skeletal muscle (Takeshima et al., 1993). mRNA for *ryr3* has also been found in mammalian skeletal muscle (Conti et al., 1995).

The cDNA sequences of the skeletal muscle ryanodine receptor (*ryr1*) demonstrated that the protein consists of 5,032 (from human), or 5,037 (from rabbit) amino acid residues with molecular masses of 563,584 Da (Takashima et al., 1989) or 565,223 Da (Zorzato et al., 1990), respectively. Primary structure predictions suggest the presence of several potential cytoplasmic Ca^{2+}, nucleotide, calmodulin binding, and phosphorylation sites (Takeshima et al., 1989; Zorzato et al., 1990; Hakamata et al.,1992; Nakai et al., 1990; Otsu et al., 1990).

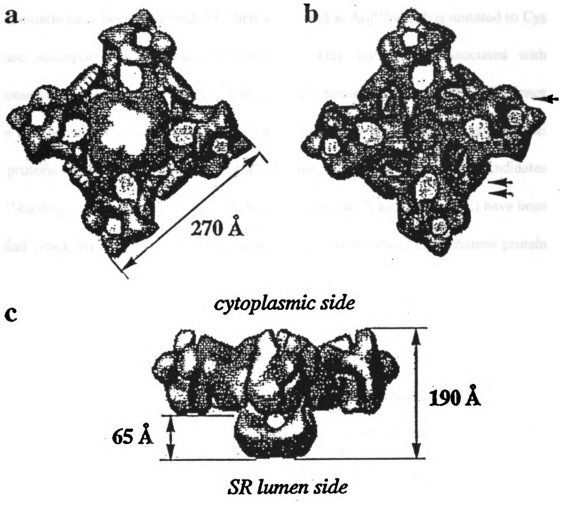


Figure 2.5 Surface representation of the 3D structure of ice-embedded Ca^{2+}-release channel in its closed state in a, bottom or as many as ten to twelve (Zorzato et al., 1990) putative transmembrane segments. The N-terminal half of the protein view, b, top view and c, side view (From Serysheva et al., 1995).

Two regions in the Ca^{2+} channel regarding regulating Ca^{2+}-induced Ca^{2+} release in skeletal muscle have been identified. The first was found at Arg^{615} which is mutated to Cys in swine susceptible to malignant hyperthermia. This mutation is associated with hypersensitivity of the Ca^{2+} channel to Ca^{2+}-induced Ca^{2+} release (Fujii et al., 1991). Evidence for the second region was obtained in $^{45}Ca^{2+}$ and ruthenium red overlay studies with trpE fusion proteins containing fragments of the Ca^{2+} channel primary structure. Three candidates for Ca^{2+}-binding sites (amino acid residues 4246-4467, 4382-4417, and 4478-4512) have been identified which may be involved in increasing the Ca^{2+} sensitivity of Ca^{2+}-channel protein (Chen et al., 1992). The experimental results from Treves et al.(1993) indicate that the Ca^{2+}-dependent gating domain of the Ca^{2+}-channel lies near the junctional SR membrane at the level of, or closely associated with, myoplasmic loop 2, corresponding to residues 4380-4625. This implies that the Ca^{2+}-gating domain is located far from the surface of the Ca^{2+}-channel, in contact with the T-tubules, the membrane compartment that generates the trigger signal for channel opening during E-C coupling.

2.2.3 Regulation of the Ca^{2+} release channel

The main cellular event controlled by Ca^{2+}-channel in skeletal muscle is E-C coupling, i.e. the increase in SR Ca^{2+} permeability triggered by muscle cell depolarization. Present physiological and biochemical evidence suggests that the vertebrate skeletal muscle Ca^{2+} release channel is under the dual control of the T-tubule depolarization (discussed in Sec. 2.1.4) and various channel modulators (discussed below). Three complementary techniques

have been used to study regulation of the Ca^{2+} channel *in vitro*. First, the Ca^{2+} release rates of passively or actively loaded triads or heavy SR vesicles have been followed by rapid filtration or chemical quenching methods. Second, the behavior of channels incorporated from SR vesicles or of purified Ca^{2+} channel proteins has been studied by single channel recording in planar lipid bilayers. Third, [^{3}H]ryanodine binding by the channel protein has been used as a probe to study the functional states of the Ca^{2+} channel.

Many compounds are known to affect SR Ca^{2+} release. Potentiators of SR Ca^{2+} release include μM Ca^{2+}, adenine nucleotides, caffeine, halothane, ryanodine at nM concentrations, sulfhydryl reagents, and calmodulin (at $[Ca^{2+}] < 0.1$ μM). Inhibitors include mM Mg^{2+}, Ca^{2+} at mM concentrations, ryanodine at μM concentrations, ruthenium red, procaine, dantrolene, spermine and calmodulin (at $[Ca^{2+}] > 1$ μM) (McPherson and Campbell, 1993). It is likely that many of these compounds affect Ca^{2+} release by direct actions on the Ca^{2+}-channel as many of the compounds affect [^{3}H]ryanodine binding to the purified Ca^{2+}-channel, thus affecting the channel conductance or open state probability of purified Ca^{2+}-channel incorporated into planar lipid bilayers (Fleischer and Inui, 1989).

A bell-shaped Ca^{2+} activation curve of Ca^{2+} efflux from heavy SR vesicles has been obtained in the absence of other regulatory ligands such as Mg^{2+} and ATP; Ca^{2+} efflux is maximal at 5-10 μM Ca^{2+} (Fill et al., 1990; Kirino et al., 1983; Meissner, 1984; Moutin and Dupont, 1988; Nagasaki and Kasai, 1983). Ca^{2+} release is almost completely inhibited at 100 nM Ca^{2+} or > 10 mM Ca^{2+}. Such a curve suggests that the Ca^{2+} release channel possesses high-affinity activating and low-affinity inhibitory Ca^{2+} binding sites. Furthermore, there is evidence which indicates that the affinity and cooperativity of interaction between high- and

low-affinity [^{3}H]ryanodine binding sites are dependent on Ca^{2+} concentration and ionic strength (Chu et al., 1990; Lai et al., 1989; McGrew et al., 1989; Meissner and El-Hashem, 1992; Mickelson et al., 1990; Pessah et al., 1987).

Mg^{2+} likely inhibits SR Ca^{2+} release by multiple mechanisms including (a) competition with Ca^{2+} for the Ca^{2+} activation sites, (b) binding to the low-affinity Ca^{2+} inhibitory sites, or (c) steric blocking of the channel as it binds to a site near the conduction pathway (Kirino, 1983; Meissner and Henderson, 1987; Nagasaki and Kasai, 1983).

Ca^{2+}-induced Ca^{2+} release is greatly potentiated by physiological (mM) concentrations of ATP (Meissner, 1984; Morii and Tonomura, 1983). Optimal channel activation was found in the presence of μM Ca^{2+} and mM ATP to give maximal Ca^{2+} release rates. Various adenine nucleotides (AMP-PCP, ADP, AMP, cAMP, adenosine, adenine) also potentiate Ca^{2+} release, which suggests that activation occurs because of binding of the nucleotide to an effector site rather than covalent modification of the channel protein via a phosphorylation reaction. (Meissner et al., 1986; Moutin and Dupont, 1988; Nagasaki and Kasai, 1983).

Caffeine increases the sensitivity of the Ca^{2+} -induced Ca^{2+}-release mechanism to Ca^{2+} and adenine nucleotides both in skinned fibers and in SR vesicles (for review see Martonosi, 1984). The increase in apparent Ca^{2+} affinity in Ca^{2+} channel in the presence of 50 mM caffeine is 20-fold. Caffeine in the millimolar range has been found to stimulate [^{3}H]ryanodine binding to skeletal Ca^{2+} release channel (Chu et al., 1990a; Zimanyi et al., 1992; Zimanyi and Pessah, 1991). This effect resulted from an increase in the ryanodine association rate without a change in the dissociation rate (Chu et al., 1990). In the presence of Ca^{2+} and Mg^{2+}, caffeine appears to increase the affinity of the activation site for Ca^{2+} (Pessah et al., 1987). The effects

of caffeine and ATP are additive, resulting in increasing maximum rate of Ca^{2+} release (Nagasaki and Kasai, 1983).

Whereas the cardiac RyR is an excellent substrate for the multifunctional Ca^{2+}/calmodulin protein kinase, phosphorylation of the skeletal muscle RyR by endogenous and exogenous CaM kinase was shown to have more variable effects (Chu et al., 1990; Strand et al., 1993; Witcher et al., 1991). In the skeletal muscle RyR, Ser2843 is a major target for cAMP-, cGMP- and CaM-dependent kinases (Suko et al., 1993). An inactivation of Ca^{2+} channel activity resulting from phosphorylation was observed in patch-clamp studies by Wang and Best (1992). In contrast, an activation of single channel activities by phosphorylation, which was ascribed to removal of block by Mg^{2+}, has been reported (Hain et al., 1993).

Calmodulin regulates skeletal Ca^{2+} channel by direct binding Ca^{2+}-channel instead of through a Ca^{2+}-CaM-dependent phosphorylation mechanism (Meissner, 1986; Smith et al., 1989). Further details about the CaM binding and regulation of Ca^{2+} release channel protein will be reviewed in section 2.3.2 The localization of the sites for other regulators of the Ca^{2+}-channel will be critical to the understanding of SR Ca^{2+}-channel function in E-C coupling.

2.3 Calmodulin, a Versatile Calcium Mediator Protein

2.3.1 Structure and function of calmodulin

Calmodulin (CaM) is a ubiquitous Ca^{2+} binding protein involved in a variety of cellular calcium-dependent signaling pathways. The biochemical properties reveal CaM as small, heat stable, and one of the most acidic proteins found in any tissue (Klee and Vanaman, 1982). Based on primary structural homology, CaM is a member of a family of Ca^{2+}-binding proteins that includes troponin C, parvalbumin, and myosin light chains (for review see Goodman et al., 1979). The 148 amino acid protein is a single polypeptide chain with a M_r of 16,700. Some 30% of its amino acids consist of aspartate and glutamate, accounting for the pI of 4.3. The animal CaMs sequenced to date contain no cysteine, hydroxyproline, or tryptophan. There is a high ratio of phenylalanine (8 residues) to tyrosine (2 residues) which results in displaying a distinctive ultraviolet absorption pattern, a spectrum characteristic of the fine structure of phenylalanine (Cheung, 1980). Plant CaMs typically exhibit the presence of 1 Cys and only 1 Tyr (Bazari and Clarke, 1981; Cormier, 1981).

Proteins with the functional and physicochemical properties of CaM are found in all eukaryotic organisms. CaM is found at varying concentrations in all vertebrate tissues (Klee and Vanaman, 1982). CaM from phylogenetically diverse sources have identical or at least very similar biological and biochemical properties because of the highly conserved primary structures (Cheung, 1980). Thus, CaM lacks tissue or species specificity reserved in Ca^{2+}-binding proteins with similar physicochemical properties such as troponin C (Klee and

Vanaman, 1982).

Since the discovery of CaM by Cheung (1970), it has been demonstrated that this regulatory protein mediates the cellular response to Ca^{2+} stimulation of at least 30 enzymes including Ca^{2+}-dependent protein kinases, adenylate cyclase, myosin light chain kinase, phosphodiesterase, phosphorylase kinase, phospholipase A2, plasma membrane Ca^{2+}-ATPase, guanylate cyclase, and NAD kinase (Cheung, 1980). Whereas CaM will bind to some structural proteins in the absence of Ca^{2+}, metal ions are absolutely required for its function as a regulator of most of its target enzymes *in vitro* (Means et al., 1991).

When Ca^{2+} concentration is transiently elevated in the cell in response to signal transduction mechanisms, Ca^{2+} binds to CaM with μM affinity at a stoichiometry of 4 Ca^{2+} atoms to 1 CaM molecule and this interaction produces a conformational change in CaM. The Ca^{2+}-CaM complex is now competent to interact with an acceptor or target protein. Such binding results in a further conformational change in CaM as well as structural changes in the target protein which allows expression of biological activity (Means et al., 1991).

Biophysics has revealed much about 3-dimensional structure of CaM itself, conformational change upon binding of Ca^{2+}, and interaction of CaM with its target proteins. The X-ray crystallographic structure of CaM in the Ca^{2+}-bound form shows a dumbbell-shaped molecule with two globular domains arranged in a trans configuration that each bind two Ca^{2+} ions (Fig. 2.6) (Babu et al. 1985). These domains are connected by a 26-residue, central α-helix; the middle portion, which is highly mobile, acts as a flexible tether (Barbato et al., 1992). Each Ca^{2+}-binding domain consists of two helix-loop-helix motifs which are commonly called EF-hands. This term is derived from the structural homology of this domain

Figure 2.6. Structure of calmodulin A representation of the three-dimensional crystal structure of vertebrate CaM is depicted. The amino and carboxyl termini are indicated by 'N' and 'C' respectively. The positions of the 4 bound Ca^{2+} ions are shown by black dots (From Means et al., 1991).

to the Ca^{2+}-binding helix-loop-helix unit formed by the E and F helices of parvalbumin (Kretsinger, 1980). These two pairs of helix-loop-helix are joined by a short antiparallel ß-sheet. The carboxy-terminal lobe binds Ca^{2+} with high affinity ($K_d \sim 10^{-7}$ M), the amino-terminal with lower affinity ($K_d \sim 10^{-6}$ M).

Binding of Ca^{2+} ions induces a large conformational change, which makes two solvent-exposed hydrophobic patches, one in each half of the molecule, available for target protein interaction. Each hydrophobic surface is surrounded by a polar rim which is rich in negatively charged residues. At the center of each surface, there is a deep hydrophobic cavity that is responsible for capturing an aromatic or a long aliphatic side chain of the target proteins (Ikura et al., 1992; Meador et al., 1993; Meador et al., 1992) (Fig 2.7). In addition to the extensive hydrophobic interactions, electrostatic interactions between the negatively charged residues located at the polar rim and positively charged residues of the target proteins contribute to the binding energy. Although the X-ray crystal structure of apo-CaM has not been determined, the multidimensional nuclear magnetic resonance studies on the isolated carboxy-terminal lobe have confirmed that the hydrophobic pockets are 'closed' in the absence of Ca^{2+} (Finn et al. 1993). This result has been confirmed by determination of the solution structure of Ca^{2+}-free CaM using NMR spectroscopy (Finn et al., 1995; Zhang et al., 1995; Kuboniwa et al., 1995). The removal of Ca^{2+} causes the interhelical angles of four EF-hand motifs to increase and leads to major changes in surface properties, including the closure of the deep hydrophobic cavity essential for target protein recognition. Various approaches have been used to identify CaM-binding domains in its target proteins (Billingsley et al., 1990). These include: limited proteolysis and fragment isolation by CaM affinity chromatography;

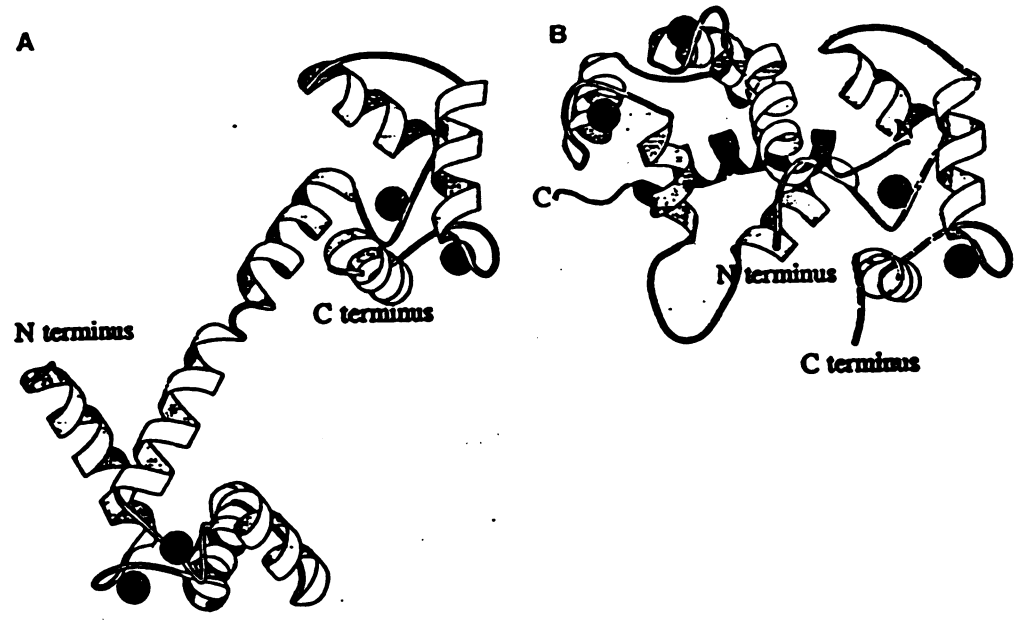


Figure 2.7 Atomic resolution structures of calmodulin and its complex with skeletal myosin light chain kinase (MLCK) peptide. (A) A ribbon diagram representation of the crystal structure of unbound Ca^{2+}-calmodulin. The four Ca^{2+} ions are shown bound to the four helix-loop-helix EF-hands. (B) The structure of Ca^{2+}-calmodulin bound to the peptide representing the calmodulin binding domain in skeletal muscle MLCK, as determined by heteronuclear multidimensional NMR in solution (From Torok and Whitaker, 1994).

photoaffinity labeling using labeled CaM; cDNA expression and deletion mapping; and the synthesis of peptides corresponding to putative CaM-binding regions. Most of the CaM-binding domains are stretches of 16-35 amino acids which, in an α-helical wheel representation, show a segregation of basic and polar residues on one side and hydrophobic amino acids on the other (O'Neil and DeGrado, 1990) (Fig. 2,8). However, the conformation of the CaM-binding domain in the native protein remains an open question. The determination of the structure of CaM bound to the synthetic CaM-binding domain of CaMKII, and the refinement of the structure of the MLCK-binding domain-CaM complex indicate that the interaction is not just hydrophobic but involves the formation of salt bridges between the basic amino-terminal half of the peptide and glutamic acid residues in the carboxyl terminus of CaM (Meador et al., 1993).

2.3.2 The Role of Calmodulin in Regulation of Ca^{2+}-Release from Channel Protein

Calmodulin was originally identified as a component of skeletal SR by Campbell and MacLennan (1982) using boiled EGTA extracts of SR vesicles. These extracts were shown to stimulate the phosphorylation of EGTA-washed SR vesicles in the same manner as calmodulin. Chiesi and Carafoli (1982) purified CaM from skeletal muscle SR using a Sepharose affinity column. Seiler et al. (1984) provided the first evidence that CaM bound to high molecular mass proteins in junctional SR vesicles isolated from skeletal and cardiac muscle which was later identified as Ca^{2+}- release channel.

Several lines of evidence suggest that CaM inhibits Ca^{2+} release activity via direct

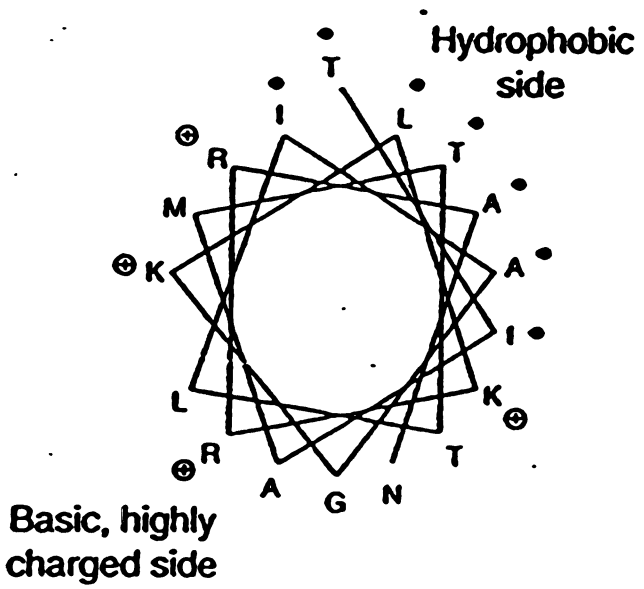


Figure 2.8 Helical-wheel projection of the calmodulin (CaM)-binding domain, showing the segregation of basic (+) and hydrophobic (•) residues to opposite sides of the helix (From James et al., 1995).

interaction with Ca^{2+}-channel instead of through a CaM-activated kinase during muscle contraction. The inhibitory effect of CaM on the Ca^{2+}-channel is Ca^{2+} concentration dependent and is reversible. In the absence of ATP, 2-10 μM CaM reduces $^{45}Ca^{2+}$ efflux rates from passively loaded skeletal SR vesicles by a factor of 2-3 in the presence of μM to mM Ca^{2+} concentrations (Meissner, 1986; Plank et al., 1988). Ca^{2+} release rates from cardiac SR are reduced by a factor of 3-6 (Meissner and Henderson, 1987). The half-maximal inhibitory concentration of CaM is between 0.1 and 0.2 μM, and maximal inhibition is observed at 1-5 μM (Meissner, 1986; Meissner and Henderson, 1987). CaM inhibits Ca^{2+}-induced Ca^{2+} release at Ca^{2+} concentrations between 0.1 and 100 μM without shifting the bell-shaped curve of Ca^{2+} dependence of release (Meissner, 1986). Single channel recordings demonstrate that 2 μM CaM, in the absence of ATP, decreases the mean open time of Ca^{2+}-release channels by 40% without having an apparent effect on single channel conductance or channel permeability properties, as measured from slope conductance and reversal potential in the presence and absence of CaM (Smith et al., 1989; Fuentes et al., 1994). [^{3}H]ryanodine binding studies reveal that CaM inhibits [^{3}H]ryanodine binding to CHAPS-solubilized and purified Ca^{2+}-channel (Fuentes et al., 1994).

Recently, it was demonstrated that CaM can also activate the Ca^{2+} release channel several fold at < 0.2 μM free [Ca^{2+}] which corresponds to the resting muscle [Ca^{2+}] condition (Tripathy et al., 1995; Buratti et al.,1995). Buratti et al. (1995) further demonstrated that the central region of the Ca^{2+}-channel, corresponding to residues 2937-3225 and 3546-3655, may contain CaM binding sites involved in the channel activation at low Ca^{2+} concentration (10^{-7} M [Ca^{2+}]). In saponin-skinned fibers, higher concentration of CaM (10 μM) potentiates Ca^{2+}

release at low Ca^{2+} concentrations (< 3 μM), while it shows an inhibitory effect at high Ca^{2+} concentration (3-30 μM) with 1 μM CaM (Ikemoto et al., 1995).

2.3.3 Identification of Calmodulin Binding Domains in Ca^{2+}-release channel

Sequence analysis of the skeletal muscle Ca^{2+}-channel identified several candidate CaM-binding sites in the C-terminal half of the protein (Fig. 2.8). Based on primary and secondary structural analysis, Takeshima et al.(1989) predicted 2 CaM binding sites at residues 3614-3637 and 4295-4325, whereas Zorzato et al.(1990) predicted 3 different sites at residues 2807-2840, 2909-2930 and 3031-3049. Marks et al.(1990) have suggested four putative CaM binding sites located at residues 2641-2657, 3362-3374, 3947-3965 and 4309-4322, by mapping the locations using limited proteolysis coupled with surface topography analysis. Brandt et al.(1992) have suggested three other candidate sites for CaM at residues 1383-1400, 1974-1996 and 3358-3374 by analysis of the calpain digestion pattern of the channel protein in the presence and absence of CaM. All these regions satisfy the motif for CaM binding sites which are basic amphiphilic helices, with the basic residues forming one face of the helix and hydrophobic residues the other face (Fig. 2.8).

Prediction of CaM binding domains have received some experimental support from the studies by several groups who employed expression of Ca^{2+}-channel fragments from corresponding cDNAs (Fig 2.9). Menegazzi et al. (1994) have defined three CaM binding regions, residues 2937-3225, 3546-3655 and 4425-4621 in rabbit skeletal muscle Ca^{2+}-channel by CaM overlay on SDS PAGE of Ca^{2+}-channel fusion proteins. The binding of CaM

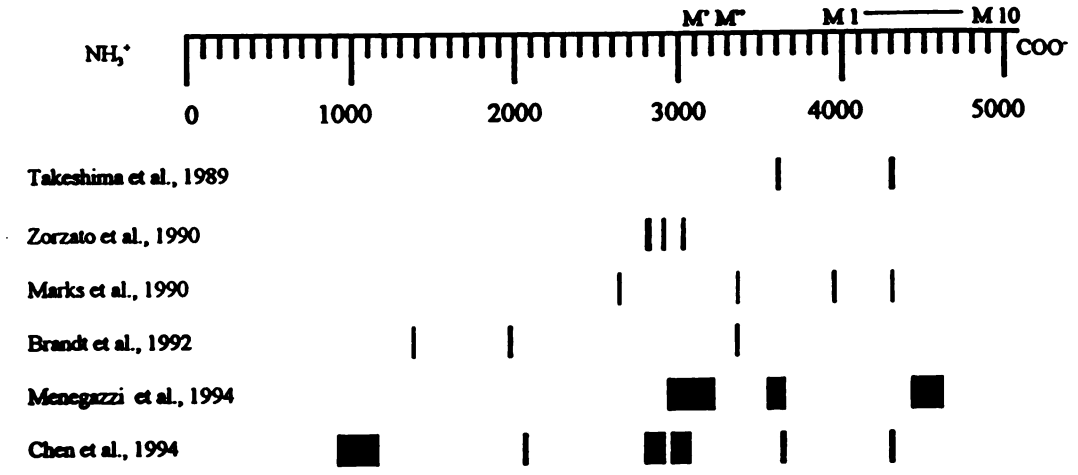


Figure 2.9 Calmodulin-binding sites in skeletal muscle sarcoplasmic reticulum Ca^{2+}-release channel. The linear sequence of the Ca^{2+}-channel is indicated by a horizontal line. The NH^{3+} and Coo^{-} termini are marked. M', M", and M1-M10 refer to predicted transmembrane sequences (Zorzato et al., 1990). The candidates of CaM-binding sites are positioned by verdical lines.

to these Ca^{2+}-channel fusion proteins are Ca^{2+} concentration dependent. Chen et al. (1994) have detected six Ca^{2+}-dependent CaM-binding domains in rabbit skeletal muscle RyR by ^{125}I-CaM overlay on TrpE fusion proteins. Strong CaM-binding domains were localized between amino acid residues 2063-2091, 3611-3642 and 4303-4328. Weaker CaM-binding domains were localized between amino acid residues 921-1173, 2804-2930 and 2961-3084. The gel overlay methods used by these groups may sometime be useful as a screening tool, but interpretation of results raise many questions. Since different degrees of renaturation of these fusion proteins on nitrocellulose membranes could affect measurement of CaM binding in the overlay assay, CaM binding sites determined by the overlay method may not represent true native binding sites in Ca^{2+} channel and this may lead to artifacts. Furthermore, although same overlay method and fusion protein same overlay method and fusion protein system is employed by both laboratories, there is little agreement on CaM-binding sites (Fig 2.9).

Efforts have been made to localize the CaM-binding sites in the three-dimensional structure of the channel protein. Wagenknecht et al. (1994), using gold-cluster-labeled CaM and electron microscopy, identified 1 CaM-binding site per subunit on the purified protein. This CaM-binding site is at least 10 nm from the transmembrane channel of the Ca^{2+}-channel protein suggesting that long-range conformational changes are involved in the modulation of the Ca^{2+} channel activity by CaM.

2.4 Malignant Hyperthermia and Porcine Stress Syndromes

2.4.1 Introduction

Malignant hyperthermia (MH) is an inherited myopathy of man and swine in which inhalational anesthetics and skeletal muscle relaxants trigger severe skeletal muscle contracture (Gronert, 1986). MH is associated with hypermetabolism and extreme elevation in body temperature which can result in death unless promptly recognized and treated with the skeletal muscle relaxant sodium dantrolene (Harrison, 1988). In man, MH is usually associated with the administration of certain anesthetic agents such as halothane or succinylcholine (Britt and Kalow, 1970). In swine homozygous for the defect, MH can also be triggered by severe stress such as that engendered by social order fighting, exercise, herding, hot environment, etc.; thus, the disease is referred to as porcine stress syndrome (PSS) (Cassens et al., 1980; Mitchell and Heffron, 1982).

Pale, soft, exudative (PSE) meat follows from the accelerated postmortem glycolysis, concomitant myolactosis, and abnormally rapid postmortem fall in muscle pH peculiar to this myopathy. This, it is proposed, alters the muscle water-holding capacity and texture by denaturation of the sarcoplasmic proteins and contractile proteins (Lawrie et al., 1958, Wismer-Pedersen and Briskey, 1961). Major economic losses in the swine industry result from the development of PSE meat that arise from postmortem manifestation of the disease in MH susceptible (MHS) pigs. However, there are some desirable traits associated with the presence of the gene for MH including leanness, muscle hypertrophy, and other desirable causes traits.

2.4.2 Abnormal sarcoplasmic reticulum Ca^{2+}-release channel in malignant hyperthermia skeletal muscle

Because Ca^{2+} is the main regulator of muscle contraction and metabolism, the defect in MH is believed to lie in abnormal Ca^{2+} regulation (Endo, 1977). The continued presence of elevated Ca^{2+} within the skeletal muscle cell results in severe muscle contracture, enhancing glycolytic and aerobic metabolism which depletes ATP, glucose, and oxygen, produces excess CO_2, lactic acid and heat, and upsets cellular and extracellular ion balances (Webb and Simpson, 1986; Simpson and Webb, 1989).

The most significant abnormality reported in MHS muscle, observed with isolated SR fractions, skinned muscle fibers, or muscle fiber bundles, is associated with the Ca^{2+}-induced calcium release mechanism of SR (Heffron and Ellis, 1985; O'Brien et al., 1986; Mickelson et al., 1986; Mickelson et al., 1988; Carrier et al., 1991). There is now considerable evidence that the primary biochemical defect in MH is associated with an abnormal SR Ca^{2+}-release channel protein. Endo et al. (1983) observed that the Ca^{2+}-induced Ca^{2+} release mechanism from MH SR was both more sensitive to Ca^{2+}, and MH SR released Ca^{2+} at a greater rate than normal SR. The data from Mickelson et al. (1986) suggest that there is no abnormality for the Ca^{2+} uptake function of Ca^{2+}-ATPase in MHS SR, that the initial phase of Ca^{2+}-induced Ca^{2+} release from MHS SR is enhanced, and that MHS SR has a different sensitivity to Ca^{2+}, Mg^{2+}, ATP and caffeine than does normal SR.

Mickelson, et al.(1988) correlated a two-fold greater Ca^{2+}-release rate in SR isolated from MHS pigs with abnormal ryanodine binding properties. The altered Ca^{2+} dependence of

[^{3}H]ryanodine binding at the low affinity Ca^{2+} site and a significantly lower K_d (95 versus 265 nM) for ryanodine in MHS Ca^{2+}-release channel than that of normal SR suggested that alterations on the SR Ca^{2+} channel may be responsible for the abnormalities in regulation of Ca^{2+} release observed in MHS muscle. Mickelson et al. (1990) further utilized [^{3}H]ryanodine binding to the Ca^{2+}-channel as a reporter of open state of the Ca^{2+} release channel, and further support the hypothesis that differences in the Ca^{2+} channel regulatory properties in response to various channel stimulators (ATP and caffeine) and inhibitors (ruthenium red and Mg^{2+}) are responsible for the abnormal Ca^{2+} releasing activity of MHS SR. The results from Fill et al. (1990) showed that normal channels were inactivated by Ca^{2+} concentrations below pCa 4, whereas MHS channels remained open at these Ca^{2+} concentrations for significantly longer times. Based on these results, the hypothesis was proposed that a defect in a low-affinity Ca^{2+} binding site was responsible to the altered gating of MHS SR channel. Based on equilibrium and kinetic evaluation of the binding of [^{3}H]ryanodine to MHS SR, Hawkes et al. (1992) demonstrated that the MH defect in pigs increases the apparent affinity of the SR membranes for [^{3}H]ryanodine by increasing the amount of high affinity sites relative to low affinity binding sites. These findings suggest that the MH defect may alter the rate at which the high affinity form of the protein converts to the low affinity form.

A single point mutation (T for C1843) in the porcine skeletal muscle ryanodine receptor gene (*ryr* 1) has been identified which results in an alteration in amino acid sequence from arginine at position 615 in normal RyR to a cysteine in the RyR of MHS pigs (Fujii et al., 1991). The following observations suggest that this single amino acid alteration is the causal mutation for MH. Molecular genetic studies have shown that this single amino acid

alteration is cosegregated with porcine MH by comparing *ryr1* genotypes, as determined by DNA-based analysis, and phenotypes, as determined by halothane challenge test which induces signs typical of MH reaction (Fujii et al., 1991; Otsu et al., 1991). Shomer et al. (1993) have demonstrated that the abnormalities in the MHS porcine Ca^{2+} release channel activity were indeed the result of a mutation in this molecule, rather than an abnormal membrane lipid environment or an unidentified regulatory proteins. Otsu et al. (1994) further confirmed that this single amino acid mutation in Ca^{2+} channel protein was causative of MH by showing that the mutated Ca^{2+} channels expressed in transfected myoblastic cells had higher sensitivity to caffeine and halothane and resulted in higher cytosolic Ca^{2+} determined by the fluorescence calcium indicator indo-1 compared to wild type Ca^{2+} channel.

Shomer et al. (1994) reported that although Ca^{2+} regulation of Ca^{2+} release channel activity is altered, the Arg^{615} to Cys^{615} mutation of the porcine Ca^{2+} release channel does not affect the conductance or ion selectivity properties of the channel. Treves, et al. (1994) report that the presence of the Arg-to-Cys point mutation in the recombinant RyR Ca^{2+} channel expressed in COS-7 transfected cells causes abnormal cytosolic Ca^{2+} transients in response to 4-chloro-m-cresol, an agent capable of eliciting in vitro contracture of MHS muscles. Their results suggest that substituting Cys for Arg^{615} in the primary structure of the RyR is sufficient to alter the intracellular Ca^{2+} homeostasis of eukaryotic cells.

It is apparent from a number of studies that the Ca^{2+}-dependent regulation of the SR Ca^{2+} release channel is altered in MH. However, it is not clear if the interaction of other compounds which can regulate this channel such as caffeine, calmodulin, Mg^{2+} and adenine nucleotide, is also altered in MHS SR. It has been reported that Arg^{615} is located on the

surface of the native Ca^{2+}-release channel and is likely near important Ca^{2+} channel regulatory sites (Mickelson et al., 1992). Therefore this mutated amino acid may cause the alteration of the modulators binding to ryanodine receptor and results in the abnormal regulation of Ca^{2+}-release from SR channel. The sensitivity of skeletal muscle contracture by caffeine is increased in the MHS pig (Britt, 1987; Gronert, 1986). Mickelson et al. (1990) further reported that in the presence of optimal Ca^{2+}, MHS SR [^{3}H]ryanodine binding was more sensitive to caffeine and ATP stimulation and less sensitive to ruthenium red or Mg^{2+} inhibition than was normal SR. However, the altered caffeine sensitivity of MHS muscle contracture does not directly result from Ca^{2+} channel mutation in MH SR (Shomer et al., 1994). The direct evidence concerning the functional role and interaction of other channel modulators such as calmodulin, Mg^{2+} on the mutated Ca^{2+} channel protein in altering Ca^{2+}-release properties of the channel protein in MH is insufficient.

CHAPTER 3

CALMODULIN INTERACTION WITH THE SKELETAL MUSCLE SARCOPLASMIC RETICULUM CALCIUM CHANNEL PROTEIN

3.1 Introduction

Contraction and relaxation of skeletal muscle are governed by changes in myoplasmic [Ca^{2+}]. Elevation of myoplasmic [Ca^{2+}], which triggers contraction, occurs when an action potential induces Ca^{2+} release from the sarcoplasmic reticulum (SR) via a Ca^{2+}-release channel (Martonosi, 1984; Inesi, 1985). Upon cessation of the action potential, Ca^{2+}-release is inhibited, the SR Ca^{2+}-pump protein reduces myoplasmic [Ca^{2+}] by pumping it back into the SR, and the muscle relaxes. Although the Ca^{2+} uptake process has been described in considerable detail, the molecular mechanisms involved in Ca^{2+}-release are, by comparison, poorly understood.

Regulation of Ca^{2+}-release channel activity has been investigated by following Ca^{2+} efflux from isolated SR membrane vesicles (Nagasaki & Kasai, 1983; Ikemoto et al., 1985; Meissner et al., 1986, Meissner, 1986) as well as by single-channel recordings of the activity of the Ca^{2+}-release channel protein incorporated from SR vesicles into planar lipid bilayers (Smith et al., 1985; Smith et al., 1986a; Smith et al., 1986b). Results of these experiments

indicated the presence of a Ca^{2+}-channel protein in SR whose activity was stimulated by μM Ca^{2+}, caffeine, adenine nucleotides, and nM concentrations of the plant alkaloid ryanodine; channel activity was inhibited by nM Mg^{2+}, calmodulin (CaM), ruthenium red, and μM concentrations of ryanodine.

Recently several groups have isolated a homotetrameric protein of subunit M_r > 450,000 which bound ryanodine and, when incorporated into planar lipid bilayers, exhibited Ca^{2+} channel activity nearly identical to that of intact SR vesicles and native SR Ca^{2+}-channels (Inui et al., 1987; Lai et al., 1988; Imagawa et al. 1987). The purified ryanodine receptor/Ca^{2+}-channel protein also displayed strikingly similar morphology to the junctional foot structure which spans the gap between the T-tubule and junctional SR (Inui et al., 1987; Lai et al., 1988). On the basis of this evidence, the ryanodine receptor protein was proposed to be synonymous with the Ca^{2+}-release channel and the junctional foot of SR vesicles.

Calmodulin is a ubiquitous Ca^{2+}-binding protein which regulates the activity of at least 30 known enzymes and other proteins in response to changes in cellular Ca^{2+} concentration (Klee and Vanaman, 1982; Means et al., 1991) Previous studies have shown that CaM inhibits the Ca^{2+} release rate from SR vesicles by 2-3 fold in skeletal muscle (Meissner, 1986; Plank et al., 1988) and up to 6-fold in cardiac muscle (Meissner & Henderson, 1987). Subsequently, Smith et al., (1989), using the planar lipid bilayer-vesicle fusion technique, demonstrated that the inhibitory effect of CaM on both skeletal and cardiac SR channel proteins resulted from reduction of the open time probability via direct binding of CaM to the channel protein.

These results have led to the hypothesis that the role of CaM in regulation of the SR

Ca^{2+}-channel activity is that of a partial feedback inhibitor of Ca^{2+}-release (Meissner, 1986, Smith et al., 1989). However, in the absence of direct binding data on the interaction of CaM with the channel protein, it is difficult to assess the physiological role of CaM in regulation of Ca^{2+}-release activity. These studies were initiated to define conditions under which CaM binds to the channel protein and thus, would be capable of regulating its activity. Our results suggest that in skeletal heavy SR, the most abundant receptor for CaM is the Ca^{2+}-channel protein. CaM binds to the channel protein with high affinity, even in the presence of EGTA. In the physiological range of KCl concentrations, binding of CaM is enhanced in the presence of 0.1 mM $CaCl_2$, and further enhanced by inclusion of 1 mM $MgCl$. Correlation of ryanodine binding data with CaM-binding data suggests that there are multiple CaM-binding sites on each channel protein subunit, and that the affinities of these binding sites for CaM change in response to metal ion concentrations.

3.2 Experimental procedures

3.2.1 Materials. Benzophenone-4-maleimide and rhodamine-X-maleimide were purchased from Molecular Probes (Junction City, OR). Na[^{125}I] and [^{3}H]-ryanodine were obtained from DuPont-NEN (Boston, MA). Ryanodine was purchased from Calbiochem (La Jolla, CA). Wheat germ was a gift from International Multifoods (Minneapolis, MN).

3.2.2 Preparation of Calmodulin and its Derivatives. CaM was purified from wheat germ using the procedure described previously (Strasburg et al., 1988). For crosslinking experiments, CaM was iodinated with [^{125}I] at the sole tyrosine residue (Tyr 139), followed by site-specific modification at Cys 27 with the photoactivatable crosslinker, benzophenone-4-maleimide (Strasburg et al., 1988). For fluorescence experiments, purified CaM was derivatized at Cys 27 with rhodamine-X-maleimide as described for I-EDANS (Strasburg et al., 1988).

3.2.3 Calmodulin Crosslinking. Affinity labeling of CaM-binding proteins in SR vesicles was performed by incubating in darkness 0.1 μM [^{125}I]-Bz-CaM with 100 μg of SR vesicles in 100 μL of 20 mM Hepes pH 7.5, and 0.2 M NaCl. $CaCl_2$, $MgCl_2$ and EGTA were included as indicated in Figure legends. The mixtures were placed in plastic microcentrifuge tubes on ice, the tubes were covered with a plate of glass, and the samples were illuminated for 20 minutes in a Stratalinker 1800 photoreactor (Stratagene Corp., La Jolla, CA) equipped with lamps of λ_{max} = 254 nm. After photolysis of the mixture, the samples were centrifuged in a

Beckman TL-100 centrifuge at 100,000 x g_{max} for 20 minutes. The membrane pellets were resuspended in water, SDS was added to produce a final concentration of 1%, and the samples were subjected to polyacrylamide gel electrophoresis using 5-20% linear gradient gels (Laemmli, 1970). The gels were dried and placed with Kodak Omat XAR-5 X-ray film in autoradiography cassettes equipped with Dupont Lightning Plus intensifying screens.

3.2.4 Preparation of Sarcoplasmic Reticulum Vesicles. Skeletal muscle heavy SR vesicles were isolated from longissimus muscle of pigs obtained from the Michigan State University swine farm or from the Yorkshire swine herd at the University of Minnesota using the procedure of Mickelson et al., (1986) with the modifications that 1 mM EGTA, 0.1 mM PMSF, 1 μg/ml of aprotinin and 1 μg/ml leupeptin were included in the homogenization buffer. Light SR vesicles were prepared from rabbit longissimus muscles according to the procedure of Fernandez et al. (1980).

3.2.5 Fluorescence Anisotropy Measurements. Rhodamine maleimide-labeled CaM (Rh-CaM) binding to the channel protein in SR vesicles was monitored by fluorescence anisotropy measurements using an SLM 4800 spectrofluorometer modified with data acquisition hardware and operating system from On-Line Instrument Systems (Bogart, GA).

Samples were held in a thermostatted cell block maintained at 22°C. The excitation wavelength of Rh-CaM was 580 nm, monochromator slits were set at 8 nm, and emitted light was isolated using Schott RG-610 filters. During titrations samples were allowed to equilibrate for 5 minutes after each addition of Rh-CaM or SR. All samples included 1 μg/ml

aprotinin, 1 μg/ml leupeptin and 0.1mM PMSF to inhibit proteolysis during the experiment.

All fluorescence measurements were performed using semi-micro, quartz fluorescence cuvettes (4 mm x 10 mm). Prior to fluorescence experiments the cells were rinsed with 1 mg/ml bovine serum albumin to minimize Rh-CaM adsorption to the walls of the cuvette. Following this treatment, the measured anisotropy value for Rh-CaM was independent of concentration over the range of 1 nM to 1000 nM, indicating that there was negligible Rh-CaM adsorption to the cuvettes.

$$f_b = \frac{(A_m - A_f)}{A_m(1-q) + q(A_b) - A_f} \qquad (1)$$

The anisotropy of a ligand (Rh-CaM) is directly proportional to the fraction of ligand bound to the receptor (Ca^{2+}-channel protein). Thus, if A_f is the anisotropy of free Rh-CaM and A_b is the anisotropy of the fully bound ligand, then the fraction bound, f_b, is determined from: where A_m is the measured anisotropy for a given ligand concentration, and q, the change in quantum yield, is the ratio of fluorescent intensity of bound species over that of the free species. If the change in quantum yield is negligible upon binding of ligand, then equation (1) reduces to:

$$f_b = \frac{(A_m - A_f)}{(A_b - A_f)} \qquad (2)$$

The fraction of ligand bound, and the concentrations of bound and free Rh-CaM are readily calculated. Values of K_d and B_{max} were calculated using the computer program Enzfitter, a

nonlinear regression analysis program of R.J. Leatherbarrow (Biosoft, Cambridge, UK). Data were fit to a one-ligand or two-ligand binding model to obtain the best fit.

The anisotropy of unbound Rh-CaM, A_f, was measured in the absence of SR. The anisotropy of the fully bound species, A_b, was obtained by titration of Rh-CaM with SR vesicles, followed by curve-fitting using the Enzfitter computer program for a single class of ligand binding sites.

Corrections for light scattering and background fluorescence were made by application of the equation:

$$A = f_1 A_1 + f_2 A_2 \qquad (3)$$

where A, A_1 and A_2 are the anisotropies of the sample, the blank, and the corrected sample, respectively. The fractional contributions, f_1 and f_2, of these species were calculated from the intensities measured with the excitation monochromator in the vertical position and the emission monochromator at 55°. The corrected sample anisotropy, therefore, is that value in the absence of background interference.

3.2.6 Calmodulin Content of Heavy SR Vesicles. Heavy SR vesicles were suspended to a concentration of 1 mg/ml in 20 mM imidazole buffer, pH 7.4. The suspension was incubated for 5 minutes in a boiling water bath, and centrifuged 15 minutes at 100,000 x g_{max} in a Beckman TL-100 centrifuge. Aliquots of the CaM-containing supernatant were used in the erythrocyte membrane ghost CaATPase assay described below.

Preparation of porcine erythrocyte membrane ghosts and assays for CaM-stimulable

activity of the CaATPase of the erythrocyte ghosts were based on the procedures described by Thatte et al. (1987). Inorganic phosphate was determined by the method of Rockstein and Herron (1951). A standard curve was prepared for CaATPase activity as a function of wheat germ CaM concentration; CaM content of heavy SR vesicles was determined from the standard curve using aliquots of the boiled SR supernatants. CaM-stimulable CaATPase activity of the boiled supernatants was defined as the ATPase activity in the presence of 0.1 mM $CaCl_2$ minus that of an equal aliquot in the presence of 1 mM EGTA.

3.2.7 Biochemical Assays. SR protein concentrations were determined by the Lowry method of (Lowry et al., 1951) using bovine serum albumin as the standard. Ryanodine binding activity of SR vesicles was determined according to the method of Mickelson et al. (1988). Ca^{2+} titration of CaM/channel protein complexes were performed using an EGTA/NTA buffer. Free Ca^{2+} concentrations were calculated using the computer program of Perrin and Sayce (1967).

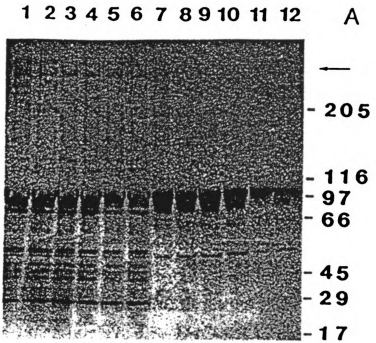
3.3 Results

3.3.1 Calmodulin Content of Heavy SR Vesicles. The CaM content of porcine heavy SR vesicles was determined by heating SR samples to release CaM, centrifugation to remove insoluble material, and measuring CaM activity in an erythrocyte membrane ghost CaATPase assay. The CaM content of our preparations of heavy SR ranged from 15-33 pmol/mg SR protein. Prior extractions of SR vesicles with EGTA did not significantly affect these results, indicating that the endogenous CaM was tightly bound and likely non-exchangeable.

3.3.2 [^{3}H]-Ryanodine Binding to SR Vesicles. The binding activity of [^{3}H]-ryanodine to heavy SR vesicles, determined by Scatchard plot analysis, show a B_{max} value of 10.6 ± 0.9 pmol/mg for our preparations. These results were similar to those obtained by Mickelson et al., (1988). The ryanodine-binding activity of the rabbit skeletal muscle light SR vesicles was 0.1 pmol/mg.

3.3.3 Identification of Calmodulin-binding proteins in SR vesicles. Purified heavy SR vesicles from porcine skeletal muscle and purified light SR from rabbit skeletal muscle were incubated with the affinity-labeling derivative [^{125}I]-Bz-CaM to identify the receptor proteins for CaM. The autoradiogram (Figure 3.1) of the gel electrophoretogram indicates that the major complex formed in the heavy SR fraction was a doublet of M_r > 450,000 which corresponds to CaM plus the channel protein subunit (Seiler et al., 1984). This complex was obtained in the presence of EGTA or Ca^{2+} at each Mg^{2+} concentration examined and suggests

Figure 3.1 Mg^{2+} and Ca^{2+} dependence of affinity labeling of skeletal muscle heavy and light SR with [^{125}I]-Bz-CaM. [^{125}I]-Bz-CaM was incubated with skeletal muscle heavy SR vesicles (A and B, lanes 1-6) or light SR vesicles (A and B, lanes 7-12) in 0.2 M NaCl, 20 mM Hepes buffer (pH 7.0) plus the following components: Lanes 1,7: 1 mM EGTA; lanes 2,8: 1 mM EGTA, 1 mM $MgCl_2$; lanes 3,9: 1 mM EGTA, 10 mM $MgCl_2$; lane 4,10: 0.1 mM $CaCl_2$; lane 5,11: 0.1 mM $CaCl_2$, 1 mM $MgCl_2$; lane 6,12: 0.1 mM $CaCl_2$, 10 mM $MgCl_2$. A) Coomassie-blue stained gel; B) Autoradiogram of dried gel. C) Inhibition of affinity labeling of skeletal muscle heavy SR vesicles by [^{125}I]-Bz-CaM with unlabeled CaM. Skeletal muscle heavy SR vesicles (1 mg/ml) were incubated with 0.1 μM [^{125}I]-Bz-CaM in the presence of 0.2 M NaCl, 20 mM Hepes buffer, pH 7.5, 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ and varying amounts of unlabeled CaM. The mixtures were separated by electrophoresis on a 5-20% acrylamide gradient gel, and the crosslinked products were identified by autoradiography of the gel. Lanes 1-10 represent 0, 0.05, 0.1, 0.5, 1, 2, 4, 6, 8, 10, μM of unlabeled CaM, respectively. Arrow indicates position of the channel protein subunit in the gel, and of the channel subunit/[^{125}I]-Bz-CaM complex in the autoradiograms.


1 2 3 4 5 6 7 8 9 10 11 12
A
205
116
97
66
45
29
17

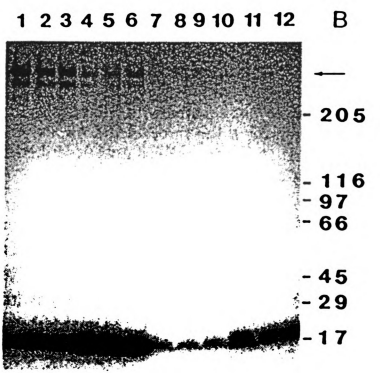
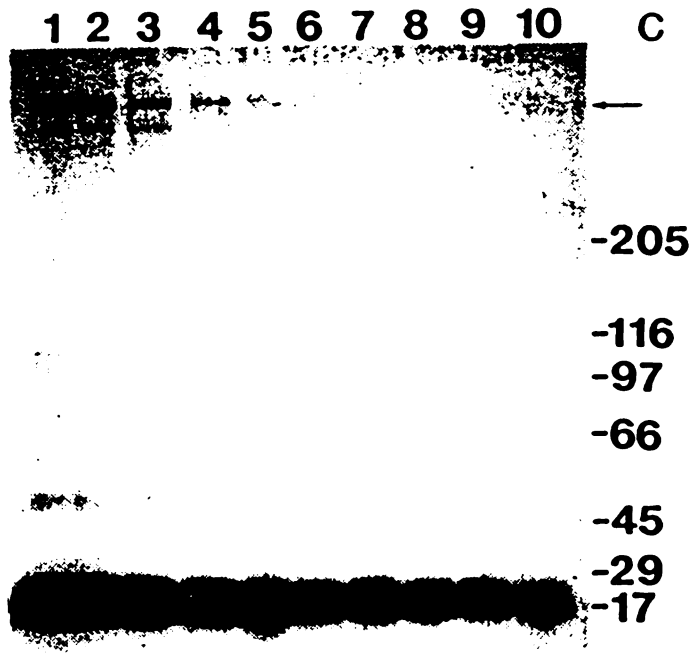

1 2 3 4 5 6 7 8 9 10 11 12
B
205
116
97
66
45
29
17

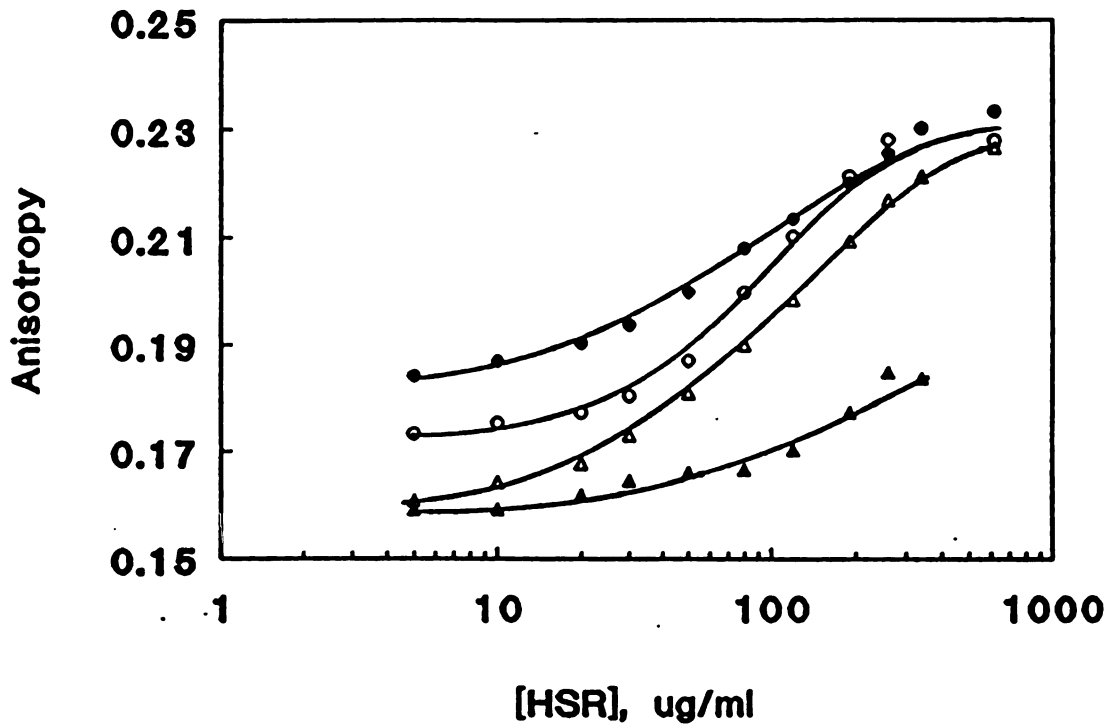
Figure 3.1--cont'


1 2 3 4 5 6 7 8 9 10 C
-205
-116
-97
-66
-45
-29
-17

that CaM could bind to the channel protein at [Ca^{2+}] in resting muscle. In contrast, at the same protein concentrations and under the same conditions, there was no apparent affinity labeling of light SR (Figure 3.1B). In order to demonstrate that the binding of CaM to the Ca^{2+}-channel protein was specific, affinity labeling experiments were conducted in the presence of increasing concentrations of unlabeled CaM. The [^{125}I]-Bz-CaM was readily displaced from the channel protein by the unlabeled CaM (Figure 3.1C), with crosslinking eliminated at unlabeled CaM concentrations greater than 1 μM.

3.3.4 Titration of Rh-CaM with SR Vesicles. Since the affinity labeling experiments indicate that Ca^{2+} channel protein is the most abundant receptor for CaM in our heavy SR preparations, fluorescence anisotropy could be used to characterize CaM interaction with the channel protein in native SR vesicles. Heavy SR vesicles were titrated into Rh-CaM under three different metal ion conditions: 1) +1 mM EGTA; 2) +0.1 mM $CaCl_2$; 3) +0.1 mM $CaCl_2$, and 1 mM $MgCl_2$. In each case, titration of SR vesicles into Rh-CaM resulted in a large increase in fluorescence anisotropy attributableto the increased molecular mass of the Rh-CaM/Ca^{2+}-channel protein complex (Figure 3.2). That the Rh-CaM was indeed binding to the channel protein is supported by the affinity labeling experiments (Figure 3.1B) and the following control experiments. The increase in anisotropy was reversed by addition of a large excess of unlabeled CaM (not shown). Titration of light SR vesicles into Rh-CaM resulted in a slight increase in fluorescence only at high SR concentrations (Figure 3.2). Furthermore, titration of Rh-CaM with SR vesicles, which were first treated with trypsin (1:50 w/w, 1 hour at 37°C), resulted in no change in anisotropy (not shown), indicating that there were

Figure 3.2 Titration of Rh-CaM with skeletal heavy and light SR vesicles under different divalent ion conditions. The sample medium contained 10 nM Rh-CaM, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 plus one of the following conditions: 1 mM EGTA (●); 0.1 mM $CaCl_2$ (○); 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$, (△) in a starting volume of 1 mL. Heavy SR: (●, ○, △,); light SR (▲). The Rh-CaM sample was titrated with SR vesicles in parallel with a buffer blank containing the same media minus Rh-CaM. Corrections were made for light scattering as described in "Experimental Procedures".


0.25
0.23
0.21
0.19
0.17
0.15
Anisotropy
1
10
100
1000
[HSR], ug/ml

negligible non-specific Rh-CaM interactions with the membranes and that light scattering corrections were valid.

Data from these titrations (Figure 3.2) were used to determine the anisotropy values for the free Rh-CaM species (A_f) and for the fully bound Rh-CaM/Ca^{2+}-channel protein complex (A_b). Normally these values are obtained from the limits of the titration curves. However, values for A_b could be slightly underestimated in these experiments because of excessive light scattering at high concentrations of SR vesicles (>1 mg/ml). Instead, A_b values were calculated by extrapolation using the Enzfitter program applied for a single ligand binding site on Rh-CaM. The A_b values obtained for each metal ion condition averaged 0.2435. The A_f values obtained from 10 nM samples of Rh-CaM under the various metal ion conditions in the absence of added SR, were 0.1808 (+ 1 mM EGTA), 0.1728 (+ 0.1 mM $CaCl_2$), and 0.1585 (+ 0.1 mM $CaCl_2$, + 1 mM $MgCl_2$). There was no significant change in fluorescence intensity upon binding of Rh-CaM to the channel protein (q = 1.0); therefore the fraction of Rh-CaM bound was calculated using Eq. 2.

3.3.5 Ionic Strength Dependence of the Binding of Rh-CaM to SR Vesicles. Our initial experiments (not shown) were conducted in the absence of added KCl and showed that in the presence of EGTA, there was no change in Rh-CaM anisotropy upon titration with SR vesicles. The results thus suggested that in the absence of adding KCl there was no binding of Rh-CaM to the channel protein at $[Ca^{2+}] < 50$ nM. However, in the presence of KCl there was an increase in fluorescence anisotropy observed upon titration of SR into Rh-CaM in the presence of EGTA (Figure 3.2). To determine whether binding of Rh-CaM to the skeletal SR

Ca^{2+}-channel protein could be dependent upon ionic strength, the anisotropy for fixed concentrations of Rh-CaM and SR vesicles was measured as a function of KCl concentration. The chosen concentrations of SR (86 μg) and Rh-CaM (10 nM) correspond to the approximate midpoints of the titration curves in Figure 3.2. As the salt concentration was varied, either an enhancement or inhibition of binding would be observed by an increase or decrease in anisotropy, respectively. The KCl titration data (Figure 3.3) clearly show that in the presence of EGTA, the binding of Rh-CaM to the channel protein was highly ionic strength-dependent, increasing from <10% Rh-CaM bound at 3 mM KCl to 45% bound at 0.2 M KCl. At [KCl] >0.3 M, the anisotropy rapidly declined, indicating decreased binding of Rh-CaM to the Ca^{2+} channel protein. In the presence of 0.1 mM Ca^{2+} plus 1 mM Mg^{2+}, titration of KCl into the SR/Rh-CaM mixture also indicated a significantly higher affinity of Rh-CaM for the Ca^{2+}-channel protein, with a maximum at about 0.3 M KCl. However, in contrast to the EGTA conditions, binding of Rh-CaM to the channel protein did not significantly decrease at higher KCl concentrations.

3.3.6 Ca^{2+} Dependence of the binding of Rh-CaM to SR vesicles. Ca^{2+} is required for the binding of CaM to most of its known receptors (Klee et al., 1982), although in a few cases the affinity of CaM for its receptor decreases with elevated [Ca^{2+}] (Cimler et al., 1985; Masure et al., 1986). Therefore, to define optimal [Ca^{2+}] for CaM/channel protein interaction, the Ca^{2+}-dependence of the binding of Rh-CaM was determined. The optimal [Ca^{2+}] for Rh-CaM/channel protein interaction was dependent on the $MgCl_2$ concentration (Figure 3.4). In the absence of Mg^{2+}, the optimal [Ca^{2+}] for enhanced binding of Rh-CaM to the channel

Figure 3.3. KCl dependence of the binding of Rh-CaM to SR vesicles under different divalent ion conditions. The sample buffer contained 86 μg heavy SR vesicles, 10 nM Rh-CaM, 0.3 M sucrose, 50 mM PIPES, pH 7.0 and either 1 mM EGTA (●) or 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (○) in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. % CaM bound was calculated from measured anisotropy as described in "Experimental Procedures"; 100% bound corresponds to 229 pmol/mg in the presence of EGTA and 80 pmol/mg in the presence of calcium plus magnesium.

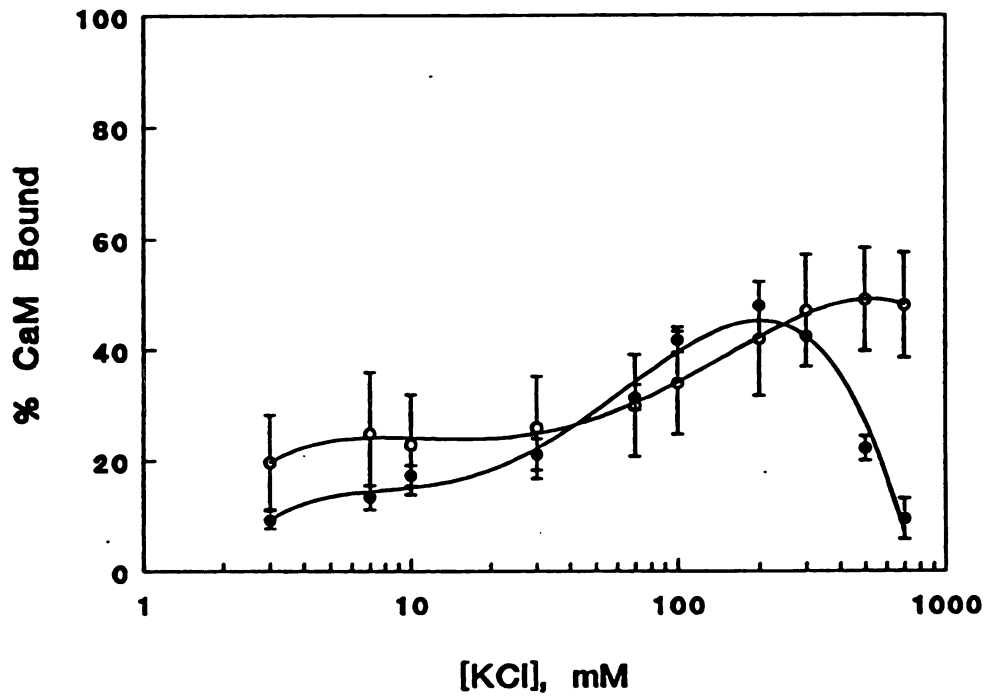
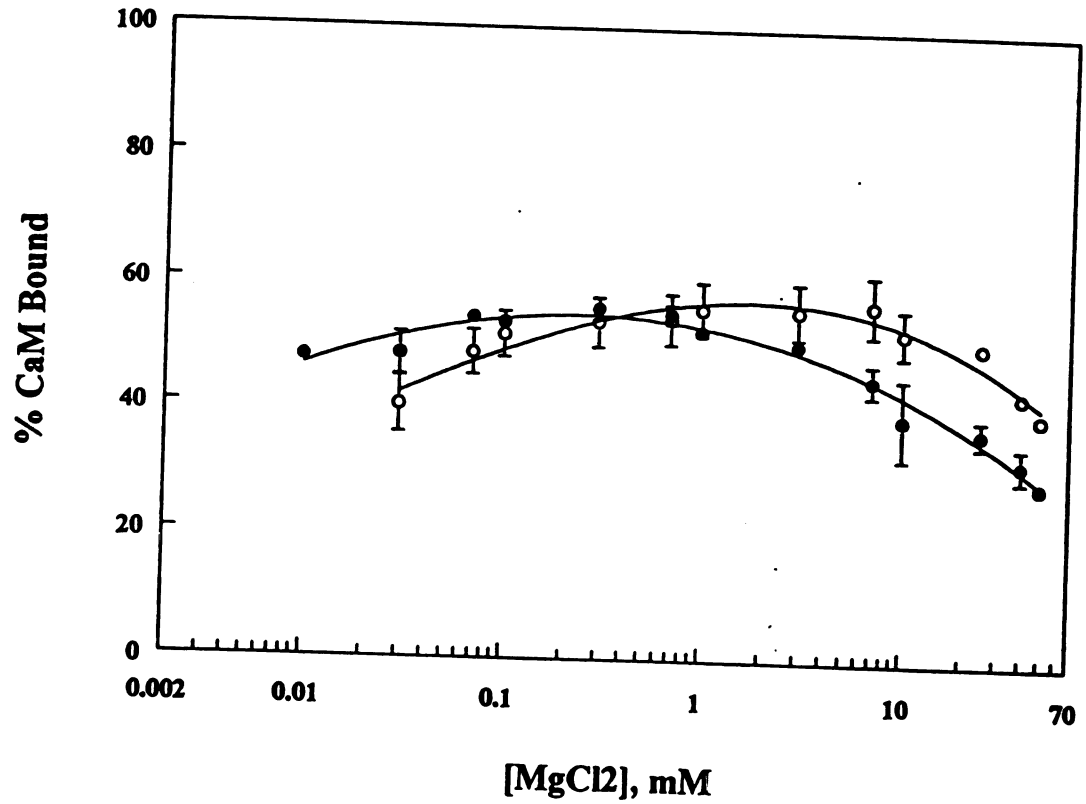

100
80
60
40
20
0
% CaM Bound
1
10
100
1000
[KCl], mM

Figure 3.5 Titration of Rh-CaM/SR vesicles with $MgCl_2$. The sample medium contained 86 μg heavy SR vesicles, 10 nM Rh-CaM, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 0.1 mM $CaCl_2$ (O) or 1 mM EGTA (●) in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. % CaM bound was calculated from measured anisotropy as described in " Experimental Procedures"; 100% bound corresponds to 80 pmol/mg in the presence of calcium and 229 pmol/mg in the presence of EGTA.


100
80
60
40
20
0
% CaM Bound
0.002
0.01
0.1
1
10
70
[MgCl2], mM

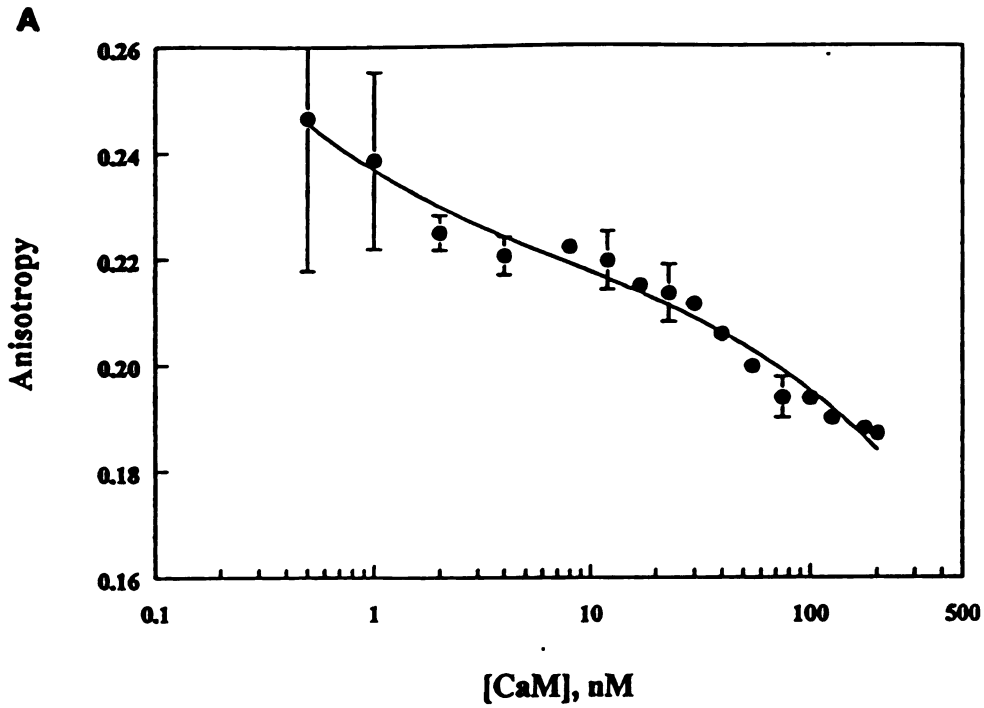
protein was approximately 1 μM, whereas in the presence of 1 mM Mg^{2+}, the optimum [Ca^{2+}] for Rh-CaM binding to the channel protein was approximately 50 μM (Figure 3.4).

3.3.7 Mg^{2+}-dependence of the Binding of Rh-CaM to SR Vesicles. Previous results (Meissner et al., 1986) indicated that Mg^{2+} is an antagonist of the Ca^{2+} release activity in heavy SR vesicles. To determine whether Mg^{2+} might alter the interaction of CaM with the Ca^{2+}- channel protein, the anisotropy of Rh-CaM was determined as a function of [Mg^{2+}] in the presence or absence of 0.1 mM Ca^{2+}. Binding of Rh-CaM to the channel protein was slightly enhanced as the [Mg^{2+}] was increased to 0.5 mM (+EGTA) or 1 mM (+Ca^{2+}) as indicated by the increase in fluorescence anisotropy. At [Mg^{2+}] above 1 mM (+EGTA) or above 7 mM (+Ca^{2+}), binding was significantly inhibited (Figure 3.5).

3.3.8 Rh-CaM/Ca^{2+}-Channel Protein Binding Equilibrium. Having established optimal salt and divalent metal ion concentrations, Rh-CaM was titrated into fixed concentrations of SR vesicles to determine binding capacity and affinity of the skeletal SR Ca^{2+}-channel in SR vesicles for Rh-CaM under the following conditions: 1) 1 mM EGTA; 2) 0.1 mM $CaCl_2$; 3) 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$. A_f and A_b values were used to calculate the fraction of Rh-CaM bound to the channel protein in SR vesicles for each point in the titration.

Results of titrations conducted in the presence of EGTA are indicative of a single class of binding sites on the SR Ca^{2+}-channel protein for CaM (Figure 3.6 and Table 3.1). Scatchard analysis of the data yields a dissociation constant, K_d of 8.6 ± 0.8 nM and a binding capacity B_{max} of 229 ± 7 pmol/mg of SR protein. In the presence of 0.1 mM $CaCl_2$, the

Figure 3.6. Titration of skeletal heavy SR vesicles with Rh-CaM in the presence of EGTA. A) Anisotropy plot of titrations of heavy SR with Rh-CaM. B) Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot Rh-CaM binding to SR vesicles. The sample medium contained 90 μg of heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 1 mM EGTA in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. CaM bound was calculated from measured anisotropy as described in "Experimental Procedures".


A
Anisotropy
0.26
0.24
0.22
0.20
0.18
0.16
0.1
1
10
100
500
[CaM], nM

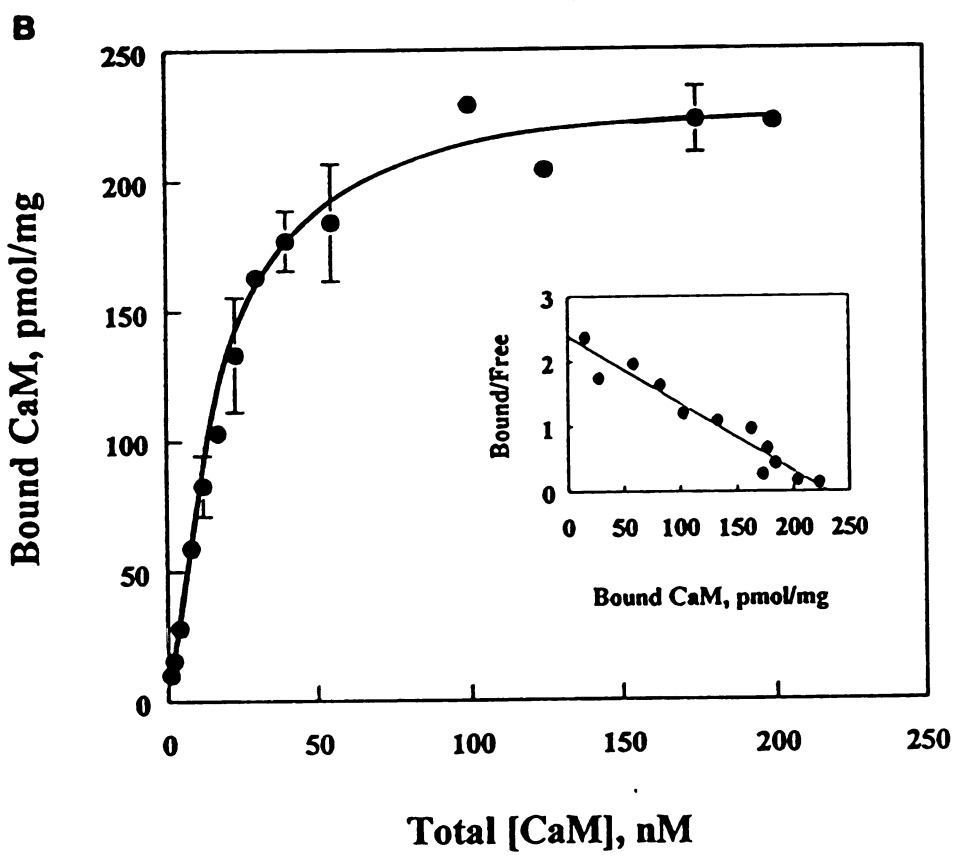

B
Bound CaM, pmol/mg
250
200
150
100
50
0
0
50
100
150
200
250
Total [CaM], nM
Bound/Free
3
2
1
0
0
50
100
150
200
250
Bound CaM, pmol/mg

Figure 3.5 Titration of Rh-CaM/SR vesicles with $MgCl_2$. The sample medium contained 86 μg heavy SR vesicles, 10 nM Rh-CaM, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 0.1 mM $CaCl_2$ (O) or 1 mM EGTA (●) in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. % CaM bound was calculated from measured anisotropy as described in " Experimental Procedures"; 100% bound corresponds to 80 pmol/mg in the presence of calcium and 229 pmol/mg in the presence of EGTA.

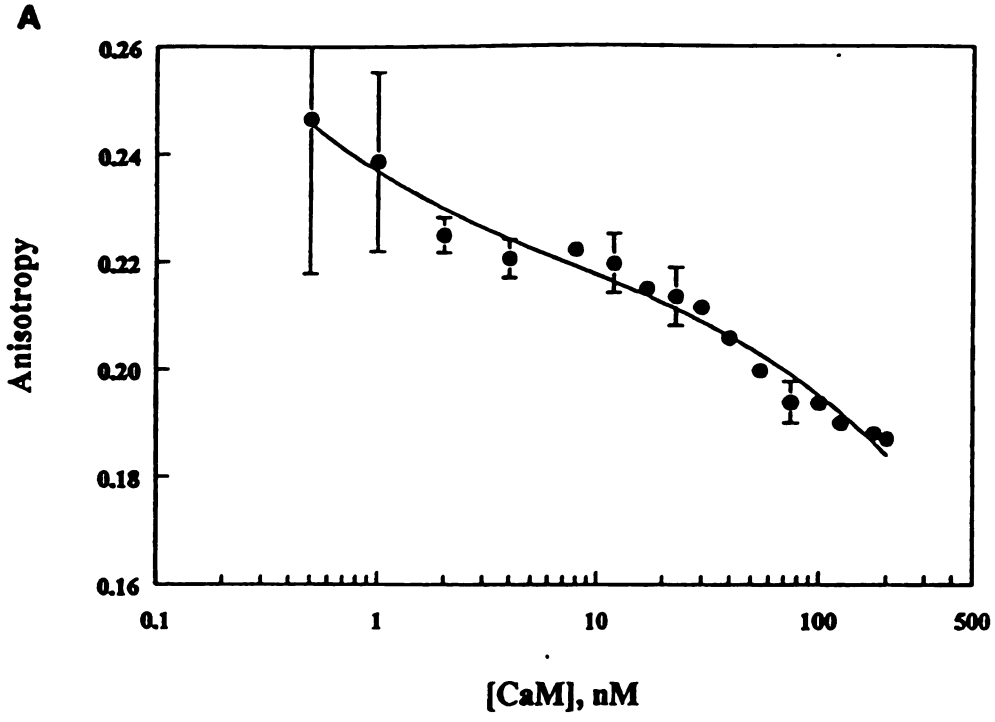
Table 3.1 Equilibrium Constants for Rh-CaM Interaction with the Ca^{2+}-channel Protein in SR Vesicles*

	B_{max1} pmol/mg	K_{d1} nM	B_{max2} pmol/mg	K_{d2} nM
+1 mM EGTA	229±7	8.6±0.8	-	-
+0.1 mM $CaCl_2$	54±7	3±1.1	166±28	239±102
+0.1 mM $CaCl^2$ +1 mM $MgCl_2$	10.0±0.8	0.1±0.03	70±2	17±1

* Data were obtained from titrations of SR vesicles with Rh-CaM in the presence of 0.3 m KCl, 50 mM pipes, pH 7.0, and divalent ion conditions as listed below. Data are means ± S.E.of 3 preparations each.

titration data are consistent with two classes of ligand binding sites on the channel protein for CaM (Figure 3.7 and Table 3.1). The high affinity class of sites has K_{d1} = 4.3 ± 1.1 nM and B_{max1} =54 ± 7 pmol/mg; the results of low affinity binding class site shows K_{d2} = 239 ± 102 and B_{max2} = 166 ± 28 pmol/mg. In the presence of 0.1 mM Ca^{2+} plus 1 mM Mg^{2+}, there is a dramatic shift in the binding capacity of the high affinity class of sites (Figure 3.8 and Table 3.1). The high affinity binding site has a B_{max1} = 10.0 ± 0.8 pmol/mg and K_{d1} = 0.10 ± 0.03 nM; the lower affinity class of sites has K_{d2} of 17 ± 1 nM and a B_{max2} = 70 ± 2 pmol/mg.

Figure 3.6. Titration of skeletal heavy SR vesicles with Rh-CaM in the presence of EGTA. A) Anisotropy plot of titrations of heavy SR with Rh-CaM. B) Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot Rh-CaM binding to SR vesicles. The sample medium contained 90 μg of heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 1 mM EGTA in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. CaM bound was calculated from measured anisotropy as described in "Experimental Procedures".


A
Anisotropy
0.26
0.24
0.22
0.20
0.18
0.16
0.1
1
10
100
500
[CaM], nM

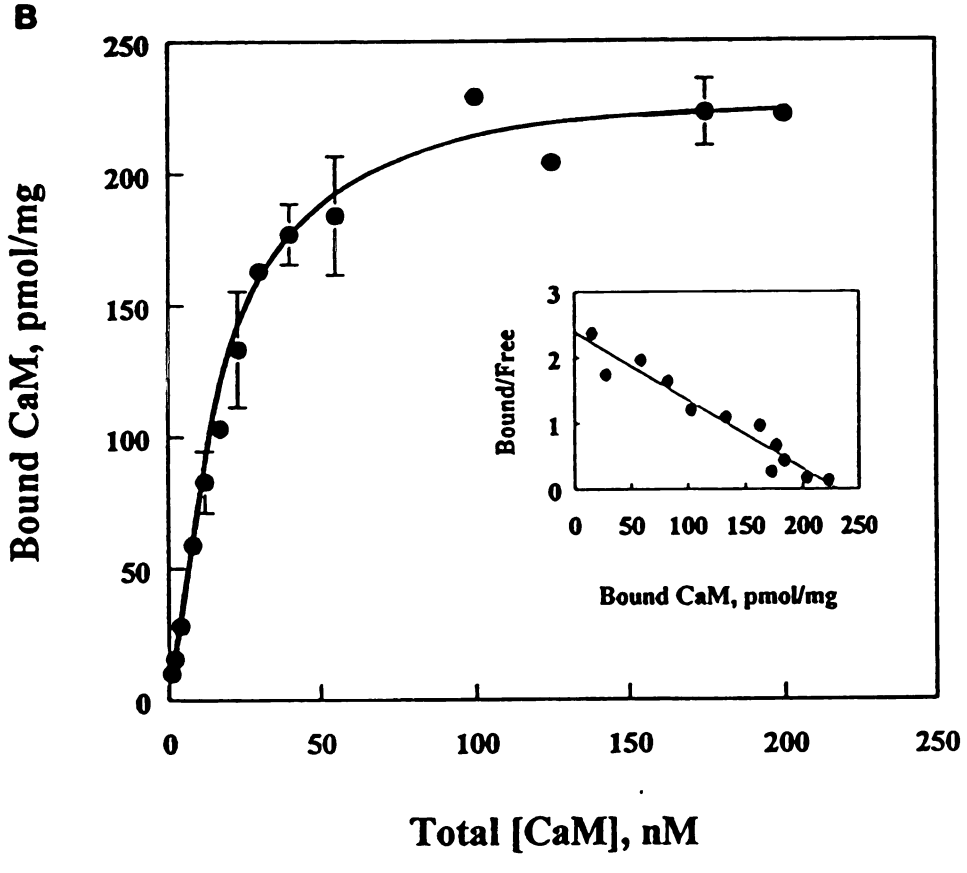

B
Bound CaM, pmol/mg
250
200
150
100
50
0
0
50
100
150
200
250
Total [CaM], nM
Bound/Free
3
2
1
0
0
50
100
150
200
250
Bound CaM, pmol/mg

Figure 3.7 Titration of skeletal heavy SR with Rh-CaM in the presence of $CaCl_2$. A) Anisotropy plot of titrations of heavy SR with Rh-CaM. B) Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot of binding of Rh-CaM to SR vesicles. The sample medium contained 150 μg heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 0.1 mM $CaCl_2$ in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. CaM bound was calculated from measured anisotropy as described in " Experimental Procedures".

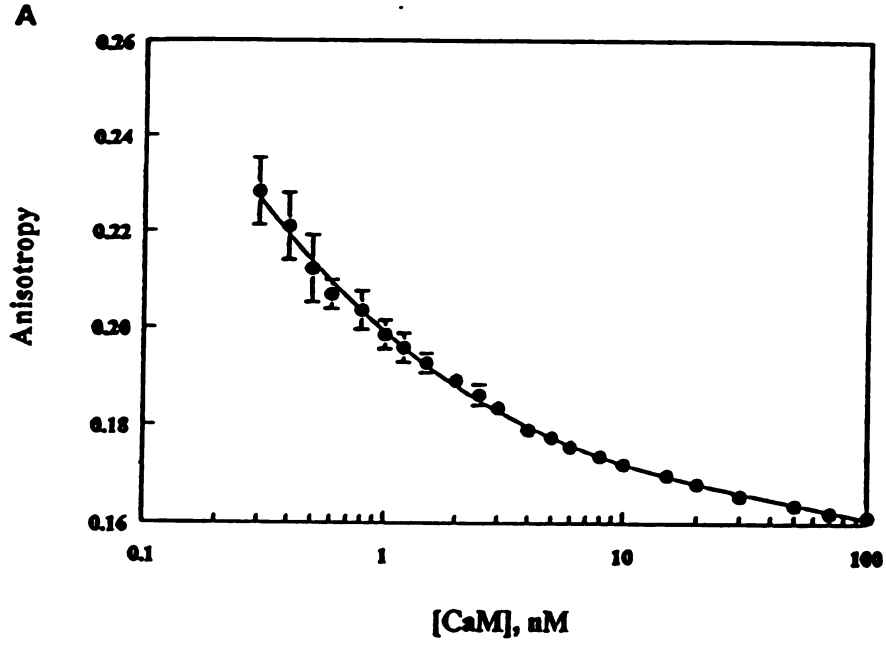
Table 3.1 Equilibrium Constants for Rh-CaM Interaction with the Ca^{2+}-channel Protein in SR Vesicles*

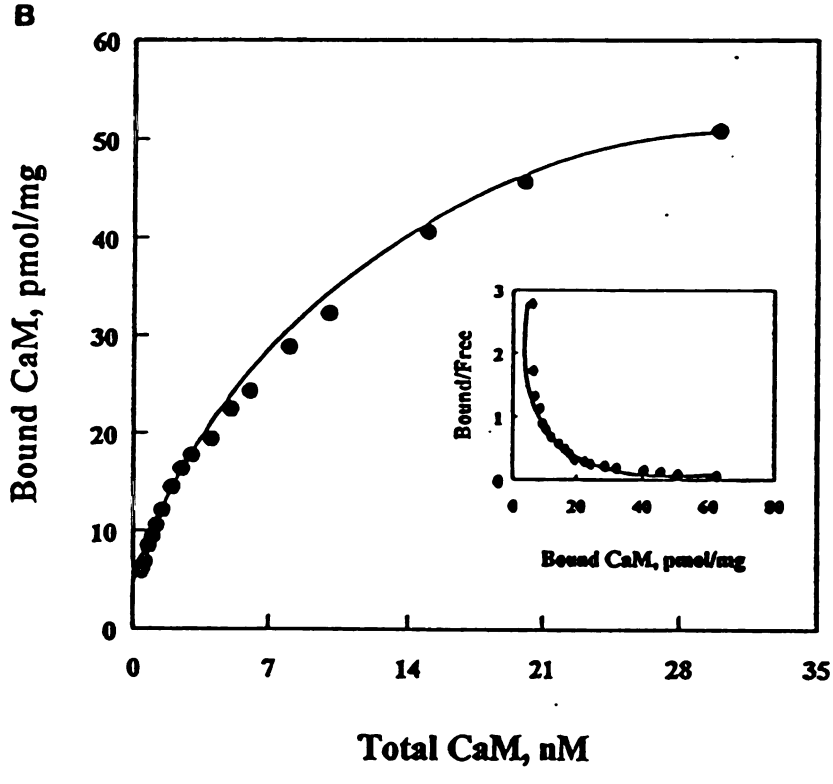
	B_{max1} pmol/mg	K_{d1} nM	B_{max2} pmol/mg	K_{d2} nM
+1 mM EGTA	229±7	8.6±0.8	-	-
+0.1 mM $CaCl_2$	54±7	3±1.1	166±28	239±102
+0.1 mM $CaCl^2$ +1 mM $MgCl_2$	10.0±0.8	0.1±0.03	70±2	17±1

* Data were obtained from titrations of SR vesicles with Rh-CaM in the presence of 0.3 m KCl, 50 mM pipes, pH 7.0, and divalent ion conditions as listed below. Data are means ± S.E.of 3 preparations each.

titration data are consistent with two classes of ligand binding sites on the channel protein for CaM (Figure 3.7 and Table 3.1). The high affinity class of sites has K_{d1} = 4.3 ± 1.1 nM and B_{max1} =54 ± 7 pmol/mg; the results of low affinity binding class site shows K_{d2} = 239 ± 102 and B_{max2} = 166 ± 28 pmol/mg. In the presence of 0.1 mM Ca^{2+} plus 1 mM Mg^{2+}, there is a dramatic shift in the binding capacity of the high affinity class of sites (Figure 3.8 and Table 3.1). The high affinity binding site has a B_{max1} = 10.0 ± 0.8 pmol/mg and K_{d1} = 0.10 ± 0.03 nM; the lower affinity class of sites has K_{d2} of 17 ± 1 nM and a B_{max2} = 70 ± 2 pmol/mg.

Figure 3.8. Titration of skeletal heavy SR with Rh-CaM in the presence of $CaCl_2$ plus $MgCl_2$. A) Anisotropy plot of titration of heavy SR with Rh-CaM. B) Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot analysis of binding of Rh-CaM to SR vesicles. The sample medium contained 50 μg of heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$ in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. CaM bound was calculated from measured anisotropy as described in " Experimental Procedures".


A
Anisotropy
0.26
0.24
0.22
0.20
0.18
0.16
0.1
1
10
100
[CaM], nM


B
Bound CaM, pmol/mg
60
50
40
30
20
10
0
0
7
14
21
28
35
Total CaM, nM
Bound/Free
3
2
1
0
0
20
40
60
80
Bound CaM, pmol/mg

3.4 Discussion

It has been previously shown that CaM partially inhibits Ca^{2+} release from SR vesicles (Meissner, 1986; Plank et al., 1988) and lowers the open state probability of the channel protein in planar lipid bilayers (Smith et al., 1989). The present studies were conducted to characterize the interaction of CaM with the Ca^{2+}-channel protein in heavy SR vesicles by defining conditions in which CaM may bind to and thus potentially regulate channel protein activity.

The porcine heavy SR vesicles used in this study contained a small but significant amount of CaM. The fact that subsequent EGTA washes of the vesicles prior to the endogenous CaM assay did not significantly reduce CaM levels suggests that the endogenous CaM is non-exchangeable and tightly bound to proteins such as phosphorylase kinase (Eibschutz et al., 1984). Our results for endogenous CaM are slightly higher than those obtained by Meissner (1986) who obtained 6-12 pmol/mg of protein for rabbit skeletal muscle heavy SR vesicles. The differences may result from species differences and in methods for determination of endogenous CaM abundance.

Affinity labeling experiments were conducted to identify the CaM-binding proteins in our SR preparations. Wheat germ CaM, iodinated at Tyr 139, was derivatized at Cys 27 (located in the N-domain of CaM) with the photoaffinity label benzophenone-4-maleimide (Strasburg et al., 1988). This probe is reactive with any methylene groups of amino acid residues in the proximity of the label, and thus, proteins which bind within 1.0 nm of the labeled Cys on CaM are readily crosslinked and identified by autoradiography. Our results

indicated that the major receptor for CaM in purified heavy SR preparations was the Ca^{2+}-channel protein and that the binding was specific (Figure 3.1). Light SR vesicles from rabbit skeletal muscle were devoid of Ca^{2+}-channel protein as indicated by the low ryanodine-binding activity, absence of a stained band in the gel, and absence of affinity-labeled channel product in the autoradiogram (Figure 3.1). The amount of crosslinked product in heavy SR was somewhat greater in the presence of 1 mM EGTA than in the presence of 0.1 mM $CaCl_2$. However, crosslinking should not be regarded as a quantitative method. Differences in amount of crosslinked product could reflect actual differences in the amount of CaM bound, but the fluorescence data (Table 3.1) indicated that this was not always the case. Formation of transient, low affinity complexes are quickly locked in a covalent crosslink upon excitation by light. Another explanation for the decreased yield in the presence of Ca^{2+} is decreased efficiency of crosslinking owing to a Ca^{2+}-dependent conformational change in the vicinity of the crosslinker on CaM or a Ca^{2+}-dependent conformational change in the channel protein.

Seiler et al. (1984) first observed that the primary CaM receptor in heavy SR was a protein doublet corresponding to the channel protein (Lai et al., 1988). Their experiments were done using mammalian CaM derivatized with the photoaffinity label, methyl-4-azidobenzimidate which labels up to 4 different lysine residues on CaM (Klevit & Vanaman, 1984).

In contrast to these studies, Vale (1988) observed one major CaM receptor (60 kDa), and six minor CaM receptors (M_r > 200, 148, 125, 41, 33, and 23 kDa) in SR membranes of rabbit skeletal muscle. The discrepancy may be largely attributable to the fact that Vale used a preparation of SR which was not fractionated on sucrose gradients. That preparation

therefore, would likely have comprised a more diverse membrane fraction which contained many more CaM binding proteins than our preparations or those of Seiler et al (1984). Furthermore, it is possible that proteolysis of the channel protein yielded fragments in Vale's preparations which bound CaM. Our preparation employed protease inhibitors to minimize this possibility.

Since there was one major receptor for CaM present in our heavy SR preparations, fluorescence spectroscopic techniques could be used to obtain quantitative data on the interaction of CaM with channel in SR vesicles. Wheat germ CaM was chosen for derivatization because it possesses a single sulfhydryl residue for chemical modification (Toda et al., 1985). Rhodamine-x-maleimide was chosen for labeling of wheat germ CaM because the rhodamine-CaM adduct retains biological activity, it has a high quantum yield, and its emission maximum is in the red portion of the spectrum, thus providing a suitably strong signal in the low nM concentration range of labeled CaM such that the light scattering contribution to the total signal from the membrane vesicles is minimized (Mills et al., 1988; Strasburg et al, 1988).

CaM is known to regulate more than 30 different proteins and enzymes in a Ca^{2+}-dependent manner; i.e., Ca^{2+} first binds to CaM inducing a conformational change in CaM which results in binding of the Ca_4CaM complex to the target protein and modulation of activity (see Klee & Vanaman, 1982, for review). An exception to this mechanism is the interaction of CaM with the neural specific protein P-57 or neuromodulin, in which binding of CaM to this receptor protein is enhanced in the presence of EGTA and reduced in the presence of Ca^{2+} (Andreasen et al., 1983; Masure et al., 1986; Cimler et al. 1985). A novel

aspect emerging from our fluorescence studies on CaM binding to the channel protein is that, in the concentration range of 0.1-0.3 M KCl (i.e., in the range of physiological ionic strength), Rh-CaM bound with high affinity to the channel protein in the presence of EGTA. KCl had a dramatic effect on the affinity of the channel protein for CaM. At low ionic strength in the presence of EGTA, binding of CaM to the channel protein was minimal (Figure 3.3). Titration of a mixture of Rh-CaM plus SR vesicles with KCl resulted in strong enhancement of the affinity of the channel protein for Rh-CaM, possibly owing to an ionic strength-dependent conformational change in the channel protein. Some enhancement of binding was also noted when the KCl titration was conducted in the presence of 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$, although a greater fraction of Rh-CaM was bound initially and there was little change in affinity at high [KCl]. These results are complemented by the affinity labeling experiments (Figure 3.1B) which show CaM crosslinking to the channel protein in the presence of EGTA. Together, these data suggest that CaM binds to the skeletal SR Ca^{2+}-channel protein with high affinity (K_d = 8.6 nM) at the [Ca^{2+}] present in resting muscle (<100 nM), suggesting that the mechanism of CaM interaction with and regulation of the skeletal SR Ca^{2+} channel protein is different from either of the classes of CaM receptors described previously. Although CaM may bind to the skeletal SR Ca^{2+}-channel in the presence of EGTA, Ca^{2+} is required for CaM to exert its inhibitory effect on Ca^{2+}-release (Smith et al., 1989).

Although it is difficult to accurately determine stoichiometry of binding of Rh-CaM to the channel protein in the SR vesicles, a reasonable estimate may be made based on the ryanodine-binding activity of the preparations. The functional unit of the channel protein is a tetramer of identical subunits (Lai et al., 1989), each of M_r = 565,000 (Takeshima et al.,

1989; Zorzato et al., 1990). One mole of ryanodine specifically binds with high affinity per mole of channel protein tetramer, resulting in negatively cooperative interaction between subunits (Lai et al., 1989). Since our heavy SR preparations averaged 10.6 pmol/mg of [^{3}H] ryanodine-binding activity, this suggests that the channel protein subunit concentration is 42.4 pmol/mg SR protein. Scatchard analysis of the Rh-CaM binding data in the presence of EGTA indicates a single class of CaM-binding sites with a B_{max} of 229 pmol/mg, which is consistent with 5-6 moles of CaM- binding sites per subunit.

In the presence of 0.1 mM Ca^{2+}, there are two classes of binding sites with a B_{max1} for the high affinity class of CaM-binding sites of 54 pmol/mg, consistent with approximately 1 CaM binding site per subunit, and B_{max2} for the low affinity class of CaM-binding sites of 166 pmol/mg corresponding to approximately 4 CaM binding site per subunit.

When 1 mM Mg^{2+} was included with 0.1 mM Ca^{2+}, there was a dramatic shift in the interaction of CaM with the channel protein. The B_{max1} for the high affinity class of CaM-binding sites was 10 pmol/mg. The Rh-CaM binding capacity of this class of sites was in close agreement with the [^{3}H]-ryanodine-binding data, suggesting that in the presence of both Ca^{2+} and Mg^{2+}, there is approximately 1 mole of CaM bound with high affinity per tetramer. The lower affinity class of sites showed a B_{max2} of 70 pmol/mg corresponding to approximately 2 lower affinity Rh-CaM site per subunit. The effect of inclusion of Mg^{2+} on CaM-binding may cause a conformational change in the channel protein in high affinity CaM binding sites such that there would be only one high affinity site per tetramer. Alternatively, in the presence of Mg^{2+}, binding of one CaM to the channel protein may induce a cooperative, allosteric effect on the channel protein resulting in decreased affinity of the other sites.

These estimates of CaM stoichiometry are based on the affinity labeling data which suggest that the channel protein is the major receptor for CaM in heavy SR. We cannot exclude the possibility that small amounts of other CaM-binding proteins contribute to the total observed binding. However, the abundance of the channel protein in heavy SR, and the limited CaM binding by light SR vesicles (Figure 3.2) suggest that most of the CaM binding to the SR vesicles is via the channel protein.

These studies provide the first experimental data on the stoichiometry of binding of CaM to the skeletal SR Ca^{2+}-channel protein. Previous studies employed sequence analysis to locate potential CaM-binding sites in the channel protein sequence based on the predicted requirement of a basic amphiphilic helix for CaM target proteins (O'Neil & DeGrado, 1990). Takeshima et al., (1989) predicted 2 CaM binding sites in the vicinity of residues 3614-3637 and 4295-4325, whereas Zorzato et al. (1990) predicted 3 different sites at residues 2775-2807, 2877-2898, and 2998-3016. Brandt et al. (1992), reported that CaM inhibits calpain digestion of the skeletal SR channel protein. Analysis of the digestion pattern of the channel protein in the presence and absence of CaM, coupled with the use of computer algorithms to identify consensus sequences for calpain digestion and CaM binding, led to the identification of 3 more candidate sites for CaM binding: residues 1383-1400, 1974-1996, and 3358-3374.

Our experimental data suggest that there may be as many as 5-6 CaM binding sites per subunit and that the affinity of each CaM site depends on the divalent metal ion concentration. Our data further indicate that CaM binds to the channel protein under conditions comparable to that of resting muscle (< 100 nM $CaCl_2$). This suggests that there may be a different structural element from that of a basic amphiphilic helix for CaM binding

in the channel protein, making predictions of CaM-binding domains based on sequence analysis difficult. Experiments are in progress in our laboratory to identify the CaM-binding domains in the channel protein.

CHAPTER 4

ALTERED CALMODULIN REGULATION OF THE SARCOPLASMIC RETICULUM CALCIUM RELEASE CHANNEL PROTEIN IN MALIGNANT HYPERTHERMIA-SUSCEPTIBLE PIGS

4.1 Introduction

Malignant hyperthermia (MH) is an inherited myopathy characterized by an accelerated skeletal muscle metabolism, muscle contracture, and rapidly elevated body temperature in response to triggering agents such as halogenated anesthetics (for review see Gronert, 1986). The major biochemical defect in MH is associated with alteration of the Ca^{2+} release mechanism via the sarcoplasmic reticulum (SR) (Kim et al., 1984; Mickelson et al., 1986; Mickelson et al.,1988). Abnormal functions of native, detergent-purified, or reconstituted Ca^{2+}-channel protein in malignant hyperthermia susceptible (MHS) SR include altered ryanodine binding properties (Mickelson et al., 1990), altered single-channel activity

in planar lipid bilayers (Shomer et al., 1993), increased Ca^{2+} efflux rate by channel activators (Ca^{2+}, caffeine, ATP, halothane) and a lower luminal Ca^{2+} threshold for induction of Ca^{2+} release (O'Brien et al., 1986; Ohnishi et al.,1987; Carrier et al., 1991; Louis et al., 1992). The abnormal regulation of Ca^{2+} in skeletal muscle from MHS swine results from a point mutation (Arg615 to Cys615) in the SR Ca^{2+}-channel protein (Fujii et al., 1991).

SR Ca^{2+}-release channel protein activity is modulated by numerous physiological and pharmacological agents including Ca^{2+}, Mg^{2+}, caffeine, adenine nucleotides, etc. (Martonosi, 1984). The response of the mutant channel protein to these modulators may be altered in MH. Caffeine increases the sensitivity of the Ca^{2+} induced Ca^{2+}-release mechanism to Ca^{2+} and adenine nucleotides both in skinned fibers and in SR vesicles (for review see Martonosi, 1984). The sensitivities of skeletal muscle contracture and Ca^{2+} release rate in skinned single muscle fibers to caffeine are increased in the MHS pig (Britt, 1987; Gronert, 1986; Endo et al., 1983; Ohta et al., 1989). Pessah et al. (1987) have shown that caffeine dramatically increases the affinity of Ca^{2+} binding sites which activate channel gating. Based on this finding a hypothesis was proposed that the caffeine binding domain directly influences the sensitivity of the Ca^{2+}-regulatory site(s) (Pessah et al.,1987). Otsu et al. (1994) provided evidence that the myoblastic cells expressing mutated Ca^{2+} release channel had higher sensitivity to caffeine-induced Ca^{2+} release from SR Ca^{2+} channel. However, Shomer et al. (1994) have provided evidence that the Arg615Cys mutation in Ca^{2+}-channel in MHS pig skeletal muscle does not appear to be directly responsible for the altered caffeine sensitivity of MHS muscle contracture.

Calmodulin, a ubiquitous calcium-binding protein, may play dual roles in regulating

channel protein activity. It has been implicated as a Ca^{2+} channel inhibitor (Meissner, 1986; Plank et al., 1988) and as an activator of the Ca^{2+} release process (Tripathy et al., 1995), depending on Ca^{2+} concentration. The inhibitory effect of CaM results from reduction of the channel open state probability via direct binding of CaM to the channel protein in the presence of > 1 μM [Ca^{2+}] (Smith et al., 1989). The channel activation effect by CaM occurs at nM Ca^{2+} concentrations < 100 nM corresponding to that of resting muscle condition. Studies on the stoichiometry of CaM-binding to the channel protein indicate that at the lower [Ca^{2+}] there is one class of high affinity CaM binding sites with 16-24 CaM molecules bound per channel protein tetramer, or 4-6 CaM molecules per subunit (Yang et al.,1994; Tripathy et al., 1995). In the presence of μM Ca^{2+}, there are two classes of CaM binding sites: one high affinity class of sites comprising 4 CaM's per tetramer and a low affinity class of sites comprising the remaining CaM molecules (Yang et al., 1994).

The objective of this study was to test the hypothesis that the altered Ca^{2+}-channel activity present in SR from MHS swine results in part, from abnormal CaM regulation of the Ca^{2+}-channel. This was examined by determining the equilibrium binding constants and stoichiometry of MHS and normal SR Ca^{2+}-channel protein with CaM under defined metal ion conditions. In addition, the effect of caffeine on the binding of CaM to SR vesicles from MHS and normal skeletal muscle was examined to determine whether the altered caffeine sensitivity of MHS SR Ca^{2+}-induced Ca^{2+} release results from an allosteric effect of caffeine on the binding of CaM to MHS SR Ca^{2+} channel. The results suggest that the binding of CaM to the channel protein in MHS SR is altered. However, the effect of caffeine on the binding of CaM to MHS and normal SR is not significantly different.

4.2 Experimental Procedures

4.2.1 Materials. Benzophenone-4-maleimide and rhodamine-X-maleimide were purchased from Molecular Probes (Junction City, OR). Na[^{125}I] and [^{3}H]-ryanodine were obtained from DuPont-NEN (Boston, MA). Ryanodine was purchased from Calbiochem (La Jolla, CA). Wheat germ was a generous gift from International Multifoods (Minneapolis, MN). Caffeine was from Sigma (St Louis, MO).

4.2.2 Preparation of calmodulin and its derivatives. CaM was purified from wheat germ using the procedure described previously (Strasburg et al., 1988). Purified CaM was derivatized at Cys 27 with rhodamine-X-maleimide as described for I-EDANS (Strasburg et al., 1988). For cross-linking experiments, CaM was iodinated with [^{125}I] at the sole tyrosine residue (Tyr-139), followed by site-specific modification at Cys-27 with the photoactivatable cross-linker benzophenone-4-maleimide (Strasburg et al., 1988).

4.2.3 Photoaffinity labeling of MHS or normal SR Ca^{2+} channel protein with [^{125}I]-Bz-CaM. [^{125}I]-Bz-CaM crosslinking with MHS or normal SR vesicles was performed by incubating in darkness 0.1 μM [^{125}I]-Bz-CaM with 100 μg of SR vesicles in 100 μL of 20 mM HEPES pH 7.5, 0.15 M NaCl, and different metal ion conditions as described in figure legend. The mixtures were placed in plastic microcentrifuge tubes on ice and the samples were illuminated for 20 min in a Stratalinker 1800 photoreactor (Stratagene Corp., La Jolla, CA) equipped with lamps of λ_{max} = 254 nM. After photolysis of the mixtures, the samples

were centrifuged in a Beckman TL-100 centrifuge at 100,000 g_{max} for 20 min. The membrane pellets were resuspended in water, SDS was added to produce a final concentration of 1%, and the samples were subjected to polyacrylamide gel electrophoresis using 5-20% linear gradient gels (Laemmli, 1970). The gels were dried and placed with Kodak Omat XAR-5 X-ray film in autoradiography cassettes equipped with Dupont Lightning Plus intensifying screens.

4.2.4 Preparation of MHS and normal skeletal muscle SR vesicles. Longissimus muscle of pigs, homozygous for either the MHS (Pietrain) or normal (Yorkshire) allele of the Ca^{2+} release channel, were obtained from the swine genetics herd maintained by the Department of Animal Science at the University of Minnesota Experimental Farm. Heavy SR vesicles were prepared using the procedure of Mickelson et al., (1986) with the modifications that 1 mM EGTA, 0.1 mM PMSF, 1 μg/ml of aprotinin and 1 μg/ml leupeptin were included in the homogenization buffer and at each subsequent purification step.

4.2.5 Fluorescence anisotropy measurements. Rhodamine maleimide-labeled CaM (Rh-CaM) binding to the channel protein in SR vesicles was monitored by fluorescence anisotropy measurements using an SLM 4800 spectrofluorometer modified with data acquisition hardware and operating system from On-Line Instrument Systems (Bogart, GA). Samples were held in a thermostatted cell block maintained at 22°C. The excitation wavelength of Rh-CaM was 580 nm, monochromator slits were set at 8 nm, and emitted light was isolated using Schott RG-610 filters. During titrations samples were allowed to equilibrate for 5 minutes

after each addition of Rh-CaM or SR. All samples included 1 μg/ml aprotinin, 1 μg/ml leupeptin and 0.1mM PMSF to inhibit proteolysis during the experiment.

All fluorescence measurements were performed using semi-micro, quartz fluorescence cuvettes (4 mm x 10 mm). Prior to fluorescence experiments the cells were rinsed with 1 mg/ml bovine serum albumin to minimize Rh-CaM adsorption to the walls of the cuvette. Following this treatment, the measured anisotropy value for Rh-CaM was independent of concentration over the range of 1 nM to 1000 nM, indicating that there was negligible Rh-CaM adsorption to the cuvettes.

The anisotropy of a ligand (Rh-CaM) is directly proportional to the fraction of ligand bound to the receptor (Ca^{2+}-channel protein). Thus, if A_f is the anisotropy of free Rh-CaM and A_b is the anisotropy of the fully bound ligand, then the fraction bound, f_b, is determined from:

$$f_b = \frac{(A_m - A_f)}{A_m(1-q) + q(A_b) - A_f} \qquad (1)$$

where A_m is the measured anisotropy for a given ligand concentration, and q, the change in quantum yield, is the ratio of fluorescent intensity of bound species over that of the free species. If the change in quantum yield is negligible upon binding of ligand, then equation (1) reduces to:

$$f_b = \frac{(A_m - A_f)}{(A_b - A_f)} \qquad (2)$$

The fraction of ligand bound, and the concentrations of bound and free Rh-CaM are readily calculated. Values of K_d and B_{max} were calculated using the computer program Enzfitter, a nonlinear regression analysis program of R.J. Leatherbarrow (Biosoft, Cambridge, UK). Data were fit to a one-ligand or two-ligand binding model to obtain the best fit.

The anisotropy of unbound Rh-CaM, A_f, was measured in the absence of SR. The anisotropy of the fully bound species, A_b, was obtained by titration of Rh-CaM with SR vesicles, followed by curve-fitting using the Enzfitter computer program for a single class of ligand binding sites.

Corrections for light scattering and background fluorescence were made by application of the equation:

$$A = f_1 A_1 + f_2 A_2 \qquad (3)$$

where A, A_1 and A_2 are the anisotropies of the sample, the blank, and the corrected sample, respectively. The fractional contributions, f_1 and f_2, of these species were calculated from the intensities measured with the excitation monochromator in the vertical position and the emission monochromator at 55°. The corrected sample anisotropy, therefore, is that value in the absence of background interference.

4.2.6 Biochemical Assays. SR protein concentrations were determined by the method of Lowry et al., (1951) using bovine serum albumin as the standard. Ryanodine binding activity of SR vesicles was determined according to the method of Mickelson et al. (1988).

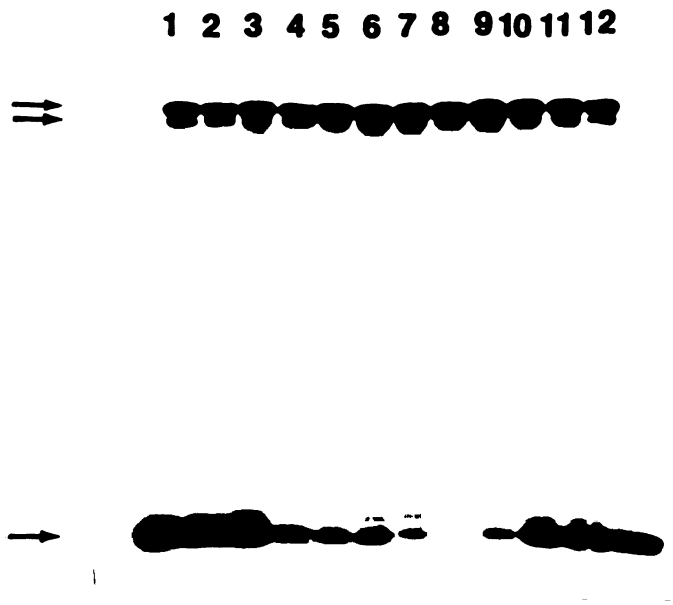
4.3 Results

4.3.1 Affinity labeling of CaM-binding proteins in MHS or normal SR vesicles. Heavy SR vesicles purified from MHS and normal porcine skeletal muscle were incubated with the affinity labeling derivative [^{125}I]-Bz-CaM under different metal ion conditions to identify CaM receptor proteins present in SR. The autoradiogram of the gel electrophoretogram (Figure 4.1) indicates that the most abundant crosslinked product in both MHS and normal heavy SR vesicles was a doublet of $M_r > 450{,}000$ which corresponds to CaM plus Ca^{2+} channel protein subunit. This cross-linking complex was formed both in the presence of 1 mM EGTA and in the presence of 0.1 mM $CaCl_2$. There were no major differences in the affinity labeling patterns between normal and MH SR.

4.3.2 [^{3}H]Ryanodine binding to MHS or normal SR vesicles. The [^{3}H]ryanodine binding capacities (B_{max}) for our MHS and normal heavy SR preparation, determined by Scatchard analysis, were 14.6 ± 0.8 and 10.6 ± 0.9 pmol/mg SR protein, respectively.

4.3.3 Titration of Rh-CaM with MHS or normal SR vesicles. Affinity labeling results indicated that Ca^{2+}-channel protein is the major CaM receptor in both MHS and normal heavy SR. Although the affinity labeling experiments yield useful information on the identity of CaM-binding components, it is difficult to obtain quantitative information on the interaction of CaM with Ca^{2+} channel protein by this method. Therefore, fluorescence anisotropy was used to define the affinity and stoichiometry of the CaM/channel protein complex formation

Figure 4.1. Affinity labeling of MHS and normal skeletal muscle heavy SR with [^{125}I]-Bz-CaM. [^{125}I]-Bz-CaM was incubated with skeletal muscle heavy SR vesicles in the presence of 20 mM Hepes pH 7.5, 0.15 M NaCl, and metal ion conditions as described below. Following initiation of crosslinking, proteins were separated by electrophoresis on a 5-20% acrylamide gradient gel, and the cross-linked products were identified by autoradiography of the gel. Lanes 1-6: normal heavy SR; Lanes 7-12: MHS heavy SR. Lanes 1,12: 0.1 mM $CaCl_2$ + 10 mM $MgCl_2$; Lanes 2,11: 1 mM $CaCl_2$; Lanes 3, 10: 0.1 mM $CaCl_2$; Lanes 4,9: 1 mM EGTA + 10 mM $MgCl_2$; Lanes 5, 8: 1 mM EGTA + 1 mM $MgCl_2$; Lanes 6,7: 1 mM EGTA.


1 2 3 4 5 6 7 8 9 10 11 12

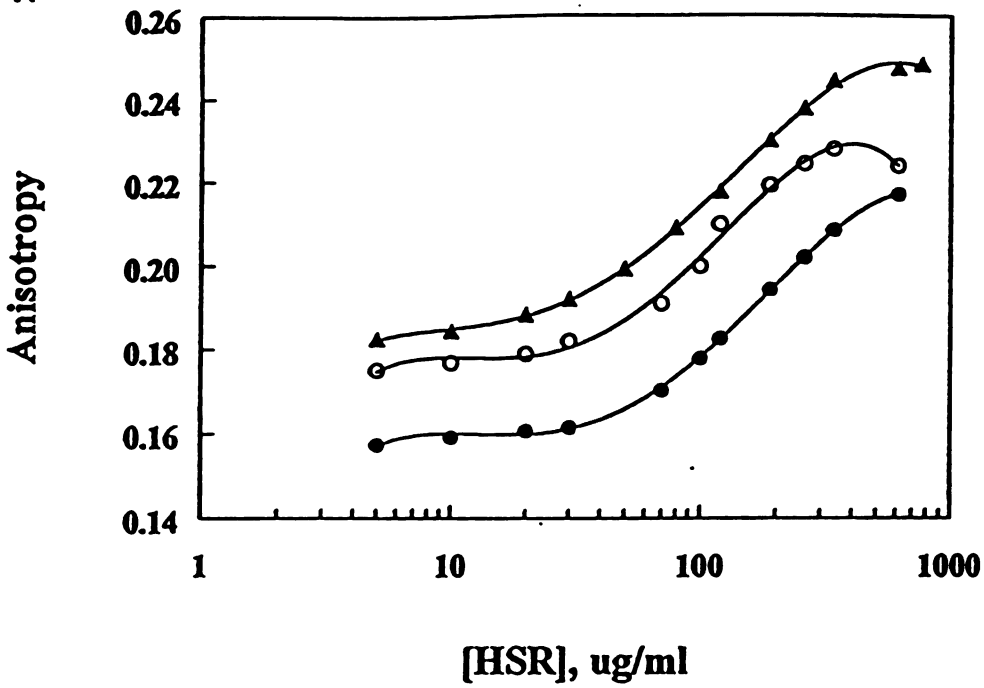
in native SR vesicles from normal and MHS porcine skeletal muscle. Heavy SR vesicles were titrated into Rh-CaM under three different metal ion conditions: (1) + 1 mM EGTA; (2) + 0.1 mM $CaCl_2$; (3) + 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. In each case, titration of SR vesicles into a solution containing Rh-CaM resulted in a large increase in fluorescence anisotropy indicating the increased molecular mass resulting from Rh-CaM binding to the channel protein (Figure 4.2).

The titration data from Figure 4.2 were used to determine the anisotropy values for the free Rh-CaM species (A_f) which is obtained in the absence of SR vesicles and for the fully bound Rh-CaM/channel protein complex (A_b). The A_b values obtained for each metal ion condition in MHS SR titrations were 0.2483 (+ 1 mM EGTA), 0.2279 (+ 0.1 mM $CaCl_2$), 0.217 (+ 0.1 mM $CaCl_2$, + 1 mM $MgCl_2$). With normal muscle SR titrations, the A_b values obtained for each metal ion condition averaged 0.2435. The A_f values obtained for Rh-CaM under the three metal ion conditions in the absence of MHS or normal SR were 0.1824 and 0.1908 (+ 1 mM EGTA), 0.175 and 0.1728 (+ 0.1 mM $CaCl_2$), 0.1573 and 0.1585 (+ 0.1 mM $CaCl_2$, + 1 mM $MgCl_2$) respectively. The subtle difference in A_f value for MH vs. normal SR results from different labeled CaM preparations used for these experiments.

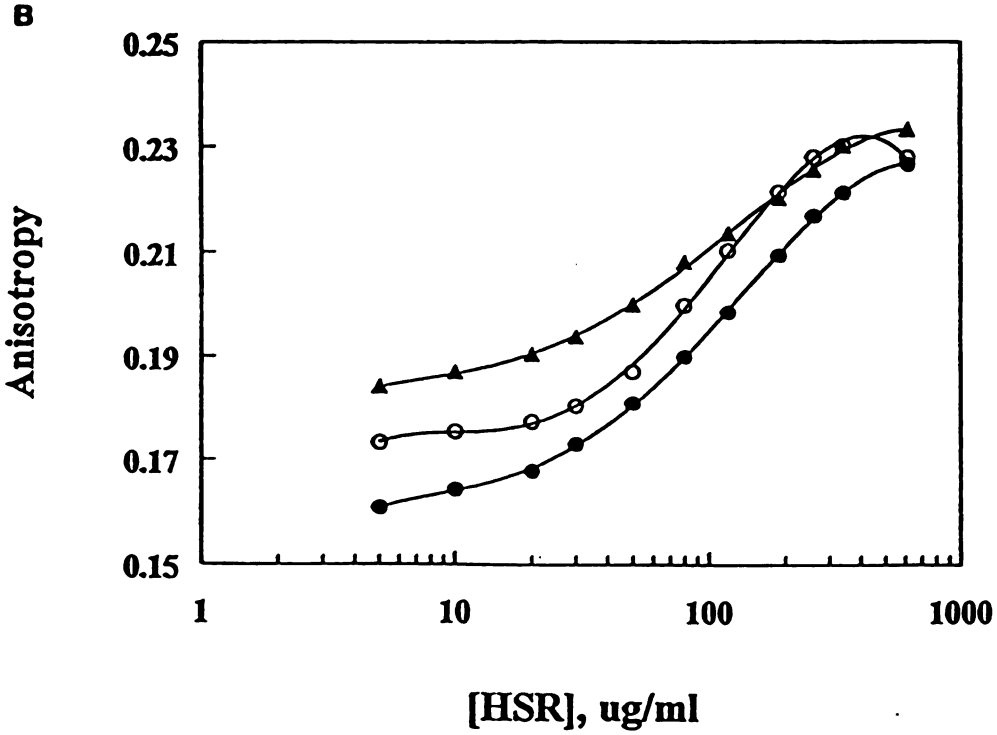
4.3.4 Rh-CaM/Ca^{2+} channel protein of MHS or normal SR binding equilibrium. Our previous studies have determined the equilibrium binding constants of CaM to the channel protein from genetically defined normal SR vesicles. In order to determine whether the altered SR Ca^{2+} channel activity in MH is associated with abnormal CaM regulation of channel activity, the affinity and stoichiometry of CaM binding to MHS SR were determined. Rh-CaM

Figure 4.2. Titration of Rh-CaM with MHS and normal skeletal heavy SR vesicles under different divalent ion conditions. The sample medium contained 10 nM Rh-CaM, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 plus one of the following conditions: 1 mM EGTA (▲); 0.1 mM $CaCl_2$ (O); 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$, (●) in a starting volume of 1 mL. Panel A and B represent SR vesicles purified from MHS and normal swine, respectively. The Rh-CaM sample was titrated with SR vesicles in parallel with a buffer blank containing the same media minus Rh-CaM. Corrections were made for light scattering as described in "Experimental Procedures".

A


0.26
0.24
0.22
0.20
0.18
0.16
0.14
Anisotropy
1
10
100
1000
[HSR], ug/ml

B


0.25
0.23
0.21
0.19
0.17
0.15
Anisotropy
1
10
100
1000
[HSR], ug/ml

was titrated into fixed concentrations of SR vesicles under the following conditions: (1) 1 mM EGTA; (2) 0.1 mM $CaCl_2$; (3) 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$. The fluorescence intensity did not change significantly upon the binding of Rh-CaM to Ca^{2+} channel protein. Therefore, the fraction of Rh-CaM bound to channel protein in SR vesicles for each titration point was calculated by anisotropy values A_f and A_b from Figure 4.2 using eq. 2. (Experimental Procedures).

Analysis of fluorescence anisotropy data from Rh-CaM titration into MHS and normal SR Ca^{2+} channel protein indicates that there are significant differences in affinity and stoichiometry of CaM/Ca^{2+} channel interaction under these three conditions. In the presence of EGTA there is a single class of CaM-binding sites on both MHS and normal SR Ca^{2+} channel protein. However, the binding capacities (B_{max}) and dissociation constants (K_d) differ substantially between these two proteins. B_{max} and K_d for MHS SR are 164 ± 4 pmol/mg and 4.2 ± 0.3 nM, respectively; for normal SR B_{max} = 229 ± 7 pmol/mg and the K_d = 8.6 ± 0.8 nM (Figure 4.3 and Table 4.1). In the presence of 0.1 mM $CaCl_2$, the binding of CaM to channel protein was shifted to two classes of ligand-binding sites. The high affinity class of binding sites displayed a B_{max1} of 45 ± 7 pmol/mg and K_{d1} of 1.6 ± 0.5 nM for MHS SR, and for normal SR B_{max1} = 54 ± 7 pmol/mg, and K_{d1} = 4.3 ± 1.1 nM. The low affinity class of binding sites shows a B_{max2} = 73.6 ± 24.9 pmol/mg and K_{d2} = 84.8 ± 81.6 nM for MHS SR, versus normal SR which shows a B_{max2} of 166 ± 28 pmol/mg and a K_{d2} of 239 ± 102 nM (Figure 4.4 and Table 4.1).

In the presence of 0.1 mM Ca^{2+} plus 1 mM Mg^{2+}, there was a dramatic shift of high affinity binding-site class to low affinity binding-sites class. The B_{max1} and K_{d1} for MHS SR

Figure 4.3. Titration of skeletal heavy SR vesicles with Rh-CaM in the presence of EGTA. Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot of Rh-CaM binding to SR vesicles. The sample medium contained 90 μg of MHS (●) or normal (○) heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 1 mM EGTA in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. CaM bound was calculated from measured anisotropy as described in "Experimental Procedures".

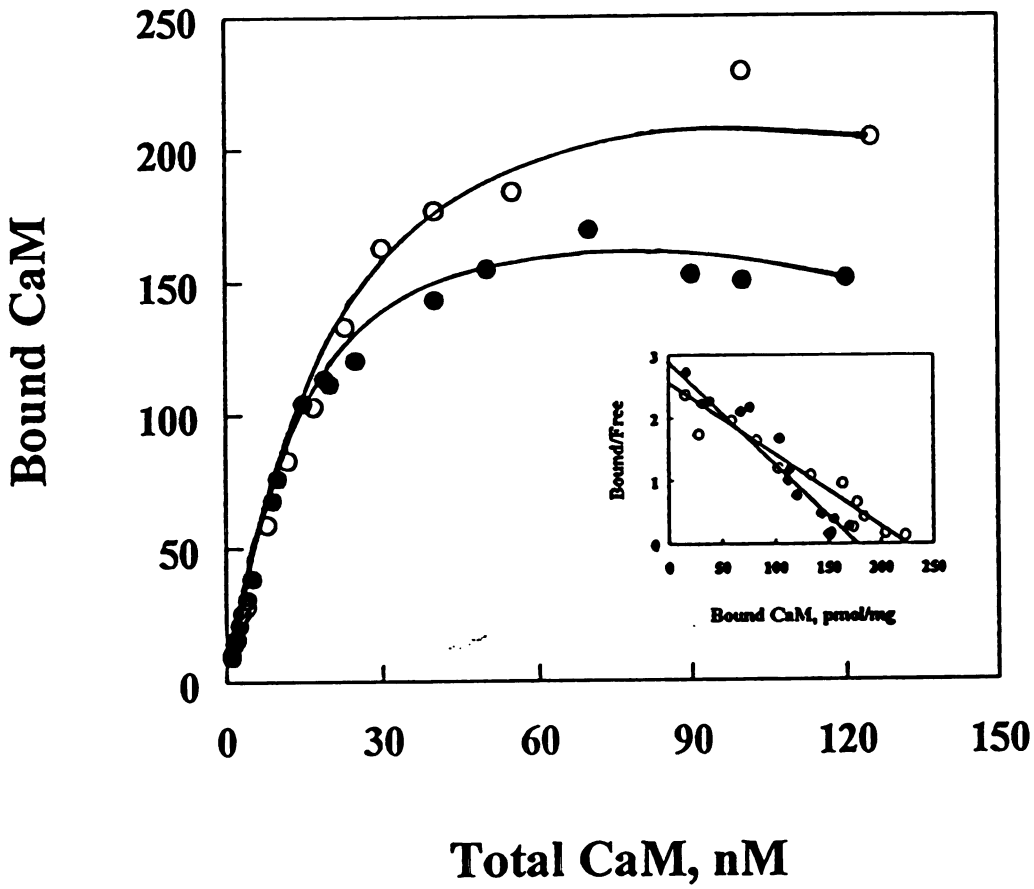

250
200
150
100
50
0
Bound CaM
0
30
60
90
120
150
Total CaM, nM
Bound/Free
Bound CaM, pmol/mg

Figure 4.4. Titration of skeletal heavy SR with Rh-CaM in the presence of $CaCl_2$. Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot of Rh-CaM binding to SR vesicles. The sample medium contained 150 μg MHS (●) or normal (○) heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 0.1 mM $CaCl_2$ in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. CaM bound was calculated from measured anisotropy as described in " Experimental Procedures".

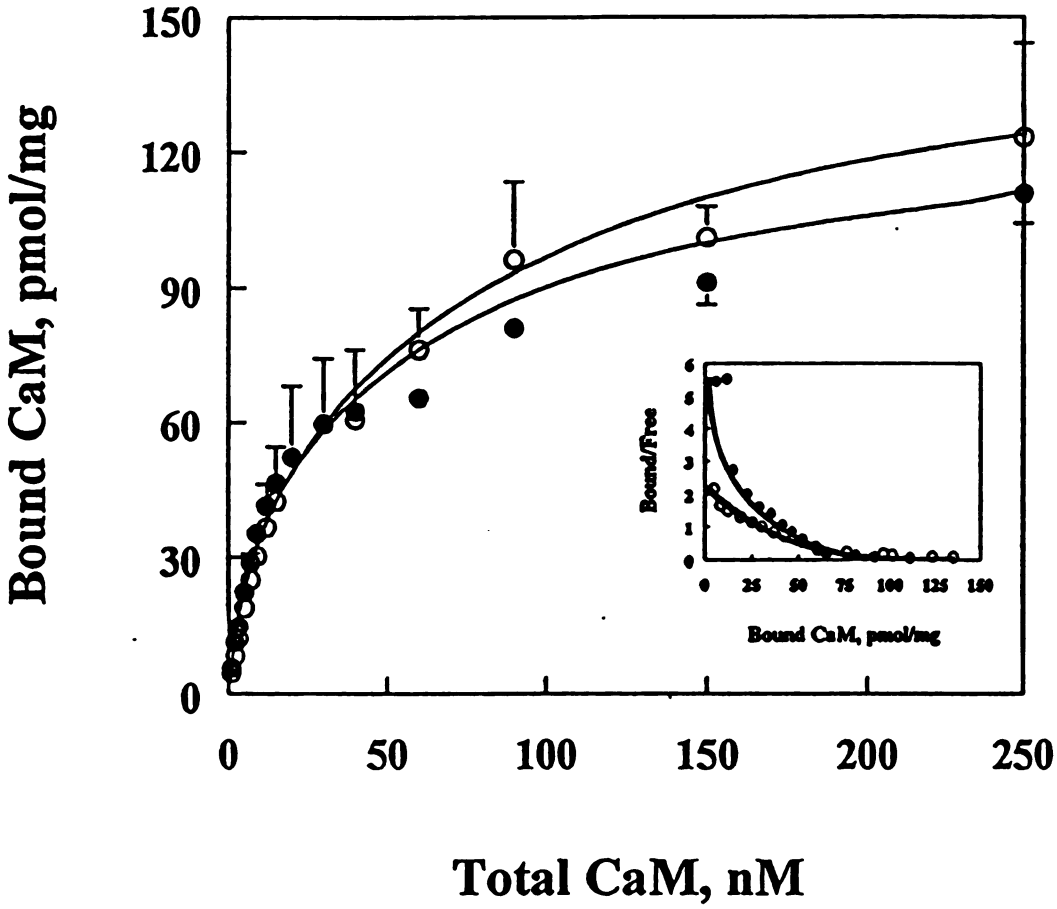

Bound CaM, pmol/mg
150
120
90
60
30
0
0
50
100
150
200
250
Total CaM, nM
Bound/Free
Bound CaM, pmol/mg

Table 4.1. Equilibrium Constants for Rh-CaM Interaction with the Ca^{2+} Release Channel Protein in Normal and MHS SR Vesicles*

	B_{max1} (pmol/mg)	K_{d1} (nM)	B_{max2} (pmol/mg)	K_{d2} (nM)
Normal				
+1mM EGTA	229±7	8.6±0.8	-	-
+0.1mM $CaCl_2$	54±7	4.3±1.1	166±28	239±102
+0.1mM $CaCl_2$ +1mM $MgCl_2$	10.0±0.8	0.10±0.03	70±2	17±1
MHS				
+1mM EGTA	164±4	4.2±0.3	-	-
+0.1mM $CaCl_2$	45±7	1.6±0.5	73.6±24.9	84.8±81.6
+0.1mM $CaCl_2$ +1mM $MgCl_2$	14.9±0.8	0.05±0.02	248±24	57.4±8.6

* Data were obtained from titrations of SR vesicles with Rh-CaM in the presence of 0.3 M KCl, 50 mM Pipes, pH 7.0, and divalent ion conditions as listed below. Data are ± SE of the means of three preparations each.

were 14.9 ± 0.8 pmol/mg and 0.05 ± 0.02 nM respectively and for normal SR, these values were 10.0 ± 0.8 pmol/mg and 0.1 ± 0.03 nM respectively. The B_{max2} and K_{d2} for MHS were 248 ± 24 pmol/mg and 57.4 ± 8.6 nM, whereas normal SR shows a B_{max2} of 70 ± 2 pmol/mg and a K_{d2} of 17 ± 1 nM (Figure 4.5 and Table 4.1).

The stoichiometry of binding of Rh-CaM to Ca^{2+}-channel protein in the SR vesicles may be estimated by dividing the Rh-CaM binding activity by the ryanodine binding activity. One mole of ryanodine binds per mole of channel protein tetramer (Lai et al., 1989); therefore, in the presence of EGTA, the CaM-binding stoichiometry of the MHS Ca^{2+}-channel protein is consistent with 11.2 mole of CaM-binding sites per mole of channel tetramer or about 3 sites per monomeric subunit (Table 4.2). In the presence of 0.1 mM $CaCl_2$, the high affinity CaM-binding site of the MHS Ca^{2+}-channel with a B_{max1} of 45 pmol/mg is consistent with 3.1 moles of CaM-binding sites per channel tetramer, or approximately one high affinity CaM binding site per subunit (Table 4.2). In the presence of Ca^{2+} plus Mg^{2+}, the high affinity CaM-binding sites in MHS SR Ca^{2+}-channel with a B_{max1} of 14.9 pmol/mg is consistent with approximately one CaM-binding site per tetramer, which is not significantly different from the number of CaM-binding sites in normal SR Ca^{2+}-channel (Table 4.2).

4.3.5 Ionic strength dependence of the binding of Rh-CaM to MHS or normal SR vesicles. Our previous results suggested that binding of Rh-CaM to the Ca^{2+} channel protein of normal skeletal muscle SR was highly ionic strength dependent (Yang et al., 1994). Although the basis for this ionic strength effect is not clear, it is likely that changes in ionic strength alter channel protein structure in such a way that affinity for Ca^{2+} is enhanced at

Figure 4.5. Titration of skeletal heavy SR with Rh-CaM in the presence of $CaCl_2$ plus $MgCl_2$. Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot of Rh-CaM binding to SR vesicles. The sample medium contained 50 μg of MHS (●) or normal (○) heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM PIPES, pH 7.0 and 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$ in a starting volume of 1 mL. Points represent the means ± S.E. of 3 preparations. CaM bound was calculated from measured anisotropy as described in " Experimental Procedures".

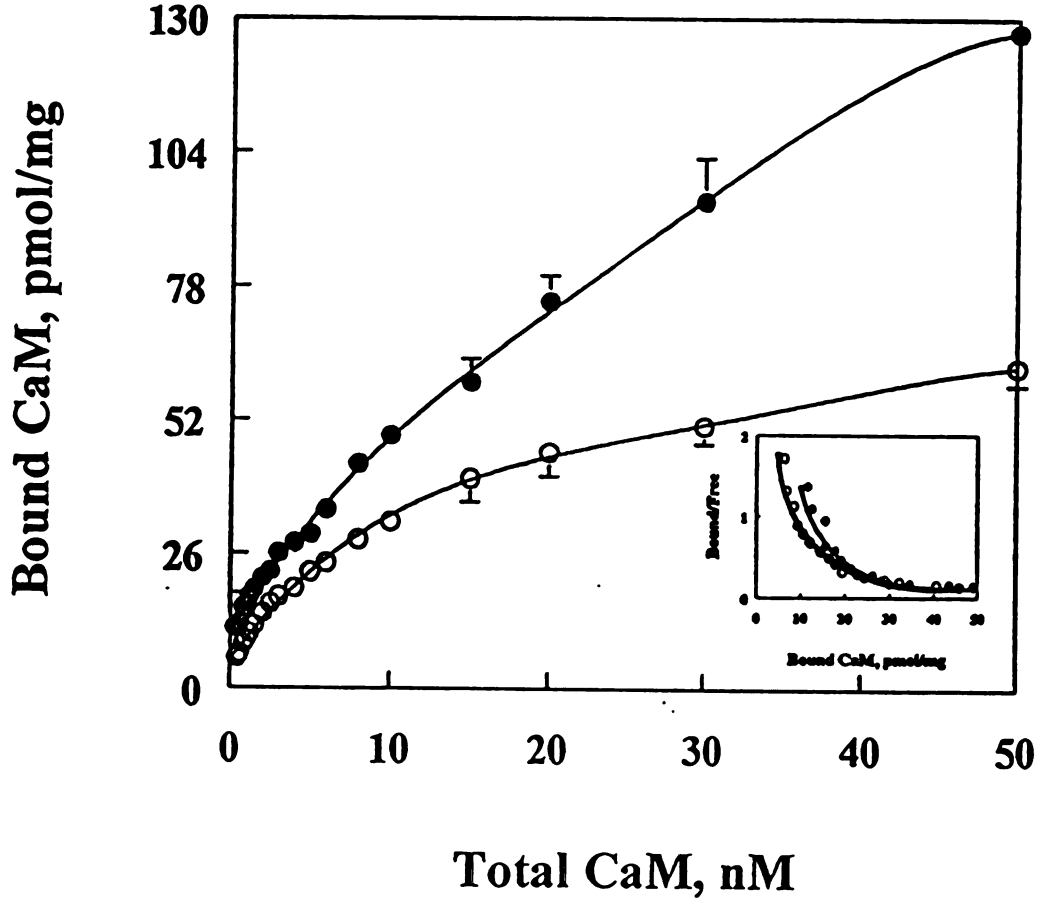

130
104
78
52
26
0
Bound CaM, pmol/mg
0
10
20
30
40
50
Total CaM, nM

Table 4.2. Stoichiometry of High Affinity Class of Rh-CaM Binding to Normal and MHS Skeletal Muscle SR Ca^{2+}-Release Channel*

	High affinity CaM binding class (moles CaM per mole of channel tetramer)	Low affinity CaM binding class (moles CaM per mole of channel tetramer)
Normal		
+1 mM EGTA	21.6	-
+0.1 mM $CaCl_2$	5.5	15.7
+0.1 mM $CaCl_2$ +1 mM $MgCl_2$	1	6.6
MHS		
+1 mM EGTA	11.2	-
+0.1 mM $CaCl_2$	3.1	5.1
+0.1 mM $CaCl^2$ +1 mM $MgCl_2$	1	17

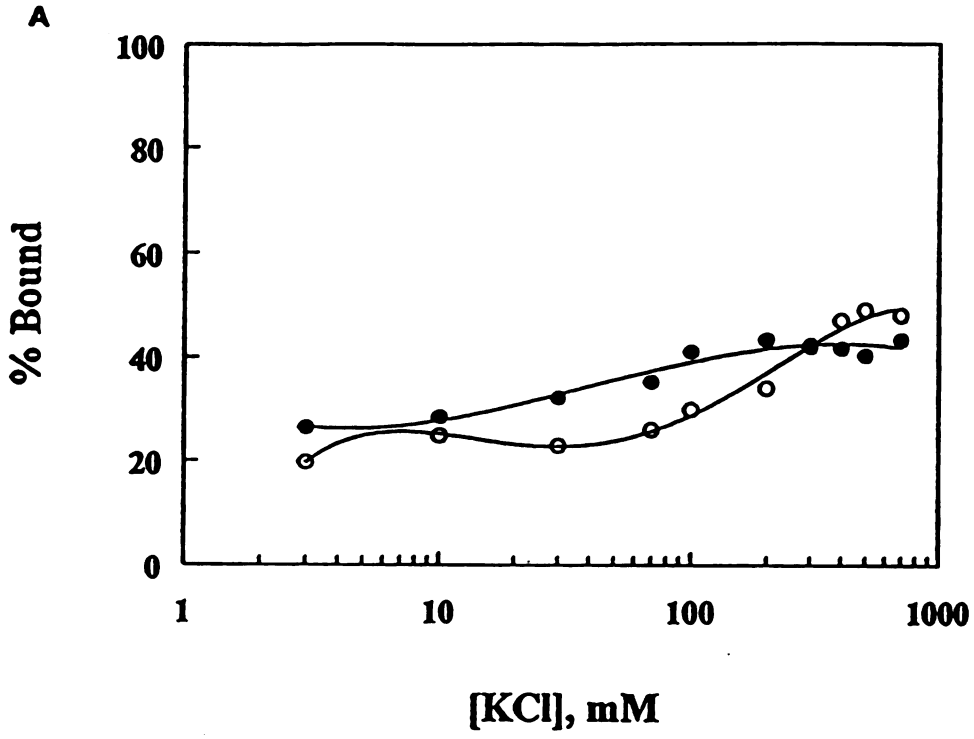
* The stoichiometries of high affinity Rh-CaM binding to normal and MHS SR Ca^{2+} channel are estimated on the base of ryanodine binding data 10.6 ± 0.9 and 14.64 ± 0.83.

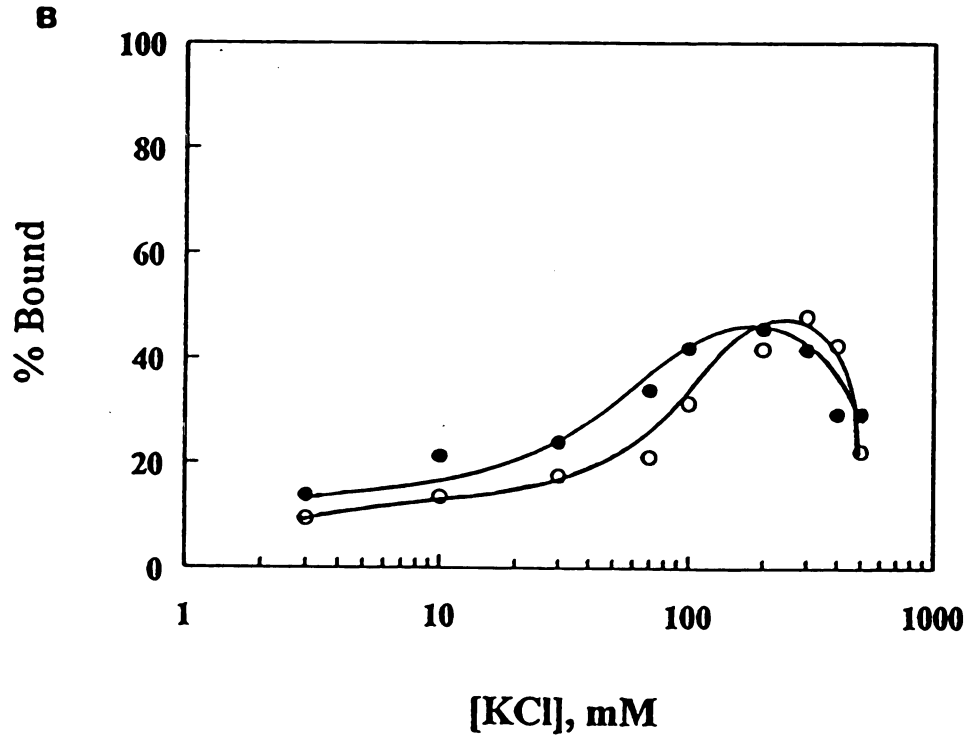
physiological ionic strength. Therefore, to determine whether the Arg615Cys mutation might have affected the ionic strength dependence of CaM binding to the channel protein, Rh-CaM binding was determined for normal and MHS SR as a function of [KCl]. The SR concentrations were fixed at levels corresponding to the concentrations of species at the mid-points of the titration curves. in Figure 4.2.

In the presence of EGTA (Figure 4.6A), the binding of Rh-CaM to both normal and MHS SR Ca^{2+} channel protein increased as KCl concentration increased reaching an optimum in the range of 0.1-0.3 M KCl. At [KCl] > 0.3 M, the binding of Rh-CaM to the channel protein rapidly decreased. In the presence of 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$, more Rh-CaM was initially bound to Ca^{2+} channel protein in the absence of KCl, and there was a gradual increase for both normal and MHS SR as KCl concentration increased from 3 mM to 0.7 M (Figure 4.6B). However, in contrast to the EGTA conditions, binding of Rh-CaM to the channel protein did not significantly decrease at higher KCl concentrations in the presence of Ca^{2+} and Mg^{2+}. The results from Figure 4.6 suggest that differences in CaM-binding between MHS and normal SR are not a result of altered ionic strength-induced structural change.

4.3.6 Caffeine dependence of the binding of Rh-CaM to MHS or normal SR vesicles. It has been suggested previously that the mutation in the MHS Ca^{2+} release channel is not directly responsible for the altered caffeine sensitivity of MHS pig muscle contracture (Shomer et al., 1995). Rather, this altered caffeine sensitivity may alter the response of the channel protein to other physiological channel modulators. To determine whether caffeine binding could affect the affinity of the normal channel protein for CaM, caffeine was titrated

Figure 4.6. KCl dependence of the binding of Rh-CaM to SR vesicles under different divalent ion conditions. The sample buffer contained 100 μg MHS (●) and 86 μg normal (○) heavy SR vesicles, 10 nM Rh-CaM, 0.3 M sucrose, 50 mM PIPES, pH 7.0 and either 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (panel A), or 1 mM EGTA (panel B) in a starting volume of 1 mL. Points were representatives of 3 preparations. % CaM bound was calculated from measured anisotropy as described in "Experimental Procedures"; 100% bound corresponds to 229 pmol/mg in the presence of EGTA and 80 pmol/mg in the presence of $CaCl_2$ plus $MgCl_2$ for normal SR measurement. For MHS SR, 100% bound corresponds to 164 pmol/mg in the presence of EGTA and 263 pmol/mg in the presence of $CaCl_2$ plus $MgCl_2$.

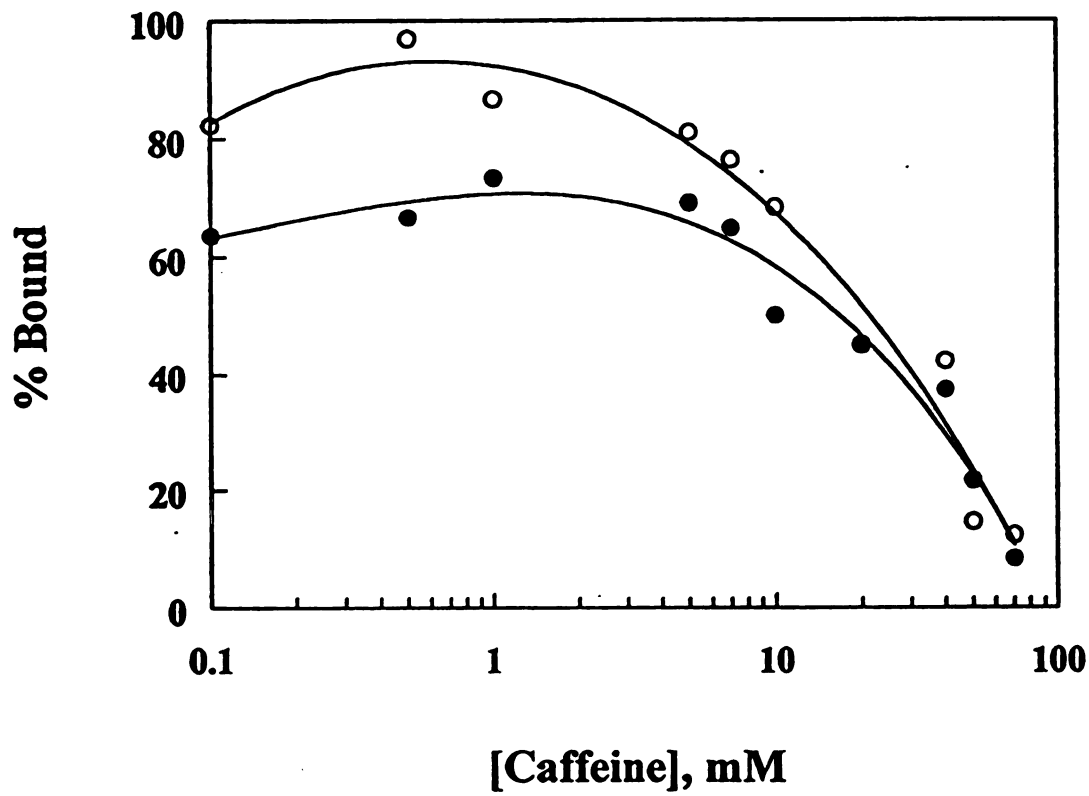

A
100
80
60
40
20
0
% Bound
1
10
100
1000
[KCl], mM


B
100
80
60
40
20
0
% Bound
1
10
100
1000
[KCl], mM

into a fixed concentration of Rh-CaM and SR from normal muscle in the presence of 100 μM $CaCl_2$ plus 1 mM $MgCl_2$. These results were compared with those obtained for MHS SR to determine whether the altered caffeine sensitivity of MHS muscle contracture might be caused by an allosteric effect on CaM binding to and regulation of SR Ca^{2+} channel protein.

Figure 4.7 indicates that as the caffeine concentration was increased from 0.1 mM to 100 mM, the fluorescence anisotropy decreased for both MHS and normal SR indicating that the binding of Rh-CaM to Ca^{2+} channel in both MHS and normal skeletal SR decreased from 90% bound to 50% bound as caffeine concentration increased. There are no significant difference between MHS and normal SR in caffeine effect on the binding of CaM to Ca^{2+}-channel.

Figure 4.7. Caffeine dependence of the binding of Rh-CaM to HSR vesicles. The sample buffer contained 100 μg of MHS (●) or normal (○) heavy SR vesicles, 10 nM Rh-CaM, 0.3 M sucrose, 50 mM Hepes (pH 7.0), 100 μM $CaCl_2$, 1 mM $MgCl_2$ in a starting volume of 1 mL. Points were representatives of 3 preparations. % CaM bound was calculated from measured anisotropy as described in "Experimental Procedures"; For MHS and normal SR, 100% bound corresponds to 263 and 80 pmol/mg, respectively.


100
80
60
40
20
0
% Bound
0.1
1
10
100
[Caffeine], mM

4.4 Discussion

A mutation in the Ca^{2+}-channel protein is responsible for the abnormalities in calcium regulation observed in MHS porcine skeletal muscle, whereas other aspects of SR structure and function are normal (Carrier et al., 1991; Fill et al., 1990; Mickelson et al., 1988; Mickelson et al., 1989; Nelson, 1983; Ohnishi et al., 1983; Ohta et al., 1989). The altered Ca^{2+}-release properties of the MHS channel protein in the muscle cell could result, in part, from aberrant regulation of channel activity by other physiological modulators of Ca^{2+} release. The intracellular Ca^{2+}-binding protein, CaM, binds to the channel protein and acts not only as an inhibitor of Ca^{2+} release (at $[Ca^{2+}] > 0.1$ μM) (Meissner, 1986; Plank et al., 1988; Smith et al., 1989; Fuentes et al., 1994), but may also play a role in activating the channel protein during the resting state of muscle (Tripathy et al., 1995; Buratti et al., 1995; Ikemoto et al., 1995). To determine whether the MH mutation alters CaM-dependent regulation of the channel protein, we compared the stoichiometry and affinity of normal and MHS channel proteins for CaM in SR vesicles.

The affinity labeling studies (Fig. 4.1) reveal that the Ca^{2+} channel protein is the major CaM-binding protein in heavy SR, and further suggest that CaM binds to the channel in the presence or absence of Ca^{2+} concentrations which correspond to muscle contraction or to the resting condition, respectively. The crosslinking experiments provide valuable information on the identity of CaM-binding proteins in SR vesicles, but the nature of the technique makes it difficult to make interpretations of a quantitative nature. In particular, changes in structure induced by metal ions may subtly change the orientation of the crosslinker relative to the

receptor protein, thus enhancing or decreasing crosslinking efficiency. Weak complexes (eg. K_d~1-1000 μM) are not readily distinguished from strong complexes (K_d~1-10 nM) because transient complexes formed during the excited state lifetime of the benzophenone probe will be covalently joined as well as strong complexes.

Fluorescence anisotropy was therefore used to obtain quantitative data on CaM-binding to SR vesicles. The fact that the channel protein is the major CaM receptor (Fig. 4.1) in both normal and MHS SR vesicles suggests that increases in anisotropy are attributable primarily to CaM binding to the channel protein. Although the autoradiographic results of MHS and normal SR do not reveal a significant difference in the amount of CaM-channel protein complex formed for reasons mentioned above, fluorescence anisotropy studies demonstrated the altered CaM binding properties with Ca^{2+} channel in MHS skeletal muscle SR compared to normal SR. The reduction in the number of CaM binding sites in MHS SR from 21 CaM mole/tetramer in normal SR to 11 mole/tetramer (Table 4.1), suggests that up to half of the high affinity of CaM binding sites are lost as a result of the mutation in MHS Ca^{2+} channel. However, the CaM binding affinity of the remaining sites did not differ significantly in MHS SR compared to normal SR at low [Ca^{2+}]. Because of differences in stoichiometry, these results imply that regulation of the channel protein by CaM in MH is somehow altered.

Normal skeletal muscle SR binds 4-5 CaM per channel protein subunit with high affinity at < 0.1 μM [Ca^{2+}] (Yang et al., 1994; Tripathy et al., 1995). The significance of CaM binding at resting Ca^{2+} levels becomes apparent from the studies of Tripathy et al. (1995) and of Buratti et al. (1995) who showed that CaM activates the skeletal muscle SR Ca^{2+} channel

at low Ca^{2+} (<0.1 μM). The reduction in number of CaM binding sites in the presence of EGTA observed in the study presented here suggests that the CaM-dependent activation process is defective in MH. Preliminary reports by O'Driscoll et al. (1996) indicate that the CaM-dependence of activation of MH SR is enhanced compared to normal as determined by ryanodine binding. In the presence of CaM, ryanodine binding by MH SR was more than double that of normal porcine SR. Since ryanodine binding is an indicator of the activated state of the channel, this result suggests that the MH mutation results in hypersensitivity of the channel protein to CaM-activation at $[Ca^{2+}] < 100$ nM. The CaM binding sites lost in MH may somehow be associated with maintaining a more stable activated state of the channel at low $[Ca^{2+}]$. Alternatively, the loss of CaM sites may be an indirect consequence of the mutation. We are presently unable to discriminate between these possibilities.

When 0.1 mM $CaCl_2$ or $CaCl_2$ plus $MgCl_2$ were included in the titration, some of CaM binding sites in Ca^{2+}-channel protein are shifted from high-affinity class to low-affinity class for both MHS and normal SR. In the presence of 0.1 mM $[Ca^{2+}]$, the stoichiometry for high affinity CaM-binding sites is reduced from 5 CaM mole per tetramer in normal SR to 3 mole per tetramer in MHS SR and the low affinity CaM-binding stoichiometry for MHS SR is reduced from 16 mole to 4 mole per tetramer. However, the binding affinity for both MHS and normal SR at either high or low affinity classes are not significantly different. Our high affinity CaM binding results are in agreement with the data of Tripathy et al.(1995) in which an increase from subμM to mM $[Ca^{2+}]$ led to the dissociation of 12 of the 16 bound CaM from Ca^{2+} channel complex with an appreciably slower time course ($T_{1/2}$ = 1 min). Ryanodine binding and single-channel measurements indicate that CaM partially inhibits Ca^{2+}-channel

activity in the presence of 50 μM to 1 mM [Ca^{2+}] and vesicle-$^{45}Ca^{2+}$ efflux study shows a rapid (<2 s) CaM inhibition of $^{45}Ca^{2+}$ efflux from SR vesicles (Tripathy et al.,1995). Therefore, the decrease of CaM binding sites in MHS SR Ca^{2+}-channel at high [Ca^{2+}] apparently results from the mutation in the Ca^{2+}-channel polypeptide which causes the release of channel inhibition by CaM and led to abnormal higher myoplasmic [Ca^{2+}].

Our data are consistent with Tripathy et al. (1995) with respect of 1 high affinity site and 3-4 low affinity sites per subunit in normal muscle channel protein. They have reported that most of the inhibitory response of CaM at contractile [Ca^{2+}] results from the high affinity site. These results suggest that the inhibitory response of CaM at high Ca^{2+} would not be affected in MH. O'Driscoll et al. (1996) support this hypothesis with evidence showing that the CaM-dependence of ryanodine binding is not altered in MH.

KCl is known to alter functional properties of the channel protein as indicated by enhanced binding of ryanodine at higher ionic strength. The unusual effect of KCl on increasing the affinity of CaM for channel protein in normal heavy SR has been previously shown (Yang et al., 1994). Since the binding equilibria of CaM to channel protein were significantly different between MHS and normal SR, we wanted to determine whether there was a differential response of channel protein binding of CaM between MH and normal SR inducible by KCl. The fact that there was no significant difference in MH vs normal SR suggests that the structural changes induced in the channel protein by higher salt concentration does not differentially affect CaM binding.

It has been reported previously that the caffeine sensitivity of MHS skeletal muscle fiber bundles to induce muscle contracture was altered, this difference has served as the basis

of the diagnostic test for MH susceptibility (Britt, 1987). However, evidence from single-channel recording showed that there was no significant difference in the caffeine sensitivities of purified MHS and normal porcine SR Ca^{2+} release channels (Shomer et al., 1994). Therefore, the increased caffeine sensitivity of the MHS skeletal muscle contracture may be caused by other channel modulators which lead to the increase of myoplasmic [Ca^{2+}]. CaM has been recently reported to activate, at resting muscle [Ca^{2+}] condition and inhibit, at higher [Ca^{2+}] condition, skeletal muscle SR Ca^{2+} channel activity.

In conclusion, our results provide direct evidence that the binding affinity and stoichiometry of CaM to the mutant Ca^{2+}-channel protein is altered in [Ca^{2+}]$_i$-dependent manner in MHS SR comparing to normal SR. The increase of intracellular [Ca^{2+}] in MHS pig skeletal muscle results, in part, from the altered CaM binding and regulation in SR Ca^{2+}-release channel.

CHAPTER 5

LOCALIZATION OF CALMODULIN BINDING DOMAINS IN THE CALCIUM RELEASE CHANNEL (RYANODINE RECEPTOR) OF SKELETAL MUSCLE SARCOPLASMIC RETICULUM

5.1 Introduction

The release of Ca^{2+} from the lumen of the sarcoplasmic reticulum (SR) into the muscle myoplasm occurs via a Ca^{2+}-release channel, also known as the ryanodine receptor (RyR), in response to transverse tubular (T-tubular) depolarization (Rios et al., 1991). This RyR/Ca^{2+}-channel corresponds morphologically to the 'foot' structures which span the gap at the triad junction between the terminal cisternae of the sarcoplasmic reticulum and the T-tubule (Inui et al., 1987a; Inui et al., 1987b; Franzini-Armstrong and Nunzi, 1983). The skeletal muscle Ca^{2+}-channel protein is a homotetrameric protein composed of four subunits of 5032 (human), or 5037 (rabbit) amino acids, each with an estimated molecular weight of 565 kDa (Takeshima et al., 1989; Zorzato et al., 1990).

The SR Ca^{2+}-channel activity is modulated by numerous compounds. The activators of SR Ca^{2+} release include Ca^{2+}, adenine nucleotides, caffeine, halothane and nM ryanodine,

whereas inhibitors include Mg^{2+}, mM Ca^{2+}, μM ryanodine, ruthenium red, and calmodulin (Meissner, 1986). Recent studies suggest that the role of CaM in regulation of the channel protein is more complex than previously thought. Four to five CaMs bind with high affinity per channel subunit at low Ca^{2+} concentration (<0.1 μM) and there is only one high affinity CaM-binding site per channel subunit at $[Ca^{2+}] > 10$ μM (Yang et al., 1994; Tripathy et al., 1995). The combination of $^{45}Ca^{2+}$ efflux measurements from SR vesicles, single-channel recording data, and [^{3}H]ryanodine binding measurements show that, at <0.2 μM Ca^{2+}, CaM activates the Ca^{2+} release channel several fold (Tripathy et al., 1995; Buratti et al., 1995; Ikemoto et al., 1995). However, at μM to mM Ca^{2+} concentrations, CaM inhibits the skeletal muscle Ca^{2+}-release channel 2-3 fold by inhibiting the open state probability of the channel (Meissner, 1986).

From molecular cloning and sequencing of the cDNA of the ryanodine receptor, a crude structural model of the ryanodine receptor has emerged. Hydropathy analysis suggests the receptor molecule has a short cytoplasmic C-terminus and either four (Takeshima et al., 1989) or ten (Zorzato et al., 1990) transmembrane domains in the C-terminal one-fifth of the molecule. The bulk of the molecular mass, the N-terminal portion (80%) of this protein, is predicted to comprise the cytoplasmic "foot" region. Three-dimensional reconstructions have been made from electron micrographs of negatively stained and frozen-hydrated, solubilized Ca^{2+}-channel. These reconstructions support the predicted structural model of ryanodine receptor, revealing a large cytoplasmic portion (29 x 29 X 12 nm) consisting of many structural domains, and a smaller transmembrane assembly projecting off of the luminal side of the SR and embedded in the bilayer membrane (Wagenknecht et al., 1989; Radermacher

et al., 1992; Radermacher et al., 1994; Serysheva et al., 1995).

Several experimental and theoretical approaches have been employed to identify the modulator binding sites in order to characterize the relationship of structure to function within the channel protein. Using predictive algorithms to identify basic amphiphilic helices, several putative CaM binding sites in the ryanodine receptor have been predicted through the analysis of the deduced primary sequence (Takeshima et al., 1989; Zorzato et al., 1990). Takeshima et al. (1989) predicted 2 CaM-binding sites at residues 3614-3637 and 4295-4325, whereas Zorzato et al. (1990) predicted 3 different sites at residues 2807-2840, 2909-2930 and 3031-3049. Experimental studies from various laboratories have also provided evidence for multiple binding sites on each subunit. Marks et al. (1990) have suggested that there are four CaM binding sites located at residues 2641-2657, 3362-3374, 3947-3965 and 4309-4322, based on experiments utilizing limited proteolysis of the channel protein coupled with surface topography analysis. Brandt et al. (1992) have suggested three other candidate sites for CaM at residues 1383-1400, 1974-1996 and 3358-3374 by analysis of the calpain digestion pattern of the channel protein in the presence and absence of CaM.

Other experimental approaches to identify and characterize CaM binding sites involve expression of RyR cDNA fragments as fusion proteins followed by SDS-PAGE and CaM overlay procedures. Using this approach, Menegazzi et al. (1994) have defined three CaM binding regions, residues 2937-3225, 3546-3655 and 4425-4621 in rabbit skeletal muscle RyR. Using the same approach, Chen et al. (1994) have localized three strong CaM-binding sites in the channel protein between amino acid residues 2063-2091, 3611-3642, and 4303-4328, and three weaker CaM-binding sites between amino acid residues 921-1173, 2804-2930

and 2961-3084 (Radermacher et al., 1994; Serysheva et al., 1995). Wagenknecht et al. (1994) have used gold-cluster-labeled CaM and electron microscopy to localize one CaM-binding site per subunit on the purified protein. Their results suggest that this CaM-binding site is at least 10 nm from the transmembrane channel of the receptor complex.

The susceptibility of the Ca^{2+} release channel to proteolysis has been used to study the relationship of primary structure and function. Mild digestion of heavy SR membranes with trypsin results in the rapid disappearance of the 564 kDa Ca^{2+} release channel protein subunit in SDS-PAGE. However, significant alterations in the sedimentation coefficient, ultrastructure, high affinity [^{3}H]ryanodine binding, and channel gating are observed only after extensive proteolytic digestion (Chu et al., 1988; Shoshan-Barmatz and Zarka, 1988; Meissner et al., 1989; Rardon et al., 1990). These results suggest that limited proteolysis of the channel protein does not substantially alter structure or function; i.e., only after extensive proteolysis does the channel protein lose its structural and functional integrity. Calcium-activated neutral proteases (CANP), also known as calpains (calcium-dependent papain-like), are a group of cysteine endopeptidases with neutral pH optima and are absolutely dependent on Ca^{2+} for catalytic activity. They have been frequently used to generate proteolytic fragments for structure-function studies (Murachi, 1983; Pontremoli and Melloni, 1986; Suzuki, 1987), and it has been shown that the Ca^{2+} release channel was the primary substrate of calpain in heavy SR membranes (Rardon et al., 1990).

Major tryptic fragments and calpain proteolysis products have been identified and positioned in the SR Ca^{2+}-release channel protein sequence (Chen et al., 1993; Brandt et al., 1992; Marks et al., 1990). Using immunoblotting analysis with site-specific antibodies, Chen

et al. (1993) identified 7 tryptic cleavage sites in Ca^{2+}-channel protein, 3 of which were not in the regions previously identified by Marks et al. (1992). Callaway et al. (1994) have isolated and partially sequenced the polypeptides arising from limited trypsin digestion of the Ca^{2+}- channel complex which results in a 28 S complex.

In this study, we have combined the approach of using limited trypsin and calpain digestion of the native Ca^{2+}-release channel in the rabbit skeletal muscle SR membranes, coupled with crosslinking of radiolabeled CaM to the channel protein to position CaM-binding sites in the native protein structure. Two CaM binding sites in the central regions of Ca^{2+}- release channel protein have been tentatively identified by immunoblotting analysis with site-specific antibodies.

5.2 Experimental Procedures

5.2.1 Materials. Benzophenone-4-maleimide was purchased from Molecular Probes (Junction City, OR). $Na^{125}I$ was obtained from DuPont-NEN (Boston, MA). Wheat germ was a generous gift from International Multifoods (Minneapolis, MN). CHAPS, CAPS, and MOPS were obtained from Sigma.

5.2.2 Preparation of rabbit skeletal heavy SR vesicles. SR membranes were prepared from rabbit back and hind leg white skeletal muscle by differential centrifugation and were further purified using sucrose gradient centrifugation (Hamilton and Tate, 1991; Rosemblatt, et al., 1981).

5.2.3 Preparation of CaM and its derivatives. CaM was purified from wheat germ using the procedure described previously (Strasburg et al., 1988). Purified CaM was iodinated with ^{125}I at the sole tyrosine residue (Tyr-139), followed by site-specific modification at Cys-27 with the photoactivatable cross-linker benzophenone-4-maleimide (Strasburg et al., 1988).

5.2.4 Limited tryptic digestion of heavy SR vesicles. Heavy SR was trypsinized at 37 °C for 5 min in 0.3 M KCl, 0.1 mM $CaCl_2$, and 50 mM MOPS, pH 7.0. The optimal ratio for trypsin:heavy SR protein varied from prep to prep, but was usually 1:1000 to 1:500 (w/w) conditions. The reaction was quenched with a 20-fold weight excess of soybean trypsin inhibitor protein.

5.2.5 [^{125}I]-Bz-CaM cross-linking with SR vesicles. Affinity labeling of tryptic channel peptides were performed by incubating in darkness 1.0 μM [^{125}I]-Bz-CaM with 10 mg trypsin or non-trypsin treated SR vesicles in 1 mL of 20 mM Hepes pH 7.0, and 0.3 M KCl and 1 mM EGTA for 30 min. The mixtures were illuminated for 5 minutes in a Stratalinker 1800 photoreactor equipped with lamps of λ_{max} = 254 nm. After photolysis of the mixtures, a portion of the samples were subjected to SDS-polyacrylamide electrophoresis (Schagger and Jagow, 1987).

5.2.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli (1970) which has been previously described (Yang et al., 1993); and the method of Schagger and Von Jagow (1987), using 1.5 mm thick, 10% polyacrylamide separating gel (10% acrylamide, 3% bis-acrylamide), 1 M Tris-HCl, pH 8.45, 0.1% SDS, 0.13% glycerol (w/v), 10% ammonia persulfate, 10 μl TEMED and a stacking gel (4% acrylamide, 3% bis-acrylamide), 1 M Tris-HCl, pH 6.8, 0.1% SDS, 10% ammonia persulfate, 10 μl TEMED. Samples were denatured for 5 min at 95 °C in 50 mM Tris, pH 6.8, containing 4% SDS, 0.01% Serva Blue G, and 12 % glycerol. All samples were reduced with 20 mM dithiothreitol (DTT) prior to electrophoresis. The lower chamber running buffer contained 0.2 M Tris-HCl buffer, pH 8.9. The upper chamber running buffer contained 0.1 M Tris-HCl buffer, 0.1 M Tricine, 0.1% SDS, pH 8.25. Electrophoresis was conducted at 4 °C and constant voltage (80 volts). Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol, 10% acetic acid and destained with 40% methanol, 10% acetic acid. The dried gels were placed with Kodak Omat XAR-5 X-ray film

in autoradiography cassettes equipped with Dupont Lightning Plus intensifying screens. The other portion of the samples were subjected to sucrose gradient purification.

5.2.7 Calpain digestion of heavy SR vesicles crosslinked with [^{125}I]-Bz-CaM. Calpain digestion of [^{125}I]-Bz-CaM labeled SR vesicles was performed in a digestion buffer containing 0.1 mM $CaCl_2$, 50 mM NaCl, 20 mM MOPS, and 2 mM DTT (pH 7.4) at 36 °C for 1.5 hr. The calpain/SR weight ratio was 1/50 (w/w). Digestion was terminated by the addition of leupeptin to a final concentration of 10 μM. The digested mixtures were subjected to sucrose gradient centrifugation for further purification of [^{125}I]-Bz-CaM bound Ca^{2+}-channel peptides.

5.2.8 Sucrose gradient purification of [^{125}I]-Bz-CaM bound Ca^{2+}-channel proteolytic polypeptides. The proteolytic Ca^{2+}-channel/[^{125}I]-Bz-CaM complex was isolated from SR membranes by sucrose gradient centrifugation. The samples were solubilized in 2% CHAPS, followed by density gradient centrifugation through continuous 5 - 20% sucrose for 18 hr at 110,000 x g in a Beckman SW 28 rotor as described by Callaway et al. (1994). The fractions from the sucrose gradients containing the peak of [^{125}I] or [^{3}H] radiative signal were pooled and concentrated. The concentrated fractions were prepared for blotting and then subjected to N-terminal sequencing according to the method of Callaway et al. (1994).

5.2.9 Preparation of samples for amino acid sequencing and Western blots. SDS-polyacrylamide gels were cast 24 h in advance. Sodium thioglycolate (0.1 mM) was added to the upper cathode running buffer to scavenge free radicals. After electrophoresis, the

separated bands of protein were transferred to Immobilon P-SQ membranes (Millipore Corp., Bedford, MA) for sequencing or Immobilon P membranes for immunoblot at 20 Volts in 5% methanol, 10 mM CAPS (pH 11.0) transfer buffer at 4 °C overnight. The bands were stained with Coomassie Brilliant Blue and cut out for sequencing according to the method of LeGendre and Matsudaira (1989).

5.2.10 N-terminal sequencing. Sequencing was performed by Dr. Richard Cook at Baylor College of Medicine.

5.2.11 Western blots. The transfer membranes were blocked with 5% Blotto (Bio-Rad), PBS-0.1% Tween 20 for 1 h at 37 °C or 16 h at 4 °C. Membranes were then incubated for 90 min at room temperature with the primary antibody diluted in 3% BSA, PBS-0.1% Tween 20. After three washes with PBS-0.1% Tween 20, membranes were incubated with alkaline phosphatase-conjugated goat antirabbit IgG antiserum (Cappel, Durham, NC), washed five times with PBS-0.1% Tween 20, and developed with alkaline phosphatase substrate.

5.2.12 Protein Assay. SR protein determination was carried out by the methods of Lowry et al. (1951) using bovine serum albumin as a standard.

5.3 Results

5.3.1 Affinity labeling and purification of trypsin-treated Ca^{2+}-release channel from skeletal muscle heavy SR membranes. Previous reports have indicated that there are four to five CaM binding sites per Ca^{2+}-release channel subunit in skeletal muscle SR (Yang et al., 1994; Tripathy et al., 1995). This study was conducted to localize the CaM binding domains in the channel protein. In initial studies, heavy SR membranes isolated from rabbit skeletal muscle were partially proteolyzed for 5 min with trypsin at a protein:trypsin ratio of 1000:1 (w/w) and affinity-labeled with [^{125}I]-Bz-CaM in the presence of 1 mM EGTA. The autoradiogram of SDS-PAGE indicated a significant decrease in the native Ca^{2+}-channel protein band with the appearance of five [^{125}I]-Bz-CaM binding bands with apparent molecular weights of 143 kDa, 117 kDa, 97.5 kDa, 73.2 kDa, 51.7 kDa (Fig. 5.1).

CHAPS solubilized tryptic Ca^{2+}-channel complexes labeled with [^{125}I]-Bz-CaM were isolated and purified from 5-20% sucrose gradient centrifugation. This trypsin treatment resulted in a small shift of the apparent sedimentation coefficient of the CHAPS solubilized Ca^{2+}-channel from 30 S to 28 S (Callaway et al., 1994). The fractions containing the channel protein with bound [^{125}I]-Bz-CaM were identified by gamma-counting (Fig. 5.2).

5.3.2 Identification of [^{125}I]-Bz-CaM bound tryptic Ca^{2+}-channel polypeptides from the purified 28 S complex. Two major CaM-binding polypeptides of the purified 28 S complex labeled with [^{125}I]-Bz-CaM have been identified from autoradiograms of SDS-PAGE (Fig. 5.3). The apparent molecular weights of the crosslinked products obtained in the presence of

Figure 5.1. Autoradiography of [^{125}I]-Bz-CaM bound trypsin-treated heavy SR membranes from rabbit skeletal muscle. For 100 μL reaction, 0.1 μM [^{125}I]-Bz-CaM was incubated with 100 μg trypsin-treated heavy SR in 0.3 M KCl, 1 mM EGTA, and 50 mM Hepes buffer, pH 7.0. The mixtures were separated by electrophoresis on a 5-18% gradient SDS-PAGE (Laemmli, 1970) and the cross-linked products were identified by autoradiography of the gel. Lane 1 and 2 represent trypsin-treated and control (no trypsin-treated) SR, respectively.

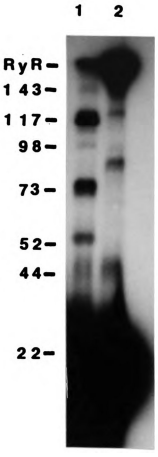

1
2
RyR
143
117
98
73
52
44
22

Figure 5.2. Sucrose gradient profile of 28 S complex of the CHAPS purified [^{125}I]-Bz-CaM labeled Ca^{2+}-release channel. The 28 S complex purification process was described in Experimental Procedures on a 16 ml linear 5-20% sucrose gradient containing 0.2% CHAPS, 300 mM KCl, 50 mM MOPS (pH 7.0). The gradient was sedimented for 18 h at 110,000 x g in a SW 28 rotor, and 20 drop fractions were collected from the bottom of the gradient. Aliquots (50µl) from each fraction were counted by a gamma-counter.

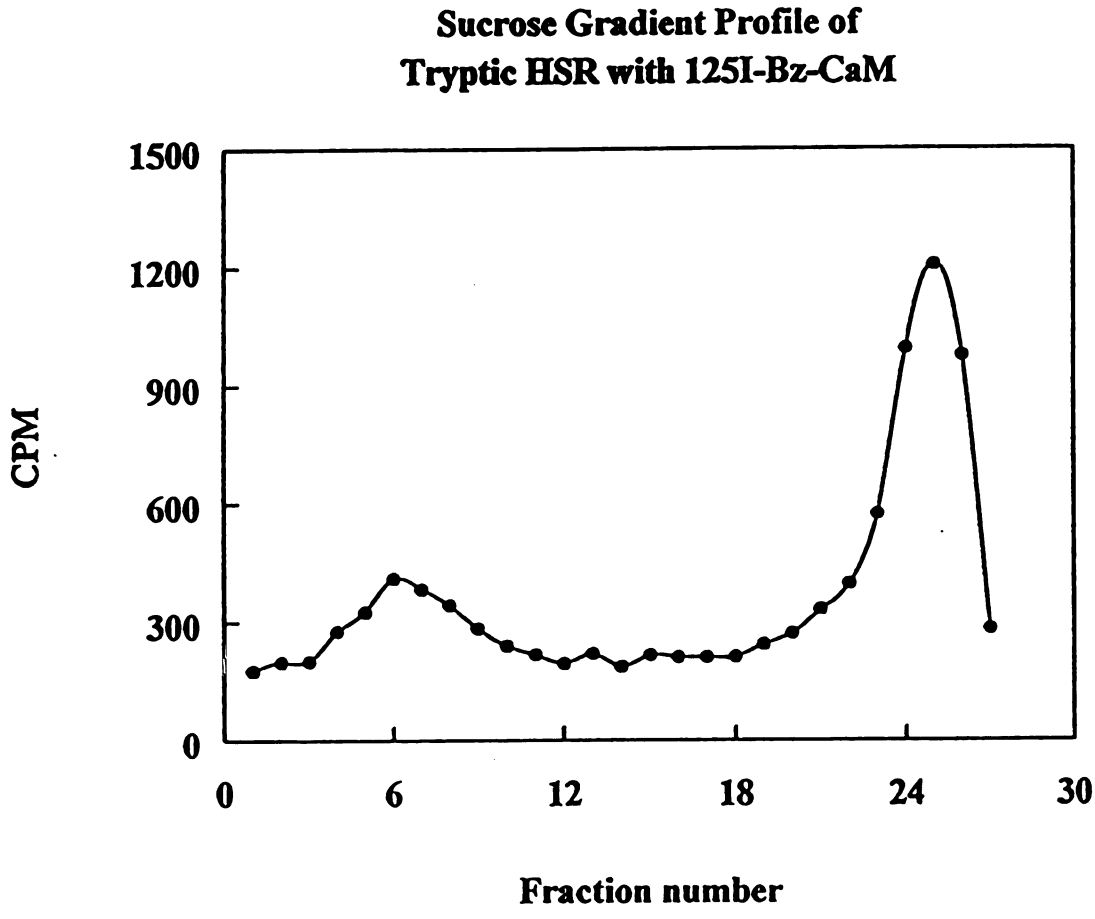
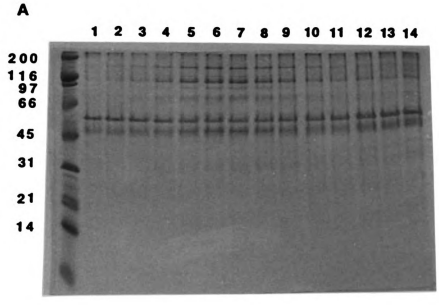

Sucrose Gradient Profile of
Tryptic HSR with 125I-Bz-CaM
CPM
1500
1200
900
600
300
0
0
6
12
18
24
30
Fraction number

Figure 5.3. Identification of the 28 S complex of the [^{125}I]-Bz-CaM bound Ca^{2+} -release channel. Aliquots (40 μL) of collected fractions from sucrose gradient were electrophoresed on a 7% SDS-PAGE (Schagger and Von Jagow, 1987) described in Experimental Procedures. Panel A and B, Coomassie-stained of gel. Panel C and D, autoradiography of dried gels from panel A and B, respectively. Lane 1-14 in panel A were from sucrose gradient fractions 1-14. Lane 15-26 in panel B were from fractions 15-26. Lane 27 was from the resuspension of SR precipitate in sucrose gradient.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
200
116
97
66
45
31
21
14

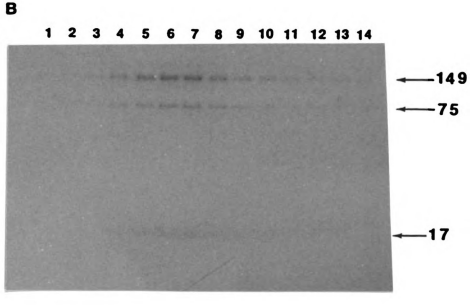
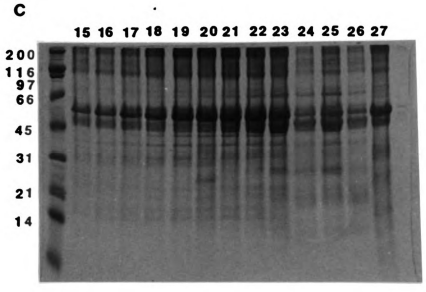
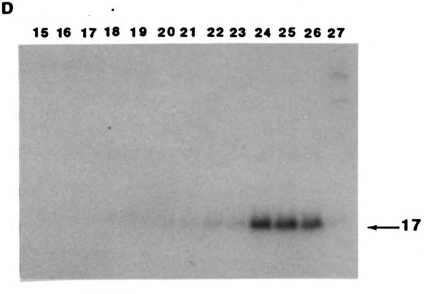

B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
149
75
17

Figure 5.3--cont'


C
15 16 17 18 19 20 21 22 23 24 25 26 27
200
116
97
66
45
31
21
14


D
15 16 17 18 19 20 21 22 23 24 25 26 27
17

1 mM EGTA were 149 kDa and 75 kDa. If one CaM binds per fragment, these complexes would correspond to CaM plus channel protein tryptic fragments of approximately 133 kDa, and 58 kDa. Based on these apparent molecular weights, two tryptic fragment candidates are suggested: the 135 kDa N-terminal fragment (residues 1-1508) and the 50 kDa fragment (residues 2401-2840) (Fig 5.4).

Fractions 5-9 containing the channel protein complex bound with [^{125}I]-Bz-CaM were pooled and subjected to SDS-PAGE (Fig. 5.3). The separated bands of protein from SDS-PAGE were transferred to Immobilon membrane, stained with Coomassie blue and the blot was subjected to autoradiography. Two [^{125}I]-Bz-CaM binding bands appeared on the autoradiogram of the membrane blot corresponding to the bands present in the autoradiogram of the gel (Fig. 5.5). Sequencing attempts on the 149 kDa [^{125}I]-Bz-CaM binding peptide excised from the Immobilon membrane failed because the N-terminus of this peptide was blocked. This result supports the possibility that this complex corresponds to the N-terminal 135 kDa fragment plus CaM because sequencing results of Callaway et al. (1994) indicated that the 135 kDa fragment isolated from the tryptic Ca^{2+}-channel 28 S complex was blocked at the NH_2 terminus (Fig 5.4). Therefore, these results suggest that one CaM-binding peptide is localized in the region between the N-terminus and residue 1508 in the amino acid sequence of Ca^{2+}-channel.

The second complex obtained from the 28 S purification had an apparent M_r of 75 kDa. Because of the proximity of this complex to the 76 kDa fragment, separation of these bands was impossible with present techniques. Because the amount of 75 kDa fragment was much less than the 76 kDa fragment in amino acid sequencing data, it is not possible to

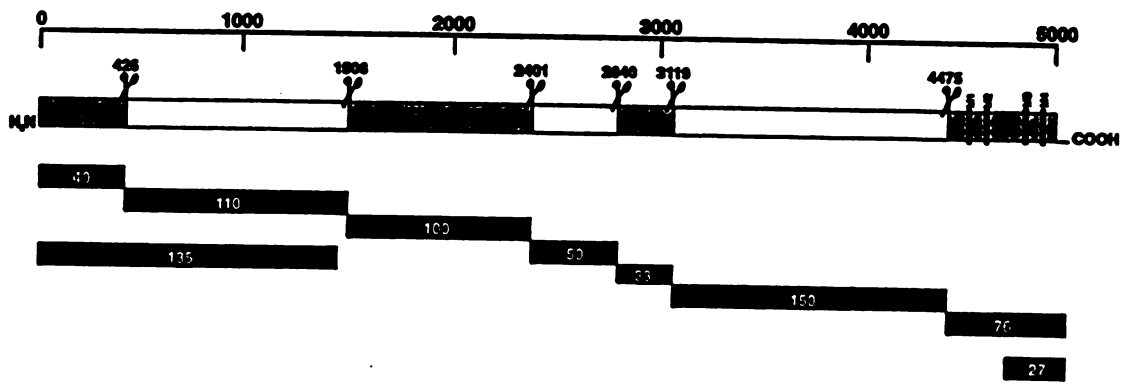
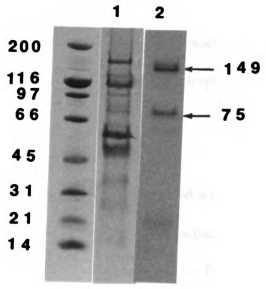


Figure 5.4. Fragmentation map of 28 S complex of tryptic SR Ca^{2+} release channel from rabbit skeletal muscle (From Callaway et al., 1994).

Figure 5.5. [^{125}I]-Bz-CaM binding tryptic fragments within the Ca^{2+} -release channel were recognized and sequenced. Fractions 5-9 from previous data were pooled and concentrated by Centricon concentrators (Amicon, Inc., Beverly, MA) after dialyzing over night in 50 mM MOPS, pH 7.0. The concentrated solution was subjected to 7% SDS-PAGE Schagger and Von Jagow, 1987) and transferred to Immobilon-P-SQ membrane and prepared for sequencing as described in Experimental Procedures. Lane 1 is Coomassie stain of transferred membrane; lane 2 is autoradiogram of transferred membrane.


1
2
200
116
97
66
45
31
21
14
149
75

confirm the identity of this band from amino acid sequencing. However, based on the molecular weight from the SDS gels and the likelihood that this tryptic Ca^{2+}-channel fragment binds 1 CaM, the site of CaM binding likely corresponds to the 50 kDa fragment which is positioned between amino acid 2401-2840 based on 28 S Ca^{2+}-channel fragmentation map (Fig. 5.4). Therefore, two tentative CaM-binding sites, amino acid residues 1-1508 and 2401-2840, in the Ca^{2+}-release channel have been positioned from tryptic digests and photoaffinity labeling.

5.3.3 Purification of calpain digested Ca^{2+}-channel labeled with [^{125}I]-Bz-CaM. In order to complement the tryptic digestion results, we performed calpain digestion following [^{125}I]-Bz-CaM cross-linking with SR membranes. This work was performed in collaboration with Dr. Hamilton and Dr. Yili-Wu at Baylor College of Medicine. Following affinity labeling with [^{125}I]-Bz-CaM, the Ca^{2+}-channel/[^{125}I]-Bz-CaM complex was labeled with [^{3}H]ryanodine and subjected to calpain digestion and the proteolytic fragments were purified on 5-20% sucrose gradients. The sucrose gradient profile of purified proteolytic products is shown in Figure 5.6. Aliquots from fractions 6-10, 23 and 24 were subjected to 5% SDS-PAGE. The Coomassie stained gel of the calpain digested channel protein and its autoradiogram are shown in Figure 5.7. Autoradiography of calpain-digested Ca^{2+}-channel bound with [^{125}I]-Bz-CaM demonstrated that there were four polypeptides which bound [^{125}I]-Bz-CaM. The apparent molecular weights of these complexes corresponded to 480 kDa, 365 kDa, 210 kDa, and 130 kDa (Fig. 5.8B). It has been reported that, based on the results of affinity labeling and immunoblots, calpain-digested Ca^{2+}-channel was initially degraded into an N-terminal 173

Figure 5.6. Sucrose gradient profile of calpain digested Ca^{2+}-release channel labeled with [^{125}I]-Bz-CaM (From Yili Wu at Baylor College of Medicine). The purification process of calpain digested Ca^{2+}-channel/[^{125}I]-Bz-CaM was described in Experimental Procedures. Gradients were sedimented for 18 h at 110,000 x g in a SW 28 rotor, and 20 drop fractions were collected from the bottom of the gradient. Aliquots (50μl) were counted for radioactivity by scintillation counter.

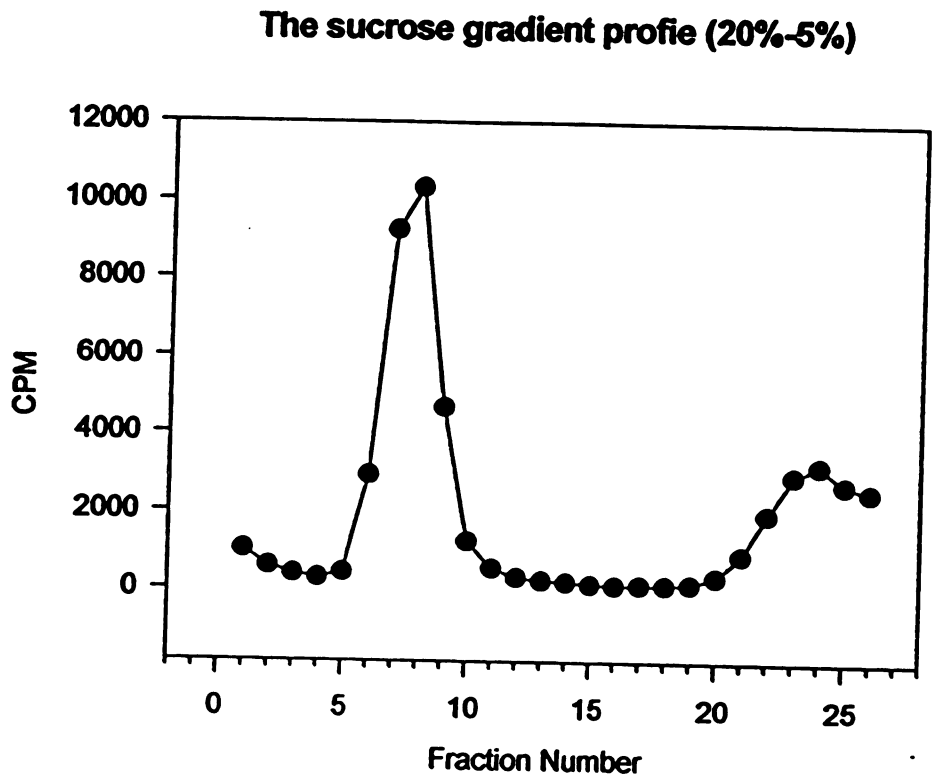
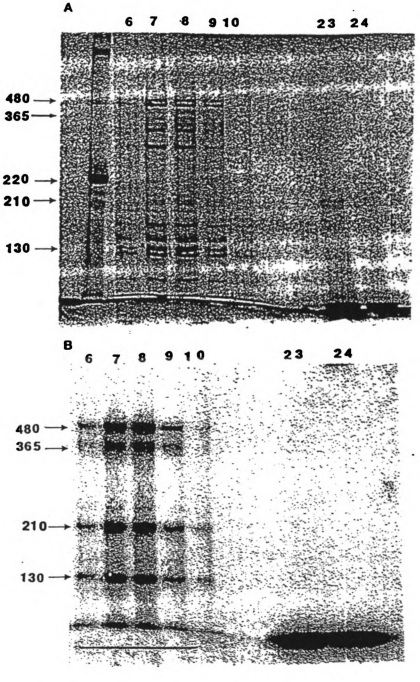
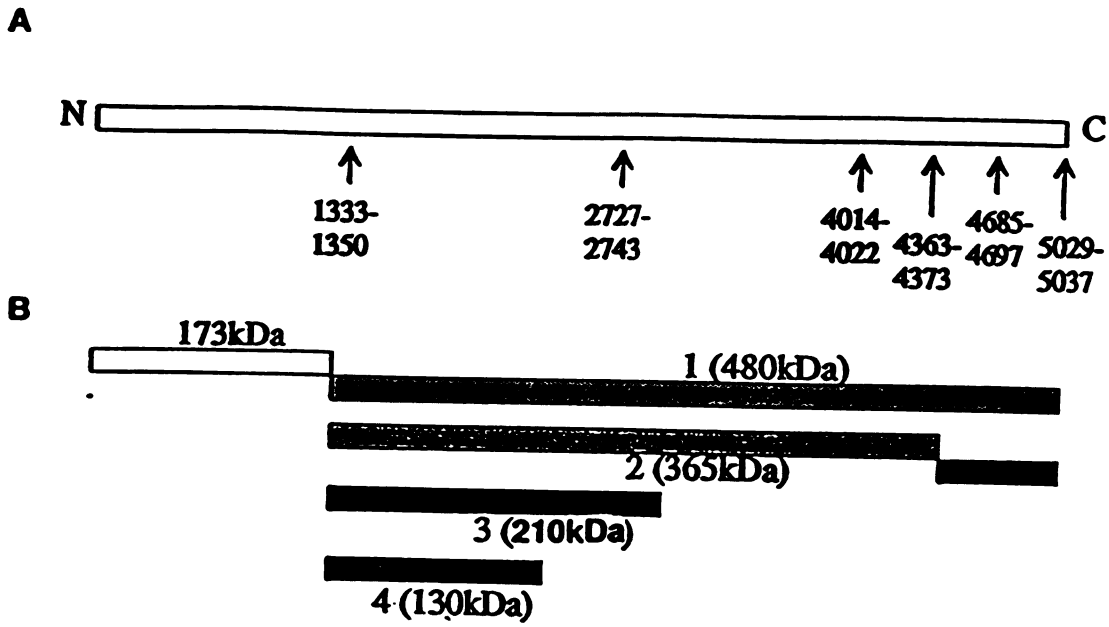

The sucrose gradient profie (20%-5%)
12000
10000
8000
6000
4000
2000
0
CPM
0
5
10
15
20
25
Fraction Number

Figure 5.7. SDS-PAGE and autoradiography of sucrose gradient purified calpain digested Ca^{2+}-release channel labeled with [^{125}I]-Bz-CaM (From Dr. Yili Wu at Baylor College of Medicine). Panel A, Coomassie stain of 5% SDS-PAGE from collected fractions 6-10, 23 and 24 in Figure 5.6. Panel B, autoradiography of dried gel from panel A.



(From Dr. Yili Wu at Baylor college of Medicine).

Figure 5.8. Calpain proteolytic fragments of Ca^{2+}-release channel identified by immunoblots (From Dr. Yili Wu at Baylor College of Medicine). Panel A is the primary structure of Ca^{2+}-release channel. Site-specific antibodies against to various regions in Ca^{2+}-channel are shown by the arrows. Panel B is summary of calpain proteolytic Ca^{2+}-channel fragments bound to [^{125}I]-Bz-CaM.


A
N
C
1333-
1350
2727-
2743
4014-
4022
4363-
4373
4685-
4697
5029-
5037
B
173kDa
1 (480kDa)
2 (365kDa)
3 (210kDa)
4 (130kDa)

kDa fragment and a C-terminal 480 kDa fragment which was further cleaved to a 365 kDa fragment at the N-terminus and a smaller fragment at the C-terminus with M_r about 100 kDa (as shown in Fig. 5.8 B) (Brandt et al., 1992; Gilchrist et al., 1992; Rardon et al., 1990). Our results showed that there were no [^{125}I]-Bz-CaM crosslinked products corresponding to the N-terminal 173 kDa fragment nor the C-terminal 100 kDa fragment. These results suggest that the CaM-binding sites are located between the C-terminal end of 173 kDa fragment and the N-terminal end of 100 kDa fragment (see Fig. 5.8 B).

5.3.4 Identification of [^{125}I]-Bz-CaM bound calpain digested Ca^{2+}-channel fragments by immunoblots. Site-specific antibodies against specific regions (as shown in Fig. 5.8 A) of the Ca^{2+}-channel were used as probes for immunoblotting to establish the identify of the calpain digested fragments and crosslinked products. The immunoblots of [^{125}I]-Bz-CaM/Ca^{2+}-channel digested with calpain (data not shown) indicated that antibody (1333-1350) recognized both the 210 kDa and 130 kDa bands and both bands were labeled with [^{125}I]-Bz-CaM (data not shown). However, antibody (2727-2743) only recognized the 210 kDa fragment and not the 130 kDa fragment. These results imply that both the 210 kDa and 130 kDa fragments begin at the C-terminal end of 173 kDa fragment which means the 210 kDa fragment is the degraded product of the 365 kDa fragment and the 210 kDa fragment which has been further cleaved to the 130 kDa fragment. Since the autoradiogram did not show the remaining degraded product (M_r = 80 kDa) from the 210 kDa fragment labeled with [^{125}I]-Bz-CaM, the CaM binding sites are thus suggested to localize in the 130 kDa fragment which comprises amino acid residues 1333 to 2515 (as shown in Figure 5.8 B).

The calpain-digested 130 kDa fragment is localized between residues 1333 and 2515 which comprises two [^{125}I]-Bz-CaM bound tryptic fragments, residues 1-1508 and residues 2400-2840 from Figure 5.5. Therefore, in combining results of the trypsin and calpain digests, CaM binding sites have been localized more precisely to amino acids 1333-1508 and 2400-2515 in the skeletal muscle SR Ca^{2+}-channel (Fig. 5.9).

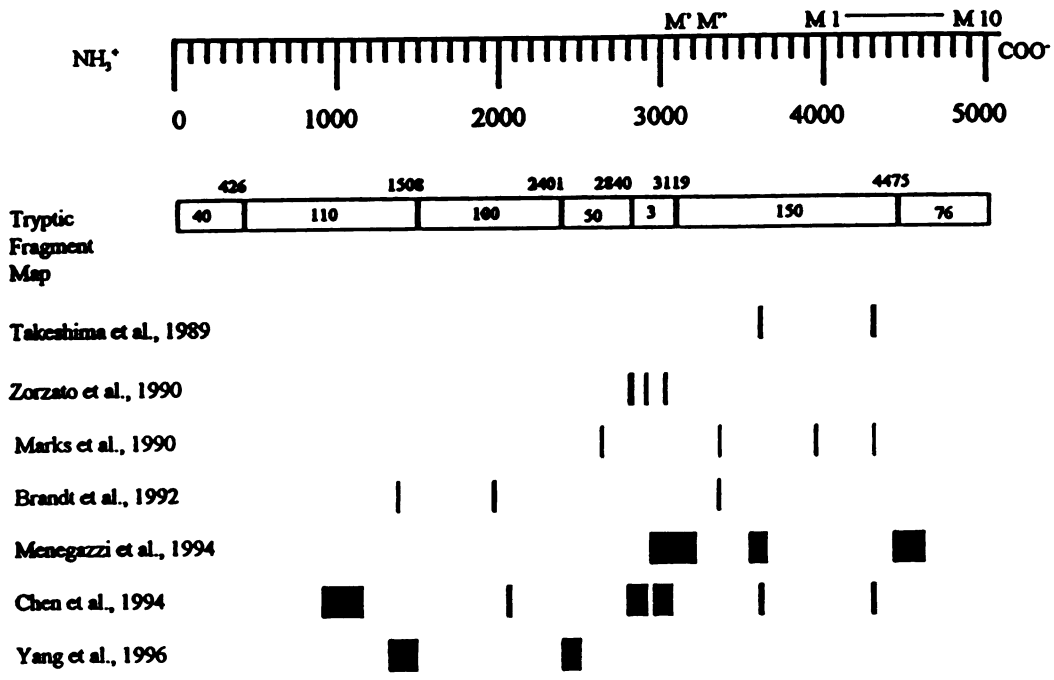


Figure 5.9 CaM-binding sites in skeletal muscle SR Ca^{2+}-release channel. The linear sequence of the Ca^{2+}-channel is indicated by a horizontal line. The NH^{3+} and COO^- termini are marked. M', M", and M1-M10 refer to predicted transmembrane sequences (Zorzato et al., 1990). The tryptic fragments are represented by open boxes with molecular masses (x1000) inside the boxes. The candidates of CaM-binding sites are positioned by verdical lines.

5.4 Discussion

Limited proteolysis has previously provided insights into structure-function relationship within the channel protein (Chu et al., 1988; Shoshan-Barmatz and Zarka, 1988; Meissner et al., 1989; Rardon et al., 1990; Chen et al., 1993; Callaway et al., 1994). Seven major tryptic sensitive regions within Ca^{2+}-release channel have been identified and positioned by site-specific antibodies (Chen et al., 1992) and amino acid sequencing (Marks et al., 1990; Callaway et al., 1994) as shown in Figure 5.4.

Calpain also catalyzes specific and limited cleavage of substrate including enzymes, myofibrillar proteins, membrane proteins, cytoskeletal proteins, and receptor proteins (Dayton et al., 1976; Mellgran, 1987; Puca et al., 1977; Vedeckis et al., 1980). It has been suggested that CaM-binding proteins are good substrates for calpain which recognizes the PEST (proline, glutamic acid, serine, threonine-rich) sequence for binding sites in substrate proteins (Wang et al., 1989). The Ca^{2+}-release channel protein has eight PEST sequences (Brandt et al., 1992). One of the calpain cleavage sites in Ca^{2+}-release channel has been suggested at residues 1383-1400 which was predicted to be near a CaM-binding site (Brandt et al., 1992; Shoshan-Barmatz et al., 1994). In this work, we used combined photoaffinity labeling and immunoblotting approaches with partial trypsin and calpain proteolysis of Ca^{2+} release channel in the SR membrane to localize and identify CaM binding sites in the Ca^{2+} channel.

Wheat germ CaM was iodinated at Cys-27 with the UV sensitive crosslinker benzophenone-4-maleimide which can react with any methylene groups of amino acid residues in the proximity of the label. Thus, polypeptide residues which are within 1.0 nm of the

labeled Cys-27 are readily cross-linked. Therefore, it is likely that crosslinked polypeptides either constitute the CaM-binding sites, or are in very close proximity to CaM.

When crosslinking in the presence of 1 mM EGTA, at least 5 tryptic fragments from SR membrane bound [^{125}I]-Bz-CaM (Fig 5.1). These results are consistent with previous Rh-CaM/Ca^{2+}-channel binding stoichiometry data which suggest there are 4-5 CaM binding sites per channel subunit (Yang et al., 1994; Tripathy et al. 1995). Two of these CaM-binding fragments in the tryptic Ca^{2+}-channel have been further purified from SR membrane by sucrose gradient centrifugation and identified by autoradiography of SDS-PAGE. These two purified fragments had apparent molecular weights of 149 kDa and 117 kDa which corresponded to the 117 kDa and 73 kDa fragments, respectively in Figure 5.1. The difference of the apparent molecular weights between these two results were due to different SDS-PAGE systems which caused the difference of polypeptides mobility shift in the gels.

There were three [^{125}I]-Bz-CaM labeled fragments missing from the sucrose gradient purified 28 S complex (Fig. 5.3) when compared to the results before purification (Fig 5.1). The loss of [^{125}I]-Bz-CaM bound 51.7 kDa fragment in the autoradiogram of sucrose gradient purified 28 S complex fractions might result from dissociation of this fragment from 28 S complex during purification process and diffusion into gradient solution. The other two missing bands might be due to insufficient radioactive intensity of the [^{125}I]-Bz-CaM in the autoradiogram. After subtracting CaM's molecular weight, the 149 kDa and 117 kDa fragments labeled with [^{125}I]-Bz-CaM are suggested to correspond to the 135 kDa and 50 kDa fragments in the Ca^{2+}-channel fragmentation map (Fig. 5.4) which are localized between residues 1-1508 and 2400-2840.

To complement the trypsin digestion results, we further used a more selective protease, calpain, that degrades primarily the Ca^{2+}-channel protein in the heavy SR. Photoaffinity labeling with [^{125}I]-Bz-CaM and immunoblotting with site-specific antibodies were used to identify calpain digested Ca^{2+}-channel fragments bound to CaM. The autoradiogram demonstrated that there were 4 calpain-digested Ca^{2+}-channel fragments labeled with [^{125}I]-Bz-CaM after sucrose gradient purification.

It has been reported that calpain digested the Ca^{2+}-channel in an ordered sequence of susceptible sites; that is, the first site must be cleaved before the second site becomes exposed (Brandt et al., 1992; Gilchrist et al., 1992; Shoshan-Barmatz et al., 1994). Our results were in agreement with these reports. Calpain initially cleaved the intact Ca^{2+}-channel monomer into peptides of 173 (N-terminal end) and 480 kDa (C-terminal end) which was subsequently cleaved into 100 and 365 kDa fragments. The 365 kDa peptide was cleaved to 210 kDa and then further, into a 130 kDa fragment (Figure 5A). Therefore, our calpain digested fragments labeled with [^{125}I]-Bz-CaM can be traced from 480 kDa to 365 kDa and then to 210 kDa and finally to the 130 kDa fragment. Our immunoblots of calpain-digested Ca^{2+}-channel suggested the N-terminus of this 130 kDa fragment which labeled with [^{125}I]-Bz-CaM spanned amino acid residues 1333-1350 and the end of C-terminus was at residue 2515. This result is in agreement with one of the calpain cleavage sites in the Ca^{2+}-release channel, residues 1383-1400, and was predicted to be near a CaM-binding site (Brandt et al., 1992; Shoshan-Barmatz et al., 1994). In combining calpain-digested results with the results form trypsin digestion, two CaM-binding domains were localized to residues 1333-1508 and 2400-2515.

Menegazzi et al. (1994) and Chen et al. (1994) have tentatively identified several Ca^{2+}-

dependent CaM-binding sites in rabbit skeletal muscle Ca^{2+}-channel protein with different experimental approaches. The CaM-binding sites in SR Ca^{2+}-channel in our results are different from Menegazzi et al. (1994) and Chen et al. (1994), and possible reasons for these discrepancies can be explained as follows. Both groups identified CaM binding regions in Ca^{2+}-channel protein through the use of expressed fusion proteins using cDNAs encoding fragments of the rabbit skeletal muscle Ca^{2+}-release channel protein and the use of gel overlay procedures. Since different degrees of renaturation of these fusion proteins on nitrocellulose membranes could affect CaM binding activity in the overlay assay, CaM binding sites determined by the overlay method may not represent true CaM binding sites in Ca^{2+}-channel. Furthermore, the overlay method may not have detected all types of CaM-binding sites. Thus, it is possible that other Ca^{2+}-dependent or Ca^{2+}-independent CaM-binding sites may exist in the native Ca^{2+}-channel. Finally, fusion proteins only cover 90% of the length of the Ca^{2+}-channel amino acid sequence (Chen et al., 1994) and CaM-binding sites located in or near the regions that are not covered by fusion proteins would not have been detected in the overlay study.

This study is the first to identify CaM-binding sites in the native channel protein structure. Efforts are continuing to narrow down the CaM-binding sites in the native structure and to correlate structure with inhibitory or activating effects on Ca^{2+}-channel activity.

CHAPTER 6

OVERALL CONCLUSIONS AND FUTURE RESEARCH

I. Overall conclusions

1. There are multiple CaM-binding sites on each channel protein subunit and the affinities of these CaM-binding sites depend on the concentration of Ca^{2+} and Mg^{2+}. The binding of CaM to the SR Ca^{2+}-channel is regulated by modulators of the Ca^{2+}-channel activity itself, and this novel regulation is likely to be important in the mechanism of excitation-contraction.

2. The equilibrium binding constants and stoichiometry of CaM to MHS SR Ca^{2+}-release channel are altered compared to normal skeletal muscle. Our data are consistent with our hypothesis that the altered binding equilibrium of CaM to MHS Ca^{2+}-channel results, in part, abnormal regulation of SR Ca^{2+} release activity. However, the effects of ionic strength and caffeine on the binding of CaM to both MHS and normal SR are not significantly different.

3. Two CaM-binding sites, amino acid residues 1333-1508 and 2400-2515, in the central regions of Ca^{2+}-release channel protein from rabbit skeletal muscle have been identified.

II. Future research

1. Characterization of the physiological role of CaM binding to MHS skeletal SR Ca^{2+}-release channel by different experimental approaches such as single channel recording, ryanodine binding study and [$^{45}Ca^{+2}$] efflux study.

2. Identification of CaM-binding sites in SR Ca^{2+}-release channel from MHS skeletal muscle. Loss of specific CaM-binding sites may be associated with altered functional activity.

3. Further examination of the physiological function of these two identified CaM-binding sites on Ca^{2+}-channel gated by binding to various channel modulators such as ryanodine, caffeine, Ca^{2+} and Mg^{2+} etc.

CHAPTER 7

BIBLIOGRAPHY

Andreasen, T.J., Luetje, C.W., Heideman, W., and Storm, D.R. 1983. Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. Biochemistry 22:4615-4618.

Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R. and Cook, W.J. 1985. Three-dimensional structure of calmodulin. Nature 315:37-40.

Barbato, G., Ikura, M., Kay, L.E., Pastor, R.W., Bax, A. 1992. Backbone dynamics of calmodulin studies by 15N relaxation using inverse detected two-dimensional NMR spectroscopy: the central helix is flexible. Biochemistry 31:5269-5278.

Bazari, W.L. and Clarke, M. 1981. Characterization of a novel calmodulin from *Dictyostelium discoideum*. J. Biol. Chem. 256:3598-3603.

Bendall, J.R. 1952. Effect of the 'Marsh factor' on the shortening of muscle fibre models in the presence of adenosine triphosphate. Nature 170:1058-1060.

Bendall, J.R. 1953. Further observations on a factor (the 'Marsh' factor) effecting relaxation of ATP-shortened muscle-fibre models, and the effect of Ca and Mg ions upon it. J. Physiol. 121:232-254.

Beringer, T. 1976. A freeze-fracture study of sarcoplasmic reticulum from fast and slow muscle of the mouse. Anat. Rec. 184:647-663.

Berridge, M.J. 1993. Inositol tris-phosphate and calcium signaling. Nature 361:315-325.

Billingsley, M. L., Polli, J.W., Pennupacker, K.R. and Kincaid, R.L. 1990. Identification of calmodulin-binding proteins. Methods Enzymol. 184:451-467.

Block B.A., Imagawa, T., Campbell, K.P. and Franzini-Armstrong, C. 1988. Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. J. Cell Biol. 107:2587-2600.

Brandt, N.R., Caswell, A.H., Brandt, T. and Mellgren, R. 1992. Mapping of the calpain proteolysis products of the junctional foot protein of the skeletal muscle triad junction. J.

Membrane Biol. 127:35-47.

Britt, B.A. and Kalow, W. 1970. Malignant hyperthermia: a statistical review. Can. Anaesth. Soc. J. 17:293-315.

Britt, B.A. (Editor) 1987. Malignant hyperthermia. Boston, MA. Nijhoff.

Buratti, R., Prestipino, G., Menegazzi, P., Treves, S. and Zorzato, F. 1995. Calcium dependent activation of skeletal muscle Ca^{2+} release channel (ryanodine receptor) by calmodulin. Biochem. Biophys. Res. Commun. 213:1082-1090.

Callaway, C., Serysheva, A., Wang, J.P., Slavik, K.J., Needleman, D.H., Cantu III, C., Wu, Y., Jayaraman, T., Marks, A.R., and Hamilton, S.L. 1994. Localization of the high and low affinity [^{3}H]ryanodine binding sites on the skeletal muscle Ca^{2+} release channel. J. Biol. Chem. 269: 15876-15884.

Campbell, K. P., Franzini-Armstrong C. and Shamoo, A.E. 1980. Further characterization of light and heavy sarcoplasmic reticulum vesicles. Biochem. Biophys. Acta 602:97-116.

Campbell, K.P. and MacLennan, D.H. 1981. Purification and characterization of the 53,000-dalton glycoprotein from the sarcoplasmic reticulum. J. Biol. Chem. 256: 4626-4632.

Campbell K.P. and MacLennan, D.H. 1982. A calmodulin-dependent protein kinase system from skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 257:1238-1246.

Campbell, K.P., MacLennan, D.H., Jorgensen, A.O. and Mintzer, M.C. 1983. Purification and characterization of calsequestrin from canine cardiac sarcoplasmic reticulum and identification of the 53,000 dalton glycoprotein. J. Biol. Chem. 258:1197-1204.

Campbell, K.P., Knudson, C. M., Imagawa, T., Leung, A.T., Sutko, J.L., Kahl, S.D., Raab, C.R. and Madson, L. 1987. Identification and characterization of the high affinity [^{3}H]ryanodine receptor of the junctional sarcoplasmic reticulum Ca^{2+} release channel. J. Biol. Chem. 262:6460-6463.

Carrier, L., Vaillaz, M., & Dupont, Y. 1991. Abnormal rapid Ca^{2+} release from sarcoplasmic reticulum of malignant hyperthermia susceptible pigs. Biochim. Biophys. Acta 1064: 175-193.

Cassens, R.G., Marple, C.N., and Eikelenboom, G. 1980. Animal physiology and meat quality. Adv. Food Sci. 24:71-155.

Caswell, A.H., Brandt, N.R., Brunschwig, J.P. and Purkerson, S. 1991. Localization and partial characterization of the oligomeric disulfide-linked molecular weight 95,000 protein (triadin) which binds the ryanodine and dihydropyridine receptors in skeletal muscle triadic vesicles. Biochemistry. 30:7507-7513.

Chandler, W.K., Rakowaky, R.F. and Schneider, M.F. 1976. Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. J. Physiol. London 254:285-316.

Chen, S.R.W., Zhang, L., MacLennan, D.H. 1992. Characterization of a Ca^{2+} binding and regulatory site in the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 267:23318-23326.

Chen, S.W.R., Airey, J.A., and MacLennan, D.H. 1993. Positioning of major tryptic fragments in the Ca^{2+} release channel (ryanodine receptor) resulting from partial digestion of rabbit skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 268:22642-22649.

Chen, S.R.W., and MacLennan, D.H. 1994. Identification of calmodulin-, Ca^{2+}-, and ruthenium red-binding domains in the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 269:22698-22704.

Cheung, W.Y. 1970. Cyclic 3', 5'-nucleotide phosphodiesterase. Demonstration of an activator. Biochem. Biophys. Res.Commun. 38:533-538.

Cheung, W.Y. 1980. Calmodulin plays a pivotal role in cellular regulation. Science 207:19-27.

Chiesi, M. and Carafoli, E. 1982. The regulation of Ca^{2+}-transport by fast skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 257:984-991.

Chu, A., Sumbilla, C., Scales, D., Piazza, A. and Inesi, G. 1988. Trypsin digestion of junctional sarcoplasmic reticulum vesicles. Biochemistry 27:2827-2833.

Chu, A., Diaz-Munoz, M., Hawkes, M. J., Brush, K., Hamilton, S.L. 1990. Ryanodine as a probe for the functional state of the skeletal muscle sarcoplasmic reticulum calcium release channel. Mol. Pharmacol. 37:735-741.

Cimler, B.M., Andreasen, T.J., Andreasen, K.I., and Storm, D.R. 1985. P-57 is a neural specific calmodulin-binding protein. J. Biol. Chem. 260:10784-10788.

Conti, A., Tarroni, P., Rossi, D. and Sorrentino, V. 1995. Ryanodine receptors:isoforms expression in mammals and chicken (Abstract). Biophys. J. 68:A52.

Cormier, M.J., Charbonneau, H. and Jarrett, H.W. 1981. Plant and fungal calmodulin:Ca^{2+}-dependent regulation of plant NAD kinase. Cell Calcium 2:313-333.

Dayton, W.R., Reville, W.J., Goll, D.E. and Stromer, M.A. 1976. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. Biochemistry 15:2159-2167.

de Meis, L. and Vianna, A.L. 1979 Energy interconversion by the Ca^{2+}-dependent ATPase of the SR. Ann. Rev. Biochem. 48:275.

Dulhunty, A. and Valois, A. 1982. A freeze-fracture study of normal and dystrophic C57BL mouse muscle. Muscle Nerve. 5:425-433.

Dulhunty, A. and Valois, A. 1983. Indentations in the terminal cisternae of amphibians and mammalian skeletal muscles. J. Ultrastruct. Res. 84:34-49.

Dulhunty, A., Gage, P. and Valois, A. 1983. Indentations in the terminal cisternae of slow- and fast-twitch muscles from normal and paraplegic rats. J. Ultrastruct. Res. 84:50-59.

Ebashi, S. and Endo, M. 1968. Calcium ion an dmuscle contraction. Prog. Biophys. Mol. Biol. 18:123-183.

Ebashi, S., Kodama, A., and Ebashi, F. 1968. Troponin, I. Preparation and physiologic function. J. Biochem. 64:465-477.

Eibschutz, B., Wong, A.P., Lopaschuk, G.D., Katz, S. 1984, The presence and binding characteristics of calmodulin in microsomal preparations enriched in sarcoplasmic reticulum from rabbit skeletal muscle. Cell Calcium 5:391-400.

Endo, M., Tanaka, M., Ogawa, Y. 1970. Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned muscle fibers. Nature 228:34-36.

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. Physiol. Rev. 57:71-108.

Endo, M. 1985. Calcium release from sarcoplasmic reticulum. Curr. Top. Membr. Transp. 25:181-230.

Entman, M.L., Bick, R., Chu, A., Van-Winkle, W.B. and Tate, C.A. 1986. The mechanism of nucleotide induced calcium translocation across sarcoplasmic reticulum membranes: Evidence for a non-translocated intermediate pool of calcium. J. Mol. Cell. Cardiol. 18:781-798.

Fernandez, J.L., Rosemblatt, M., Hidalgo, C. 1980. Highly purified sarcoplasmic reticulum vesicles are devoid of Ca 2+-independent ('basal') ATPase activity. Biochim. Biophys. Acta 599:552-568.

Fill, M., Coronado, R., Mickelson, J.R., Vilven, J., Ma, J.J., et al. 1990. Abnormal ryanodine receptor channels in malignant hyperthermia. Biophys. J. 57:471-475.

Finn, B.E., Drakenberg, E. and Forsen, S. 1993. The structure of apo-calmodulin. A 1H NMR examination of the carboxy-terminal domain. FEBS Lett. 336:368-374.

Finn, B.E., Evenas, J., Drakenberg, T., Waltho, J.P., Thulin, E. and Forsen, S. 1995. Calcium-induced structural changes and domain autonomy in calmodulin. Nature Structural Biology 2:777-783.

Fleischer, S. and Inui, M. 1989. Biochemistry and biophysics of excitation-contraction coupling. Annu. Rev. Biophys. Biophys. Chem. 18:333-364.

Ford, L.E. and Podolsky, R.J. 1970. Regenerative calcium release within muscle cells. Science 167:58-59.

Franzini-Armstrong, C. 1970. Purified ryanodine receptor form skeletal muscle is the Ca-permeable pore of the calcium release channel. J. Cell Biol. 47:488-499.

Franzini-Armstrong, C. 1980. Structure of sarcoplasmic reticulum. FASEB 39:2403-2409.

Franzini-Armstrong, C. and Jorgensen, A.O. 1994. Structure and development of E-C coupling units in skeletal muscle Annu. Rev. Physiol. 56:509-534.

Fuentes, O., Valdivia, C., Vaughan, D., Coronado, R. and Valdivia, H.H. 1994. Calcium-dependent block of ryanodine receptor channel of swine skeletal muscle by direct binding of calmodulin. Cell Calcium 15:305-316.

Fujii, J., Otsu. K., Zorzato, F., Deleon, S.,Khanna, V.K., Weiler, J.E., O'Brien, P.J. and MacLennan, D.H. 1991. Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. Science 253:448-451.

Fujita, K. 1954. Action of adenosine derivatives on muscle activity. II. Glycerol treated muscle and 'relaxing factor'. Folia Pharmacol. Japon 50:183-192.

Gilchrist, J.S.C. 1992. Calcium-activated neutral protease effects upon skeletal muscle sarcoplasmic reticulum protein and calcium release. J. Biol. Chem. 267:20857-20865.

Goodman, M., Pechere, J.G., Haiech, J., and Demaille, J.G. 1979. Evolutionary diversification of structure and function in the family of intracellular calcium-binding proteins. J. Mol. Evol. 13:331-352.

Gronert, G.A. 1986. Malignant Hyperthermia. In Myology (Engel, A.G., and Banker, B.Q., eds.) pp. 1763-1784. McGraw-Hill Publications, Minneapolis, MN.

Hain, J., Schindler, H., Nath, S. and Fleischer, S. 1993. Phosphorylation of the skeletal muscle calcium release channel removes block by magnesium ions. Biophys. J. 64: A151.

Hakamata, Y., Nakai, J. Takeshima, H. Imoto, K. 1992. Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. FEBS Lett. 312:229-

235.

Hamilton, S.L. and Tate, C.H. (1991) in Cellular Calcium (McCormack, J.G. and Cobbold, P.H., eds) pp.313-343, IRL Press, Oxford.

Harrison, G.G. Malignant hyperthermia. Dantrolene-dynamics and kinetics. 1988. Br. J. Anaesth. 60:279-286.

Hasselbach, W. and Migala, A. 1987. Activation and inhibition of the calcium gate of sarcoplasmic reticulum by high-affinity ryanodine binding. FEBS Lett. 221:119-123.

Hawkes, M.J., Nelson, T.E., & Hamilton S.L. 1992. [^{3}H]Ryanodine as a Probe of Changes in the Functional State of the Ca^{2+}-release Channel in Malignant Hyperthermia. J. Biol. Chem. 267:6702-6709.

Heffron, J.J.A., Ellis, F.R. 1985. Clinical and biochemical aspects of malignant hyperthermia. Recent Adv. Anaesth Analg. 15:173-207.

Huxley, A. F. 1957. Muscle structure and theories of contraction. Prog. Biophys. 7:255.

Huxley, A.F. and Taylor, R.E. 1958. Local activation of striated muscle fibers. J. Physiol. 144:426.

Hymel, L., Inui, M., Fleischer, S. and Schindler, H. 1988. Purified ryanodine receptor of skeletal muscle sarcoplasmic reticulum forms Ca^{2+}-activated oligomeric Ca^{2+} channels in planar bilayers. Proc. Natl Acad. Sci. 85:441-445.

Ikemoto, N. 1982. Structure and function of the calcium pump protein of sarcoplasmic reticulum. Annu. Rev. Physiol. 44:297-317.

Ikemoto, N., Antoniu, B. and Meszaros, L.G. 1985. Rapid flow chemical quench studies of calcium release from isolated sarcoplasmic reticulum. J. Biol. Chem. 260: 14096-14100.

Ikemoto, T., Iino, M. and Endo, M. 1995. Enhancing effect of calmodulin on Ca^{2+}-induced Ca^{2+} release in the sarcoplasmic reticulum of rabbit skeletal muscle fibres. J. Physiol. 487:573-582.

Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B. and Bax, A. 1992. Solution structure of a calmodulin target peptide complex by multidimensional NMR. Science 256:632-638.

Imagawa, T., Smith, J.S., Coronado, R. and Campbell, K.P. 1987. Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+} -permeable pore of the calcium release channel. J Biol. Chem. 262:16636-16643.

Inesi, G. A. 1985. Mechanism of calcium transport. Ann. Rev. Physiol. 47:573-601.

Inui, M., Saito, A. and Fleischer, S. 1987a. Purification of the ryanodine receptor and identity with foot structure of junctional terminal cisternae of sarcoplasmic reticulum from fast muscle. J. Biol. Chem. 262:1740-1747.

Inui, M., Saito, A. and Fleischer, S. 1987b. Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. J. Biol. Chem. 262:15637-15642.

James, P., Vorherr, T. and Carafoli, E. 1995. Calmodulin-binding domains: just two faced or multi-faceted? TIBS 20:38-42.

Jenden, D.J. and Fairhurst, A.S. 1969. The pharmacology of ryanodine. Pharmacol. Rev. 21:1-25.

Jones, D.A. and Round, J.M. 1990. Skeletal Muscle in Health an dEisease. pp 49. Manchester University Press. Manchester, England.

Jones, L.R., Besch, H.R., Jr, Sutko, J.L. and Willerson, J.T. 1979. Ryanodine-induced stimulation of net Ca^{2+} uptake by cardiac sarcoplasmic reticulum vesicles. J.Pharmacol. Exp. Ther. 209:48-55.

Judge, M.D., Aberle, E.D., Forrest, J.C., Hedrick, H.B. and Merkel, R.A. 1989. Principle of Meat Science. pp 23. Kendall/Hunt Publishing Company. Dubuque, Iowa.

Kawamoto, R.M., Brunschwig, J.P., Kim, K.C., and Caswell A.H. 1986. Isolation, characterization and localization of the spanning protein from skeletal muscle triads. J. Cell Biol. 103:1405-1414.

Kawamoto, R.M., Brunschwig, J.P., and Caswell A.H. 1988. Localization by immunoelectron microscopy of spanning protein of triad junction in terminal cisternae/triad vesicles. J.Muscle Res. Cell Motil. 9:334-343.

Kim, D.H., Sreter, F.A., Ohnishi, S.T., Ryan, J.F., Roberts, J. Allen, P. D., Meszaros, L.G., Antoniu, B. and Ikemoto, N. 1984. Biochem. Biophys. Acta 775:320-327.

Kim, K.C., Caswell, A.H., Talvenheimo, J.A., Brandt, N.R. 1990. Isolation of a terminal cisterna protein which may link the dihydropyridine receptor to the junctional foot protein in skeletal muscle. Biochemistry. 29:9281-9289.

Kirchberger, M.A., Tada, M., Katz, A.M. 1974. Adenosine 3',5'-monophosphate-dependent protein kinase-catalyzed phosphorylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. J. Biol. Chem. 249:6166-6173.

Kirino, Y., Osakabe, M. and Shimizu, H. 1983. Ca^{2+}-induced Ca^{2+} release from fragmented sarcoplasmic reticulum : Ca^{2+}-dependent passive Ca^{2+} efflux. J. Biochem. 94:1111-1118.

Klaevit, R.E., Vanaman, T.C. 1984. Azidotyrosyl calmodulin derivatives. Specific probes for protein-binding domains. J. Biol. Chem. 259:15414-15424.

Klee, C.B. and Vanaman, T. C. 1982. Calmodulin. Adv. Protein Chem. 35:213-321.

Kretsinger, R.H. 1980. Structure and evolution of calcium-modulated proteins. CRC Crit. Rev. Biochem. 8:119-174.

Kuboniwa, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C.B. and Bax, A. 1995. Solution structure of calcium-free calmodulin. Nature Structure Biology 2:768-776.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.Y. and Meissner, G. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. Nature 331:315-319.

Lai, F.A., Misra, M., Xu, Le, Smith, H.A., and Meissner, G. 1989. The ryanodine receptor-Ca super (2+) release channel complex of skeletal muscle sarcoplasmic reticulum: Evidence for

a cooperatively coupled, negatively charged homotetramer. J. Biol. Chem. 264:16776-16785.

Lakowicz, J.R. 1983. Principles of fluorescence spectroscopy. Plenum Press.

Lattanzio, G., Schlatterer, R., Nicar, M., Campbell, K., and Sutko, J. 1987. The effects of ryanodine on passive calcium fluxes across sarcoplasmic reticulum membranes. J. Biol. Chem. 262:2711-2718.

Lawrie, R.A., Cahterum, D.P. and Hale, H.P. 1958. Abnormal low pH in pig muscle. Nature 182:807.

LeGendre, M. and Matsudaira, P. 1989. in A Practical Guide to Protein and Peptide Purification for Microsequencing (Matsudaira, P.T., ed) pp 49-69, Academic Press, San Diego, CA.

Louis, C.F., Zualkernan, K., Roghair, T. and Mickelson, J.R. 1992. The effects of volatile anesthetics on calcium regulation by malignant hyperthermia-susceptible sarcoplasmic reticulum. Anesthesiology 77:114-125.

Lowry, O.H., Rosebrough, M.J., Farr, A.L., & Randall, R.J. 1951. J. Biol. Chem. 193:265-275.

MacLennan, D.H. and Wong, P.T. 1971. Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. Proc. Natl. Acad. Sci. USA 68:1231-1235.

MacLennan, D.H., Seeman, P., Iles, g.H. and Yip, C.C. Membrane formation by the adenosine triphosphatase of sarcoplasmic reticulum. J. Biol. Chem. 246:2702-2710.

MacLennan, D.H. and Holland, P.C. 1975. Calcium transport in sarcoplasmic reticulum. Annu. Rev. Biophys. Bioeng. 4: 377-404.

MacLennan, D.H., Campbell, K.P., and Reithmeier, R.A.F. 1983 Calsequestrin, in Calcium and Cell Function, Bol. 4. Cheung, W., Ed,. Academic Press, New York, pp. 151.

Marks, A.R., Fleischer, S. and Tempst, P. 1990. Surface topography analysis of the ryanodine receptor/junctional channel complex based on proteolysis sensitivity mapping. J. Biol. Chem. 265:13143-13149.

Marsh, B.B. 1951. A factor modifying muscle fibre syneresis. Nature 167:1065-1066.

Marsh, B.B. 1952. The effects of adenosine triphosphate on the fibre volume of a muscle homogenate. Biochim. Biophys. Acta 9:247-260.

Martonosi, A.N. 1984. Mechanisms of Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle. Physiol. Rev. 64: 1240-1320.

Marty, I., Robert, M., Villaz, M., De Jongh, K.S., Lai, Y. Catterall, W.A. and Ronjat, M. 1994. Biochemical evidence for a complex involving dihydropyridine receptor and ryanodine receptor in triad junctions of skeletal muscle. Proc. Natl. Acad. Sci. USA 91:2270-2274.

Masure, H.R., Alexander, K.A., Wakim, B.T., and Storm, D.R. 1986. Physicochemical and hydrodynamic characterization of p-57, a neurospecific calmodulin binding protein. Biochemistry 25:7553-7560.

Maurer, A., Tanaka, M., Ozawa, T., Fleischer, S. 1985. Purification and crystallization of the calcium binding protein of sarcoplasmic reticulum from skeletal muscle. Proc. Natl. Acad. Sci. USA 82:4036-4040.

McGrew, S.G., Wolleben, C., Siegl, P., Inui, M., and Fleischer, S. 1989. Positive cooperativity of ryanodine binding to the calcium release channel of sarcoplasmic reticulum from heart and skeletal muscle. Biochemistry 28:1686-1691.

McPherson, P.S. and Campbell, K.P. 1993. The ryanodine receptor/Ca^{2+} release channel. J. Biol. Chem. 268:13765-13768.

Meador, W.E., Means, A.R. and Quiocho, F.A. 1992. Target enzyme recognition by calmodulin:2.4 A structure of a calmodulin-peptide complex. Science 257:1251-1255.

Meador, W.E., Means, A.R. and Quiocho, F.A. 1993. Modulation of calmodulin plasticity in molecular recognition on the basis of crystal structures. Science 262:1718-1721.

Means, A.R., VanBerkum, M.F.A., Indrani, B., Lu, K. P. and Rasmussen, C.D. 1991. Regulatory functions of calmodulin. Pharmac, Ther. 50:255-270

Meissner, G. and Fleischer, S. 1971. Characterization of sarcoplasmic reticulum from skeletal muscle. Biochim. Biophys. Acta 241:356-378.

Meissner, G., Conner, G.E. and Fleischer, S. 1973. Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+} pump and Ca^{2+} -binding protein. Biochim. Biophys. Acta 298:246-269.

Meissner G. 1984. Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum J. Biol. Chem. 159:1365-1374.

Meissner, G. 1986. Evidence of a role for calmodulin in the regulation of calcium release from skeletal muscle sarcoplasmic reticulum. Biochemistry 25:244-251.

Meissner, G. and Henderson, J.S. 1987. Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. J. Biol. Chem. 262:3065-3073.

Meissner, G., Rousseau, E. and Lai, F.A. 1989. Structural and functional correlation of the trypsin-digested Ca^{2+} release channel of skeletal muscle of sarcoplasmic reticulum. J. Biol. Chem. 264:1715-1722.

Meissner, G. and El-Hashem, A. 1992. Ryanodine as a functional probe of the skeletal muscle sarcoplasmic reticulum Ca^{2+} release channel. Mol. Cell. Biochem. 114:119-123.

Meissner, G. 1994. Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. Annu. Rev. Physiol. 56:485-508.

Mellgren, R.L. 1987. Calcium-dependent proteases: an enzyme system active at cellular membranes? FASEB J. 1:110-115.

Menegazzi, P., Larini, F., Treves, S., Guerrini, R., Qusdroni, M., and Zorzato, F. 1994. Identification and characterization of three calmodulin binding sites of the skeletal muscle ryanodine receptor. Biochemistry 33:9078-9084.

Mickelson, J.R., Ross, J.A., Reed, B.K., and Louis, C.F. 1986. Enhanced Ca^{2+} induced calcium release by isolated sarcoplasmic reticulum vesicles from malignant hyperthermia susceptible pig muscle. Biochim. Biophys. Acta 862:318-328.

Mickelson. J.R., Gallant, E.M., Litterer, L.A., Johnson, K.M., Rempel, W.E., and Louis, C.F. 1988. Abnormal sarcoplasmic reticulum ryanodine receptor in malignant hyperthermia. J. Biol. Chem. 263:9310-9315.

Mickelson, J.R., Litterer, L.A., Jacobson, B.A. and Louis, C.F. 1990. Stimulation and inhibition of [^{3}H]ryanodine binding to sarcoplasmic reticulum from malignant hyperthermia susceptible pigs. Arch. Biochem. Biophys. 278:251-257.

Mickelson, J. R., Knudson, C. M., Kennedy, C. F. H., Yang, D., Litterer, L. A., Rempel, W.E., Campbell, K.P. and Louis, C. F. 1992. Structural and functional correlates of a mutation in the malignant hyperthermia-susceptible pig ryanodine receptor. FEBS Lett. 301:49-52.

Mills, J.S., Walsh, M.P., Nemcek, K., & Johnson, J.D. 1988. Biologically active fluorescent derivatives of spinach calmodulin that report calmodulin target protein binding. Biochemistry 27:991-996.

Mitchell G. and Heffron. J.J.A. 1982. Porcine Stress Syndromes. Adv. Food Res. 28:167-230.

Morii H. and Tonomura, Y. 1983. The gating behavior of a channel for Ca^{2+}-induced Ca^{2+} release in fragmented sarcoplasmic reticulum. J. Biochem. 93:1271-1285.

Moutin, M.J. and Dupont, Y. 1988. Rapid filtration of Ca^{2+}-induced Ca^{2+} release from skeletal sarcoplasmic reticulum. J. Biol. Chem. 263:4228-4235.

Murachi, T. 1983. Calpain and calpastatine. Trends Biochem. Sci. 8:167-169.

Nagasaki, K. and Kasai, M. 1983. Fast release of calcium from sarcoplasmic reticulum vesicles monitored by chlortetracycline fluorescence. J. Biochem. 94:1101-1109.

Nakai, J., Imagawa, T., Hakamata, Y., Shigekawa, M., Takeshima H. and Numa, S. 1990. Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel FEBS Lett. 271:169-177.

Nelson, T.E. 1983. Abnormality in calcium release from skeletal sarcoplasmic reticulum of pigs susceptible to malignant hyperthermia. J. Clin. Invest. 72:862-870.

Nelson, T.E. 1987. Ryanodine: antithetical calcium channel effects in skeletal muscle sarcoplasmic reticulum. J. Pharmacol. Exp. Ther. 242:56-61.

O'Brien, P.J. 1986. Porcine malignant hyperthermia susceptibility: hypersensitive calcium-release mechanism of skeletal muscle sarcoplasmic reticulum. Can. J. Vet. Res. 50:318-328.

O'Driscoll, S., McCarthy, T.V., Eichinger, H.M., Erhardt, W., Lehmann-Horn, F.and Herrmann-Frank, A. Calmodulin sensitivity of the sarcoplasmic reticulum ryanodine receptor from normal and malignant hyperthermia susceptible muscle.(submitted to Biochem. J. 1996)

Ohnishi, S.T., Taylor, S. and Gronert, G.A. 1983. Calcium-induced Ca^{2+} release from sarcoplasmic reticulum of pigs susceptible to malignant hyperthermia: the effects of halothane and dantrolene. FEBS Lett. 161:103-107.

Ohnishi, S.T. 1987. Effects of halothane, caffeine, dantrolene and tetracaine on the calcium permeability of skeletal sarcoplasmic reticulum of malignant hyperthermic pigs. Biochim. Biophys. Acta 897:261-268.

Ohta, T., Endo, M., Nakano, T., Morohoshi, Y., Wanikawa, K. and Ohga, A. 1989. Ca^{2+}-induced Ca^{2+} release in malignant hyperthermia-susceptible pig skeletal muscle. Am. J. Physiol. 256 (Cell Physiol. 25):C358-367.

O'Neil, K.T. and DeGrado, W.F. 1990. How calmodulin binds its targets: sequence independent recognition of amphiphilic alpha-helices. Trends Biochem. Sci. 15:59-64.

Otsu, K., Willard, H.F., Khanna, V.K., Zorzato, F.,Green, N.M. and MacLennan, D.H. 1990. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J.Biol. Chem. 265:13472-13483.

Otsu, K., Khanna, V.K., Archibald, A.L. and MacLennan, D.H. 1991. Cosegregation of porcine malignant hyperthermia and a probable causal mutation in the skeletal muscle ryanodine receptor gene in backcross families. Genomics 11:744-750.

Otsu, K., Nishida, K., Kimura, Y., Kuzuya, T., Hori, M., Kamada, T. and Tada, M. 1994. The point mutation Arg^{615} - Cys^{615} in the Ca^{2+} release channel of skeletal sarcoplasmic reticulum is responsible for hypersensitivity to caffeine and halothane in malignant hyperthermia. J. Biol. Chem. 269:9413-9415.

Perrin, D.D. and Sayce, I.G. 1967. Talanta 14:833-842.

Pessah, I. N., Waterhouse, A.L. and Casida, J. E. 1985. The calcium-ryanodine receptor complex of skeletal and cardiac muscle. Biochem. Biophys. Res. Commun. 128:449-456.

Pessah, I. N., Francini, A.O., Scales, D.J., Waterhouse, A.L. and Casida, J.E. 1986. Calcium-ryandine receptor complex. Solubilization and partial characterization from skeletal muscle junctional sarcoplasmic reticulum vesicles. J. Biol. Chem. 261:8643-8648.

Pessah, I.N., Stambuk, R.A. and Casida, J.E. 1987. Ca^{2+} activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+}, caffeine and adenine nucleotides. Mol. Pharmacol. 31:232-238.

Plank, B.P., Wyskovsky, W. Hohenegger, M., Hellmann, G., and Suko, J. 1988. Inhibition of calcium release from skeletal muscle sarcoplasmic reticulum by calmodulin. Biochim. Biophys. Acta 938:79-88.

Podolsky, R.J. and Constantin, L.I. 1964. Regulation by calcium of the contraction and relaxation of muscle fibers. Fed. Proc. Fed. Am. Soc. Exp. Biol. 23:933.

Pontremoli, S. and Melloni, E. 1986. Extra lysosomal protein degradation. Annu. Rev. Biochem. 55:455-481.

Porter, K. R. and Palade G.E. 1957. Studies on the endoplasmic reticulum III. Its form and distribution in striated muscle cells. J. Biophys. Biochem. Cytol. 3:26.

Puca, G.A., Nola, E., Sica, V., and Bresciani, F. 1977. Estrogen binding proteins of calf uterus. Molecular and functional characterization of the receptor transforming factor: A Ca^{2+}-activated protease. J. Biol. Chem. 252:1358-1366.

Radermacher, M., Wagenknecht, T., Grassucci, R., Frank, J. Inui, M. 1992. Cryo-EM of the native structure of the calcium release channel/ryanodine receptor from sarcoplasmic reticulum. Biophys. J. 61:936-940.

Radermacher, M., Rao, V., Grassucci, R., Frank, J., Timerman, A.P., Fleischer, S. and Wagenknecht, T. 1994. Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. J. Cell Biol. 127:411-422.

Rardon, D.P., Cefali, D.C., Mitchell, R.D., Seiler, S.M., Hathaway, D.R. and Jones, L.R. 1990. Digestion of cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles with calpain II: Effect on the Ca^{2+} release channel. Circ. Res. 67:84-96.

Rayns, D.G., Devine, C.E. and Sutherland, C.L. 1975. Freeze-fracture studies of membrane systems in vertebrate muscle. I. Striated muscle. J. Ultrastruct. Res. 50:306-321.

Rockstein, M. and Herron, P. 1951. Anal. Chem. 23:1500-1501.

Rosemblatt, M., Hidalgo, C., Bergara, C. and Ikemoto, N. 1981. Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle. J. Biol. Chem. 256:8140-8148.

Schagger, H. And Jagow, G.V. 1987. Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166:368-379.

Schneider, M.F. and Chandler, W.K. 1973. Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. Nature 242:244-246.

Schneider, M.F. 1981. Membrane charge movement and depolarization-contraction coupling. Annu. Rev. Physiol. 43:507-517.

Schneider, M.F. 1994. Control of calcium release in functioning skeletal muscle fibers. Annu. Rev. Physiol.56:463-484.

Seiler, S., Wegener, A.D., Whang, D.D., Hathaway, D.R. and Jones, L.R. 1984. High molecular weight proteins in cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles bind calmodulin, are phosphorylated, and are degraded by Ca^{2+}-activated protease. J. Biol. Chem. 259:8550-8557.

Serysheva, I.I., Orlova, E.V., Chiu, W., Sherman, M.B., Hamilton, S.L. and van Heel, M. 1995. Electron cryomicroscopy and angular reconstitution used to visualize the skeletal muscle calcium release channel. Nature Structural Biology 2:18-24.

Shomer, N.H., Louis, C.F., Fill, M., Litterer, L.A. and Mickelson, J.R. 1993. Reconstitution of abnormalities in the malignant hyperthermia-susceptible pig ryanodine receptor. AM. J. Physiol. 264 (Cell Physiol. 33):C125-C135.

Shomer, N.H., Mickelson, J.R. and Louis, C.F. 1994a. Caffeine stimulation of malignant hyperthermia-susceptible sarcoplasmic reticulm Ca^{2+} release channel. Am. J. Physiol. 267 (Cell physiol. 36): C1253-C1261.

Shomer, N. H., Mickelson, J.R. and Louis, C.F. 1994b. Ion selectivity of porcine skeletal muscle Ca^{2+} release channels is unaffected by the Arg615 to Cys615 mutation. Biophys. J. 67:641-646.

Shoshan-Barmatz,V. and Zarka, A. 1988. Trypsin destruction of the high affinity ryanodine binding sites of the junctional sarcoplasmic reticulum. J. Biol. Chem. 263:16772-16779.

Shoshan-Barmatz,V., Weil, S., Meyer, H., Varsanyi, M. and Heilmeyer, L.M. 1994. Endogenous, Ca (2+)-dependent cysteine-protease cleaves specifically the ryanodine receptor/Ca^{2+} release channel in skeletal muscle. J. Membr. Biol. 142:281-288.

Simpson, S.P. and Webb, A.J. 1989. Growth and carcass performance of British Landrace pigs heterozygous at the halothane locus. Anim. Prod. 49:503.

Smith, J.S., Coronado, R. and Meissner, G. 1985. Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. Nature 316:446-449.

Smith, J.S., Coronado, R. and G. Meissner. 1986a. Single channel calcium and barium currents of large and small conductance from sarcoplasmic reticulum. Biophysical J. 50:921-928.

Smith, J.S., Coronado, R. and G. meissner. 1986b. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by calcium, ATP, and modulation by magnesium. J. Gen. Physiol. 88:573-588.

Smith, J.S., Imagawa, T., Fill, M., Campbell, K.P. and Coronado, R. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of the sarcoplasmic reticulum. J. Gen. Physiol. 92:1-26.

Smith, J.S., Coronado, R. and Meissner, G. 1989. Calmodulin regulation of single sarcoplasmic reticulum Ca^{2+}-release channels from cardiac and skeletal muscle. Circ. Res. 64:352-359.

Somlyo, A.V., Gonzalez-Serratos, H., Shuman, H., McClellan, G. and Somlyo, A.P. 1981. Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: an electron-probe study. J. Cell Biol. 90:577-594.

Strand, M.A., Louis, C.F. and Mickelson, J.R. 1993. Phosphorylation of the porcine skeletal and cardiac muscle sarcoplasmic reticulum ryanodine receptor. Biochem. Biophys. Biophys. Acta 1175:319-326.

Strasburg, G.M., Hogan, M., Birmachu, W., Thomas, D.D., and Louis, C.F. 1988. Site-specific derivatives of wheat germ calmodulin interactions with troponin and sarcoplasmic reticulum. J. Biol. Chem. 263:542-548.

Suko J, Maurer-Fogy, I., Plank, B., Bertel, O., Wyskovsky, W., et al. 1993. Phosphorylation of serine 2843 in ryanodine receptor-calcium release channel of skeletal muscle by cAMP-,cGMP- and CaM-dependent protein kinase. Biochim. Biophys. Acta 1175:193-206.

Sutko, J.L., Ito, K. and Kenyon, J.L. 1985. Ryanodine: a modifier of sarcoplasmic reticulum calcium release in striated muscle. Fed. Proc. 44:2984-2993.

Suzuki, K. 1987. Calcium activated neutral protease: domain structure and activity regulation. Trends Biochem. Sci. 12:103-105.

Tada, M., Yamamoto, T. and Tonomura, Y. 1978. Molecular mechanism of active calcium transport by sarcoplasmic reticulum. Physiol. Rev. 58:1-79.

Takeshima, H., Nishimura, S., Matsumoto, R., Ishida, H., Kanawa, K., Minammino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hanaoka, M., Hirose, T. and Numa, S. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature 339:439-445.

Thatte, H.S., Addis, P.B., Thomas, D.D., Bigelow, D.B., Mickelson, J.R. and Louis, C.F. 1987. Temperature-dependent abnormalities of the erythrocyte membrane in porcine malignant hyperthermia. Biochem. Med. Metab. Biol. 38:366-377.

Toda, H., Yazawa, M., Sakigama, F. and Yagi, K. 1985. Amino acid sequence of calmodulin from wheat germ. J. Biochem. 98: 833-842.

Torok, K. and Whitaker, M. 1994. Taking a long, hard look at calmodulin's warm embrace. BioEssays 16:221-224.

Treves, S., Chiozzi, P. and Zorzato. F. 1993. Identification of the domain recognized by anti-(ryanodine receptor) antibodies which affect Ca^{2+}-induced Ca^{2+} release. Biochem. J. 291:757-763.

Tripathy, A., Xu, L., Mann, G. and Meissner, G. 1995. Calmodulin activation and inhibition of skeletal muscle Ca^{2+} release channel (ryanodine receptor). Biophy. J. 69:106-119.

Vedeckis, W.V., Freeman, M.R., Schrader, W.T. and O'Malley, B.W.1980. Progesterone-binding components of chick oviduct: partial purification and characterization of a calcium-activated protease which hydrolyzes the progesterone receptor. Biochemistry 19:335-343.

Vergara, J., Tsien, R.Y., and Delay, M. 1985. Inositol 1,4,5-triphosphate: A possible chemical link in excitation-contraction coupling in muscle. Proc. Natl. Acad. Sci. USA 82:6352-6356.

Volpe, P., Salviati, G., DiVirgilio, F., and Pozzan, T. 1985. Inositol 1,4,5-triphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. Nature 316:347-349.

Wagenknecht, T., Grassuci R. Frank, J. Saito, A., Inui, M., Fleischer, S. 1989. Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum. Nature 338:167-170.

Wagenknecht, T., Berkowitz, J., Grassucci, R., Timerman, A. P., and Fleischer, S. 1994. Localization of calmodulin binding sites on the ryanodine receptor from skeletal muscle by electron microscopy. Biophys. J. 67:2286-2295.

Walker, J.W., Somolyo, A.V., Goldman, Y.E., Somolyo, A.P., and Trentham, D.R. 1987. Kinetics of smooth and skeletal muscle activation by laser pulse photolysis of caged inositol 1,4,5 triphosphate. Nature 327:249-252.

Wang, J. and Best, P.M. 1992. Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. Nature 359:739-741.

Wang, K.K.W., Villalobo, A. and Roufogalis, B.D. 1989. Calmodulin-binding proteins as calpain substrate. Biochem. J. 262:518-525.

Weber, A. and Sanadi, D.R. ed. 1966. Current topics in bioenergetics. New York: Academic Press pp.203.

Webb, A.J. and Simpson, S.P. 1986. Performance of Britich Landrace pigs selected for high and low incidence of halothane sensitivity. II. Growth and carcass traits. Anim. Prod. 43:493.

Wismer-Pedersen, J. and Briskey, E.J. 1961. Rate of anaerobic glycolysis versus structure in pork muscle. Nature 189:318.

Witcher, D.R., Kovacs R.J., Schulman, H., Cefali, D.C. and Jones, L.R. 1991. Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. J.Biol. Chem. 266:11144-11152.

Yang, H., Reedy, M.M., Burke, C. And Strasburg, G.M. 1994. Calmoculin interaction with the skeletal muscle sarcoplasmic reticulum calcium channel protein. Biochemistry 33:518-525.

Zhang, M., Tanaka, T. and Ikura, M. 1995. Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. Nature Structure Biology 2:758-767.

Zimanyi, I. and Pessah, I. 1991. Comparison of [^{3}H]ryanodine receptors and Ca^{2+} release from rat cardiac and rabbit skeletal muscle sarcoplasmic reticulum. J. Pharmacol. Exp. Ther. 256:938-946.

Zimanyi, I., Buck, E., Abramson, J., Mack, M. and Pessah, I. 1992. Ryanodine produces persistent inactivation of the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. Mol. Pharmacol. 42:1049-1057.

Zorzato, F., Fujii, J., Otsu,K., Phillips, M., Green, N.M., Lai, F.A., Meissner, G. and MacLennan. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 265:2244-2256.