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Ca^{2+} -CHANNEL PROTEIN FUNCTION AND REGULATION: POSSIBLE ALTERED Ca^{2+}-CHANNEL PROTEIN FUNCTION IN FORMATION OF PSE TURKEY

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LI-JU WANG

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Ca^{2+}-CHANNEL PROTEIN FUNCTION AND REGULATION: POSSIBLE ALTERED Ca^{2+}-CHANNEL PROTEIN FUNCTION IN FORMATION OF PSE TURKEY

By

Li-Ju Wang

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ABSTRACT

Ca^{2+}-CHANNEL PROTEIN FUNCTION AND REGULATION: POSSIBLE ALTERED Ca^{2+}-CHANNEL PROTEIN FUNCTION IN FORMATION OF PSE TURKEY

By

Li-Ju Wang

The turkey industry is experiencing pale, soft, exudative (PSE) meat quality problems which resemble that of PSE pork, a significant fraction of which is associated with a genetic defect of the Ca^{2+}-channel protein in the sarcoplasmic reticulum (SR). The aim of this research was to test the hypothesis that there is a genetic defect in the turkey SR Ca^{2+}-channel protein(s) which is responsible, in part, for the turkey PSE problem. [^{3}H]ryanodine binding studies indicated that the average Ca^{2+}-channel protein content or B_{max} from a group of commercial turkey heavy SR preparations (1.10 pmol/mg; n=7) was lower than that from a group of genetically unimproved turkeys (4.01 pmol/mg; n=7). Average channel protein contents determined from crude total membrane fraction were also significantly different between the two types of turkeys. Ca^{2+}-channel protein in heavy SR from commercial turkey heavy SR exhibited a higher binding affinity for [^{3}H]ryanodine when compared to that from unimproved turkey heavy SR (K_d=12.2 versus 20.5 nM), suggesting that differences exist in the channel protein activity or its regulation in these two populations. The [^{3}H]ryanodine

binding to the Ca^{2+} channel protein from both types of turkeys was activated at a threshold concentration of approximately 0.2 μM Ca^{2+}, and reached a plateau of binding over the range of 3 to 30 μM free Ca^{2+}, whereas it was only slightly inhibited at 1 mM Ca^{2+}. Calmodulin (CaM) affinity labeling experiments indicated that the SR Ca^{2+}-channel protein from both commercial and unimproved turkeys bind CaM in Ca^{2+}-independent fashion under physiological conditions. However, unlike the SR from unimproved turkeys, the Ca^{2+}-channel protein was not the main CaM receptor in SR from commercial turkeys. Instead, a 75 kDa protein present in most of the commercial turkey SR preparations was the most abundant CaM receptor. These results support the hypothesis that there is a genetic basis for the PSE problem in the turkey industry.

To My Beloved Father

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INTRODUCTION

The U.S. turkey industry has grown rapidly in response to increased consumer demand for lean processed meat products. To meet this demand, producers have intensely bred for growth efficiency and heavily muscled animals. In recent years, the turkey processing industry has been experiencing severe meat quality problems which closely resemble pale, soft, exudative (PSE) pork. Meat from these birds tends to be very exudative, and shows more rapid decline pH, softer texture, poorer bind and higher cooking losses compared to non-stressed birds (Sosnicki, 1993). Factors which increase the incidence of the PSE condition in turkey include stress such as exposure to heat or cold, and loading turkeys into trucks and transporting them to the slaughter plant (Froning et al., 1978). These stresses are reminiscent of the observations of factors which increase the incidence of PSE pork.

A substantial fraction of PSE pork arises from pigs with porcine stress syndrome (PSS), an inherited skeletal muscle disorder which affects 10-20% of the swine population in the U.S. (Vansickle, 1989). This syndrome results in substantial economic losses to farmers and processors, owing to death of animals from stresses of transportation , heat, crowding, etc., before reaching market, as well as losses to processors because of formation of PSE meat (Louis, 1993). While PSS has been observed in many swine breeds, the prevalence of this disorder correlates strongly with genetic selection of leaner, more heavily muscled and faster growing animals (Zhang et al.,

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Biochemical studies indicated that skeletal muscle fibers from the PSS-susceptible pig have abnormal Ca^{2+} regulation (Mickelson et al., 1986; Mickelson et al., 1988; Endo et al., 1983). The specific defect was traced to an abnormality which was subsequently identified as a point mutation in the skeletal muscle Ca^{2+}-channel protein (Arg^{615} to Cys) (Fujii et al., 1991).

The similarities in factors which produce PSE meat from turkey with that of PSE pork strongly suggests that genetic selection for desirable growth traits in both species may have resulted in inadvertent selection for a mutated form of the SR Ca^{2+}-channel resulting in undesirable meat quality characteristics. Furthermore, the fact that turkeys subjected to the same environmental stresses show different subpopulation of responses lends additional support for a genetic basis for at least some of the incidence of PSE turkey.

The hypothesis guiding this study is that a defect in Ca^{2+} regulation exists in a subpopulation of commercial turkeys which is responsible for at least some of the incidence of PSE meat. This defect may take the form of a mutation in the Ca^{2+}-channel protein resulting in altered Ca^{2+} release properties. The first objective of this study was to purify and characterize the SR from turkey skeletal muscle. The second objective was to define the biochemical basis for PSE problems associated with commercial turkey. The third objective was to define the affinity and stoichiometry of the cardiac Ca^{2+}-channel protein for CaM, as a model for behavior of one of the turkey skeletal muscle isoforms.

The dissertation was organized in a series of chapters. The first chapter is a review of the literature which forms the basis for the studies conducted for this thesis. Each subsequent chapter addresses a specific

objective as indicated above, and is organized as a manuscript with its specific introduction, materials and methods, results and discussions, and conclusion sections. The last common sections are Conclusion, Future Research, and List of References in Journal of Food Science format for the entire dissertation.

CHAPTER 1
LITERATURE REVIEW

I. The Structure of Muscle Tissue

A. Muscle Structure and Membrane Systems

Muscle tissue consists of muscle fibers, connective tissues, blood vessels, nerve fibers and blood fluid. The muscle fiber is a cell which forms the structural and functional unit of muscle tissue. The membrane surrounding the muscle fiber is called the sarcolemma. The muscle fiber comprises a highly organized network of contractile elements referred to as myofibrils (Figure 1.1).

Periodically, along the length of the fiber and around its entire circumference, invaginations of the sarcolemma form a network of tubules called transverse tubules (T tubules) which run more or less perpendicularly to the long axis of the muscle fiber (Figure 1.2). The T-tubules and cisternae form junctions with a closely meshed membrane network surrounding each myofibril called the sarcoplasmic reticulum (SR) (Figure 1.2).

The SR consists of several distinct elements: longitudinal tubules, fenestrated collar, and terminal cisternae. The longitudinal tubules are oriented in the general direction of the myofibrillar axis. The fenestrated collar forms a perforated sheet in the H zone regions of the sarcomere. The terminal cisternae are the rather large, sac-like SR structures which form junctions on both sides of the T-tubule. The structural element comprising

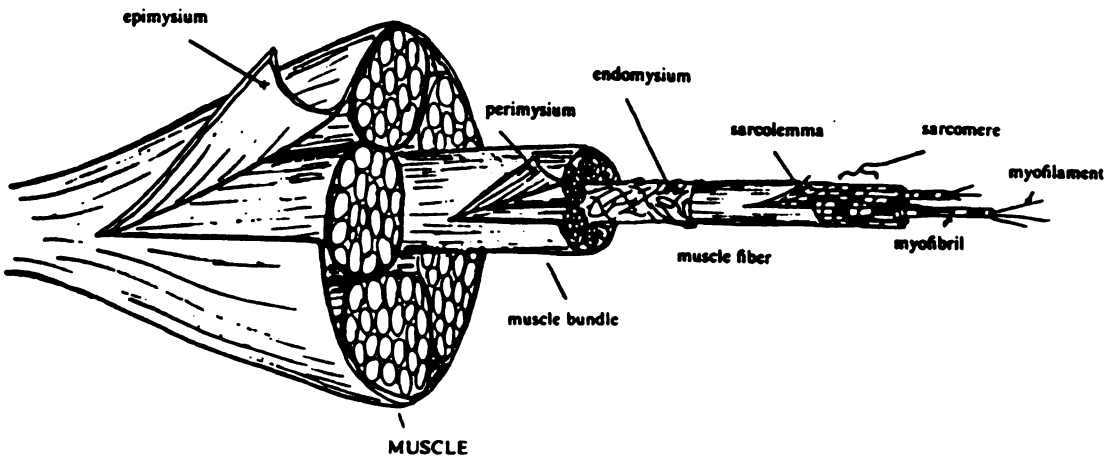


Figure 1.1 Diagrammatic representation of macroscopic and microscopic muscle structure (adapted from Chrystall, 1970).

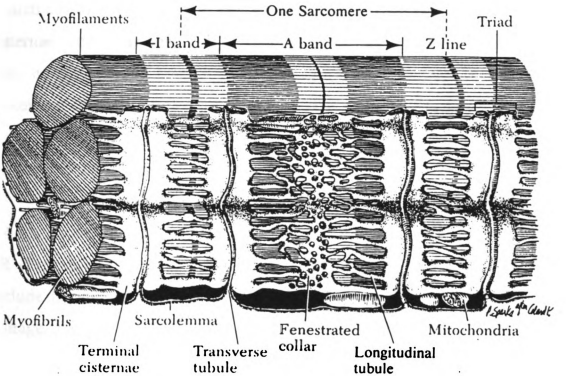


Figure 1.2 Diagrammatic representation of sarcoplasmic reticulum and T tubules, and their relation to the myofibrils of mammalian skeletal muscle (adapted from Judge et al., 1989).

the T-tubules and its adjoining cisternae is referred to as the triad.

SR can be separated into two fractions on the basis of differences in density using sucrose gradients. These fractions are referred to as light and heavy SR. The heavy SR fraction is composed of the junctional membrane (which contains calsequestrin and the Ca^{2+}-channel protein), triads, terminal cisternae, and some longitudinal tubules. The light SR fraction is almost entirely composed of longitudinal tubule membrane vesicles. The dominant protein in light SR is the Ca^{2+} pump protein (Meissner, 1975; Franzini-Armstrong, 1980).

B. The SR "Foot" Structure

The foot structure is a protein bridge which spans a gap of about 1.2-1.4 nm at the junction between the T-tubules and the terminal cisternae of SR (Franzini-Armstrong, 1970). The major proteins at the triad junction include the dihydropyridine (DHP) receptors in the T-tubule, which act as voltage-sensors, and Ca^{2+}-channel proteins of the SR, which act to release Ca^{2+} in the sarcoplasm (Figure 1.3) (Agnew, 1988; Agnew, 1989; McPherson and Campbell, 1993), as well as triadin, which is a 95 kDa protein whose function is unknown (Kim et al., 1990; Caswell et al., 1991). Analysis of the triadin amino acid sequence derived from cDNA indicates that only 47 amino acids of the protein are cytoplasmic, with the bulk of protein located in the lumen of the SR (Figure 1.3) (Knudson et al., 1993a). The lumenal region of triadin is highly positively charged. It may interact with negative charges on calsequestrin, the high capacity, moderate affinity Ca^{2+}-binding protein that is localized in the lumenal region of junctional SR (Knudson et al., 1993b).

1. Dihydropyridine (DHP) receptor

The DHP receptor is so named because this complex binds drugs

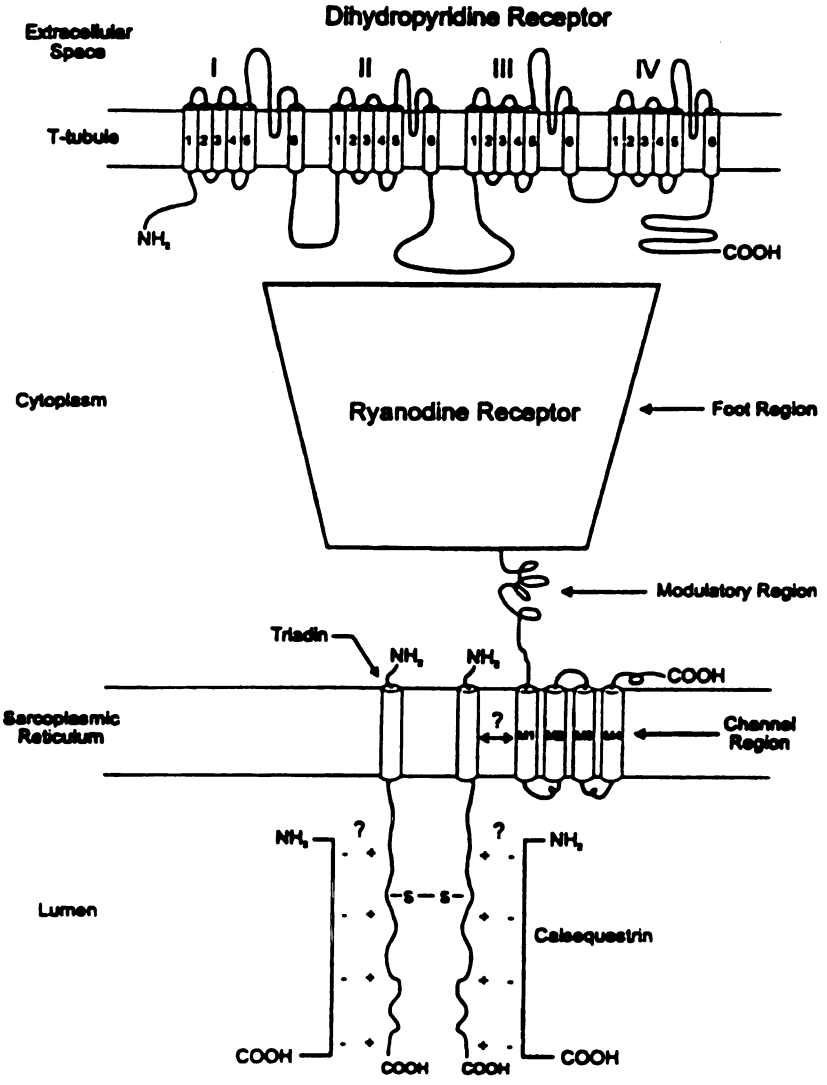


Figure 1.3 Model of the triad junction (adapted from McPherson and Campbell, 1993).

which share a common dihydropyridine basic structure. Examples of these drugs include nitrendipine, azidopine and PN200-110 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihypro-2,6-dimethyl-methyoxy-carbonylpyridine-3-carboxylate). The molecular picture of the DHP receptor is that of a large complex consisting of four or five protein subunits. Leung et al. (1988) showed that the purified DHP receptor from rabbit skeletal muscle contains four protein components of 175 kDa, 170 kDa, 52 kDa, and 32 kDa when analyzed by SDS-PAGE under nonreducing conditions. Under reducing conditions, however, it was shown that the 170 kDa subunit comprised a 140 kDa peptide linked by disulfide bonds to smaller polypeptides of 32-29 kDa (Cooper et al., 1987). Later, studies (Catterall, 1988; Campbell et al., 1988) indicated that there are five peptide subunits which constitute the DHP receptor, α_1 (175 kDa), α_2 (143 kDa), β (52 kDa), γ (32 kDa), and δ (27 kDa). The size of the α_1 subunit predicted from the cDNA clone is 212 kDa, which differs considerably from the size of 175 kDa observed after purification of DHP receptor (DeJongh et al., 1989). The α_1 subunit is the central functional component of the complex; it has four homologous domains with six proposed transmembrane segments in each domain. The α_1 subunit is present in a complex with an intracellularly disposed β subunit and a glycosylated transmembrane γ subunit. The α_1, β and γ subunits also interact with a disulfide-linked glycoprotein complex consisting of α_2 and δ subunits (Figure 1.4). The α_1 and β subunits are substrates for phosphorylation by cAMP-dependent protein kinase, which can regulate the Ca^{2+} ion conductance activity of the DHP receptor. (Catterall, 1988; Catterall, 1991).

The DHP receptor in skeletal muscle can function as a calcium channel or as a voltage sensor for excitation-contraction coupling (Agnew,

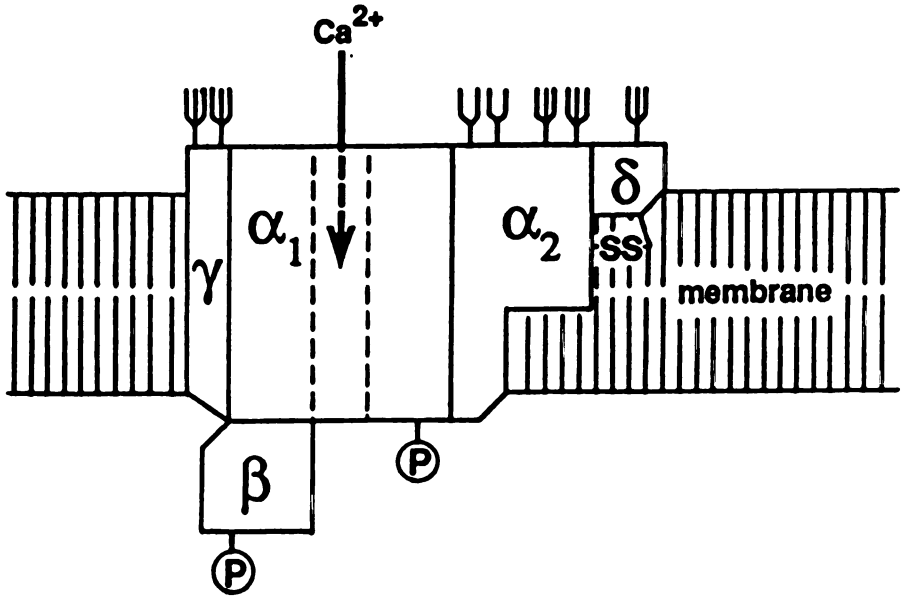


Figure 1.4 Model of the subunit structure of the dihydropyridine receptor (adapted from Catterall, 1988).

1987; Adams et al., 1990; Tanabe et al., 1990; Beam et al., 1992). As previously described, the α_1 subunit of DHP receptor has two forms in skeletal muscle: a full-length translational product (212 kDa) which is present as a minor species, and a much more abundant form that has a truncated carboxy-terminus (DeJongh et al., 1989). Recently, Beam et al. (1992) characterized the functional roles of these two proteins using murine dysgenic myotubes (muscle precursor cells). Muscular dysgenesis is a lethal autosomal recessive mutation in which skeletal muscle is paralyzed due to the failure of excitation-contraction coupling resulting from the absence of the α_1 subunit of the DHP receptor (Adams and Beam, 1990; Powell, 1990). Beam et al. (1992) cloned the full length cDNA into the dysgenic myotubes. After 2-3 days excitation-contraction coupling was restored, suggesting that only the full-length polypeptide can serve dual functions and that the truncated form can only function as a voltage sensor.

2. The SR Ca^{2+}-Channel Protein

Ca^{2+}-channel proteins from skeletal and cardiac muscle SR have been purified and biochemically characterized over the past few years. These proteins are located primarily at the terminal cisternae of the SR which forms the triad junction with T-tubules (Inui et al., 1987). The molecular weight of the Ca^{2+}-channel protein is over 2,200 kDa; and it consists of a tetramer of identical 565 kDa subunits (Lai et al., 1988; Imagawa et al., 1987). The structure of the Ca^{2+}-channel protein will be described in detail later (See The Structure of the Ca^{2+}-Channel Protein).

A protein-protein interaction between Ca^{2+}-channel proteins and DHP receptors is the mechanism believed to account for excitation-contraction coupling of skeletal muscle. Block et al. (1988) indicated that a direct interaction between the foot protein (the Ca^{2+}-channel protein) and a protein

component of junctional T-tubule membrane is observed in frog skeletal muscle using freeze-fracture images and rotary-shadowed imaging. Freeze-fracture images of the junctional T-tubule membranes demonstrate the presence of diamond-shaped clusters of particles that correspond exactly in position to the subunits of the foot proteins (or the Ca^{2+}-channel proteins). It was suggested that a voltage-dependent conformational change in the DHP receptor triggers a secondary conformational change in the SR Ca^{2+}-channel protein, leading to the opening of the channel and to an increase in myoplasmic Ca^{2+} (McPherson and Campbell, 1993; Catterall, 1991). However, at the biochemical level, a direct coupling of the Ca^{2+}-channel protein to the DHP receptor has not yet been demonstrated.

II. Excitation-Contraction Coupling

In the muscle fiber, under normal resting conditions, an electrical potential is maintained at -90 millivolts, and the concentration of free calcium in the cytosol surrounding the thick and thin filaments is very low, about 0.1 μM (Martonosi, 1984). A muscle contraction is initiated by a neuronal stimulus at the motor end plate of skeletal muscle or by an action potential transmitted along the cardiac muscle fiber. This stimulus is transferred along the sarcolemma, resulting in depolarization of the membrane. When the depolarization of the membrane is conducted by the T-tubule to the SR via the junctional foot protein, the change in polarization will trigger the Ca^{2+}-channel protein to open and to release Ca^{2+} from SR lumen to the cytoplasm, thus elevating the concentration of free Ca^{2+} into the 1-100 μM range (a $10\text{-}10^3$ fold increase) (Martonosi, 1984). The Ca^{2+} will bind to troponin C, which triggers a cascade of conformational changes in the thin filament permitting actin-myosin cross-bridge formation and

subsequent muscle contraction. Upon cessation of the action potential, the membrane is repolarized, and the SR Ca^{2+} pump (Ca^{2+}-ATPase) transfers Ca^{2+} ions back to the SR lumen using energy derived from hydrolysis of ATP, causing muscle relaxation.

The process of coupling chemical and electrical signals at the cell surface to the intracellular release of calcium and ultimate contraction of muscle fibers is termed excitation-contraction coupling. Several hypotheses exist to explain the mechanism of excitation-contraction coupling in smooth, cardiac and skeletal muscle cells. However, in each of them, both voltage-gated calcium channels (DHP receptor) in the cell surface membranes and intracellular calcium-release channel proteins in the SR play key roles.

In skeletal muscle, the most widely accepted hypothesis of excitation-contraction coupling mechanism is voltage-dependent calcium release. The molecular basis of this phenomenon involves the DHP receptor as the T-tubule voltage-sensor and the junctional foot protein as the SR Ca^{2+} release channel (Schneider and Chandler, 1973; Fleischer and Inui, 1989; Catterall, 1991; Rios et al., 1993). When the action potential arrives at the T-tubule - SR junction, it is accompanied by an outward movement of positive gating charge across the T-tubule membrane. This gating-charge movement is the electrical signature of voltage-driven conformational changes in the DHP receptor. This transmembrane conformational change might be directly linked to activation of calcium release through protein-protein interaction between the DHP receptor and the SR calcium channel (Schneider and Chandler, 1973; Pietrobon et al., 1988).

Schneider and Chandler (1973) proposed that charged molecules within the T-tubule membrane move upon depolarization and that the movement of these molecules provides a direct physical link between T-tubule

depolarization and SR Ca^{2+} release. Their hypothesis was based on the observation that a voltage-dependent charge movement, which was closely connected to muscle contraction, could be measured across the T-tubule membrane under conditions where all ionic currents were blocked. Later, it was observed that DHPs blocked change movement and SR Ca^{2+} release with similar voltage and dose dependences. The observation suggested that the charge movement was due to gating of the DHP receptor and confirmed its importance in the activation of SR Ca^{2+} release (Rios et al., 1992).

In vertebrate skeletal muscle, however, there is another hypothesis concerning the mechanism of Ca^{2+} release which involves a soluble, internal transmitter, inositol 1,4,5-trisphosphate ($InsP_3$). Vergara et al. (1985) reported the results of experiments using skinned skeletal muscle from semitendinosus muscle of the frog that suggest the $InsP_3$ may be a chemical intermediary between the T-system depolarization and the Ca^{2+} release from SR in skeletal muscle fibers. T-tubule membrane depolarization stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phosphodiesterase to form diacylglycerol and $InsP_3$. It was proposed that the latter binds to a specific receptor on the SR membrane, presumably the Ca^{2+}-channel protein or an $InsP_3$ receptor to release calcium. $InsP_3$ is rapidly deactivated by the $InsP_3$ 5-phosphatase to form inositol 1,4-biphosphate. Vergara et al. (1985) presented four pieces of evidence in support of this hypothesis. First, $InsP_3$ is generated by electrical stimulation of skeletal muscles. Second, $InsP_3$ is able to release Ca^{2+} from SR in skinned muscle fibers. Third, inhibitors of $InsP_3$ 5-phosphatase, such as 2,3-bisphosphoglycerate, Cd^{2+}, Zn^{2+}, or Ag^{+}, increase sensitivity to $InsP_3$. Fourth, drugs that inhibit $InsP_3$ release are effective blockers of excitation-contraction coupling. The polyamine antibiotic neomycin, which inhibits $InsP_3$ release, is an

effective blocker of excitation-contraction coupling. However, the Ca^{2+} release initiated by $InsP_3$ is hormonally activated and takes place on a relatively slow time scale (Caswell and Brandt, 1989). In contrast, Ca^{2+} release from skeletal muscle is activated by depolarization. It begins and is terminated within a few milliseconds of depolarization (Caswell and Brandt, 1989). Thus, current evidence does not favor the hypothesis that $InsP_3$ is involved with the skeletal muscle contraction.

On the other hand, there is strong evidence that Ca^{2+}-induced Ca^{2+} release (CICR) is the mechanism for Ca^{2+} release in heart. According to this hypothesis, a small transsarcolemmal Ca^{2+} influx via DHP receptors acts through the induction of a Ca^{2+} release from the SR. The trigger for CICR of Ca^{2+} is not a mere change of myoplasmic $[Ca^{2+}]_{free}$ outside of the SR ($\Delta[Ca^{2+}]_{free}$) but a function of the rate of change of $[Ca^{2+}]_{free}$ ($\Delta[Ca^{2+}]_{free}/\Delta t$). An increase of $[Ca^{2+}]_{free}$ to a given level induces calcium release from the SR when it is applied rapidly, whereas it induces calcium loading of the SR when it is applied slowly. From the Ca^{2+} current graph, it was observed that there were two Ca^{2+} release stages: a fast component (beat stage) and a slow component (flat stage). This suggests that the initial relatively fast component of transsacrolemmal Ca^{2+} current would trigger Ca^{2+} release; the subsequent slow component would load the SR with an amount of Ca^{2+} available for release during subsequent beats (Fabiato, 1983).

III. The Structure of the Ca^{2+}-channel Protein

As previously stated, the Ca^{2+}-channel protein is one of dozens of SR proteins and constitutes about 2-3% of the total SR protein. The localization of the Ca^{2+}-channel protein to the terminal cisternae of SR was supported by demonstrations that the plant alkaloid, ryanodine (Jenden and Fairhurst,

1969), binds to terminal cisternae but not to longitudinal SR, and that the Ca^{2+}-channel blocker ruthenium red enhances Ca^{2+} loading in terminal cisternae but not longitudinal SR (Fleischer et al., 1985). These results suggested that ryanodine could be locking a channel protein in an open state which would prevent Ca^{2+} accumulation in terminal cisternae. Identification and isolation of the Ca^{2+} -channel protein were also facilitated through the use of ryanodine which was shown to bind to the protein with high affinity and to modulate its function (Pessah et al., 1986; Seifert and Casida, 1986; Lai et al., 1989). Since the Ca^{2+}-channel protein was first isolated based on its ability to bind ryanodine, it is also called the ryanodine receptor (RYR). Recently, Carl et al. (1995) used immunofluorescence to localize the Ca^{2+}-channel protein/ryanodine receptor in developing rat skeletal muscle. The 243-9 RYR polyclonal antibody raised in rabbits specifically binds to the Ca^{2+}-channel protein in the triads. These results are consistent with the biochemical studies which indicated that the Ca^{2+}-channel protein is located in terminal cisternae of SR.

Studies of the morphology of the Ca^{2+} -channel protein have shown that it has a tetragonal or clover-leaf structure (Lai et al., 1988; Lai et al., 1989). Recently, the three-dimensional architecture of the Ca^{2+}-channel protein was determined using cryo-electron microscopy (cryo-EM), which allows structural preservation to high levels of resolution without stains or fixatives, image averaging, and conical tilt reconstruction. A three-dimensional reconstruction is then obtained from a random field of molecules (Radermacher et al., 1994; Serysheva et al., 1995). The reconstructed Ca^{2+}-channel protein shows a clear demarcation between the channel and cytoplasmic assemblies, both of which display four-fold symmetry (Radermacher et al., 1994). The cytoplasmic domain or foot protein of the

Ca^{2+}-channel protein is large (29x29x12 nm), whereas the transmembrane assembly is small, protruding 7 nm from one of its faces. A cylindrical low-density region, 2-3 nm in diameter, extends down the center of the transmembrane assembly, and possibly corresponds to the transmembrane Ca^{2+}-conducting pathway (Radermacher et al., 1994).

Stoichiometry studies by Ferguson plot analysis following SDS-polyacrylamide gel electrophoresis (PAGE) of partial and fully crosslinked and incompletely denatured complexes suggest that each Ca^{2+}-channel protein contains four identical polypeptide chains and the molecular weight of each subunit is ~ 400 kDa in rabbit skeletal muscle (Lai et al., 1989). Similar results obtained by Smith et al. (1988) indicated that the Ca^{2+}-channel protein purified by immunoaffinity chromatography from rabbit skeletal muscle is a tetramer which consists of four identical 450 kDa polypeptides. The purified Ca^{2+}-channel protein has an apparent sedimentation coefficient of 30S, indicating that it is a large, tetrameric complex with an estimated molecular mass > 10^6 (Lai et al., 1989). Likewise, in gel-exclusion chromatography, the purified Ca^{2+}-channel protein elutes as a peak corresponding to a molecular mass > 10^6 (Inui et al., 1987).

The Ca^{2+}-channel proteins from both cardiac (Otsu et al., 1990) and skeletal (Takeshima et al., 1989; Zorzato et al., 1990) SR have been sequenced from their cDNAs. Analysis of the sequence of the cardiac muscle protein indicates a molecular weight of 564,000 derived from 4969 amino acids in rabbit. The cardiac derivative is 66% identical with that of the skeletal muscle Ca^{2+} release channel, although it contains 63 fewer residues.

Analysis of the sequence of skeletal muscle Ca^{2+}-channel protein indicates that there are 10 potential transmembrane sequences in COOH-

terminal protein of the molecule. Two additional potential transmembrane sequences located nearer the center of the molecule could contribute to the formation of the Ca^{2+} conducting pore (Zorzato et al., 1990). In contrast, Takeshima et al. (1989) proposed a 4 transmembrane segment model. The cardiac Ca^{2+}-channel protein also has up to 12 potential transmembrane segments (Otsu et al., 1990).

The fact that the Ca^{2+}-channel protein activity is modulated by numerous factors (see next section) led to various efforts to predict modulator binding sites based on sequence homology. The regions of Ca^{2+}-channel protein that may interact with calmodulin, an inhibitor of Ca^{2+}-channel protein activity, are predicted at residues 2775-2807, 2877-2898, and 2998-3016 in cardiac muscle (Otsu et al., 1990). In contrast, Zorzato et al. (1990) predicted calmodulin-binding sites in skeletal muscle at residues 2807-2840, 2909-2930, and 3031-3049, whereas Takeshima et al. (1989) predicted 2 calmodulin-binding sites at 3614-3637 and 4295-4325 in skeletal muscle. In the cardiac Ca^{2+}-channel protein, a potential ATP binding domain is identified at residue 2610-2652, and a potential phosphorylation site by the cAMP-dependent protein kinase at residue 2809 was predicted based on the consensus sequence (RRXS) (Otsu et al., 1990). On the other hand, the skeletal Ca^{2+}-channel protein has several nucleotide-binding consensus sequences found at residues 4449-4454 or 4452-4457 (Takeshima et al., 1989). In addition, experimental evidence for the involvement of two Ca^{2+}-channel protein regions in regulating Ca^{2+}-induced Ca^{2+} release in skeletal muscle has been obtained. In malignant hyperthermia-susceptible pigs, the skeletal muscle Ca^{2+}-channel protein contains a cysteine instead an arginine at position 615 (Fujii et al., 1991), which alters the sensitivity of Ca^{2+}-induced Ca^{2+} release process. A second Ca^{2+}-sensitive region was obtained

in $^{45}Ca^{2+}$ and ruthenium red overlay studies with trpE fusion proteins. These findings led to the identification of three putative Ca^{2+}-binding sites at amino acid residues 4246-4467, 4382-4417, and 4478-4512 (Chen et al., 1992; Meissner, 1994).

IV. The Isoforms of the Ca^{2+}-channel Protein

Biochemical purification and cloning have revealed at least three different tissue-specific isoforms of the Ca^{2+}-channel protein in mammalian tissues. The Ca^{2+}-channel protein in skeletal muscle is referred to as the type 1 RYR (RYR1), which is expressed primarily in skeletal muscle, although it also has been found in the Purkinje cell of cerebellum (Ouyang et al., 1993) and in brain (Takeshima et al., 1993). The cardiac Ca^{2+}-channel protein (RYR2) was initially purified (Inui et al.,1987) and cloned (Otsu et al., 1990) from heart, but it is also expressed throughout the nervous system (Kuwajima et al., 1992). A third isoform (RYR3) has been cloned from brain. It is expressed primarily in brain, but is also present in smooth and skeletal muscles and some endothelial cells (Hakamata et al., 1992).

Whereas only one isoform has been detected in mammalian skeletal muscle, Sutko and colleagues first identified the existence of two isoforms (α and β) in chicken pectoral muscles (Airey et al., 1990). Subsequently, two isoforms of Ca^{2+}-channel protein in skeletal muscle have been identified in different species such as toadfish, frog, bullfrog, turtle, crocodile, and shark (Coronado et al., 1994; Ogawa, 1994). However, even in the same species, different muscles possibly contain different proportions of isoforms. For example, two isoforms (α and β) of Ca^{2+}-channel protein are expressed in approximately equal abundance in epaxial (swimming) muscles of toadfish (O'Brien et al., 1993) and blue marlin (O'Brien et al., 1995), whereas only

α isoform exists in extraocular muscle of both fish. Airey et al. (1990) indicated that the β isoform is not a proteolytic fragment of the α isoform, but is a true muscle isoform based on immunological reactivity, distinct peptide maps and immunofluorescent microscopy. The polypeptides were shown to be distinct by limited proteolysis and to be subunits of different [^{3}H]ryanodine-binding proteins by using isoform-specific antibodies; both proteins have similar distribution in the same fiber and were found to be associated with the terminal cisternae of SR by immunolabeling techniques. Additionally, the two isoforms do not necessarily exist in equal amount; both are present in equal relative amounts in microsomes from chicken breast muscle consisting of all white fiber types. However, the α isoform is more abundant than β in microsomes from chicken thigh muscle (Olivares et al., 1991).

O'Brien et al. (1995) examined the physiological properties of the α and β isoforms from different fish skeletal muscles. They found that there are differences in the calcium activation and inactivation properties of two isoforms. The α isoform, the sole isoform expressed in extraocular muscle, displays calcium concentration-dependent [^{3}H]ryanodine binding. The Ca^{2+}-channel protein is activated at 0.1 μM Ca^{2+}, shows peak activation in the 1-10 μM range, and is subsequently inhibited by ~1 mM Ca^{2+}. This feature of the α RYR isoform of fish muscle is similar to that of to the mammalian skeletal muscle (rabbit) RYR. On the other hand, the β isoform of fish skeletal muscle more closely resembles the fish and mammalian cardiac Ca^{2+}-channel protein in calcium concentration-dependent [^{3}H]ryanodine binding. The most prominent difference of the β and cardiac isoforms from α isoform is the lack of inactivation of [^{3}H]ryanodine binding and the decreased maximum open probability (P_o) in the presence of millimolar free Ca^{2+}. In

addition, [^{3}H]ryanodine binding by the α isoform is selectively inhibited by 100 μM tetracaine (70% inhibition), whereas cardiac Ca^{2+}-channel protein and the β skeletal muscle isoform are much less affected (less than 5% inhibition).

O'Brien et al. (1995) proposed a two component model for the calcium release process in nonmammalian vertebrate skeletal muscle (Figure 1.5). In this model, the skeletal-like α isoform occupies a position in SR directly coupled to the DHP receptor or voltage sensor. The β isoform is a logical candidate to be the calcium-coupled Ca^{2+}-channel protein because it has functional similarity to the cardiac Ca^{2+}-channel protein, which operates by Ca^{2+}-induced Ca^{2+} release. An influx of Ca^{2+} via the depolarization of the α isoform would trigger opening of the β channels.

Lai et al. (1992) reported that the amphibian Ca^{2+}-channel protein α and β isoforms are related to those of mammalian skeletal muscle and cardiac muscle, respectively. Immunoblot analysis shows that polyclonal antiserum to the rat skeletal Ca^{2+}-channel protein preferentially cross-reacts with the α isoform of frog skeletal Ca^{2+}-channel protein. Conversely, two monoclonal antibodies to canine cardiac Ca^{2+}-channel protein preferentially cross-react with the β isoform of frog skeletal Ca^{2+}-channel protein.

Airey et al. (1993) showed that chicken Ca^{2+}-channel protein isoforms differ with respect to CaM affinity labeling. While chicken skeletal muscle microsomes were incubated with azido[^{125}I]calmodulin, the result demonstrated that the α isoform Ca^{2+}-channel protein from chicken bound CaM to a much greater extent than the β-isoform (Airey et al., 1993).

V. The Modulators and Phosphorylation of the Ca^{2+}-channel Protein

A. Modulators of the Ca^{2+}-channel Protein

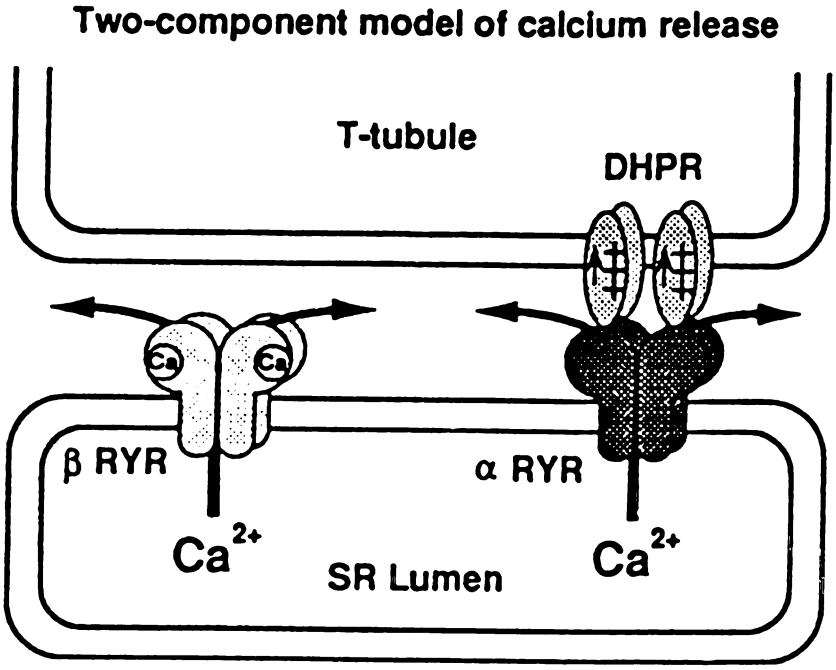


Figure 1.5 A two component model for the calcium release process in nonmammalian vertebrate skeletal muscle (adapted from O'Brien et al., 1995).

Regulation of Ca^{2+}-channel protein activity has been investigated by following Ca^{2+} efflux from isolated SR vesicles (Nagasaki and Kasai, 1983; Ikemoto et al., 1985; Meissner et al., 1986; Meissner, 1986a) and by single-channel recordings of the activity of the Ca^{2+}-channel protein incorporated from SR vesicle into planar lipid bilayers (Smith et al., 1985, 1986a, b). Results of these experiments indicate the Ca^{2+}-channel protein activity is modulated by positive effectors such as micromolar concentrations of Ca^{2+} (CICR), caffeine, adenine nucleotides and nanomolar concentrations of the plant alkaloid ryanodine, and by inhibitors such as Mg^{2+}, calmodulin, ruthenium red and micromolar concentrations of ryanodine. In addition, there are numerous other modulators for Ca^{2+}-channel protein which have been reported (Table 1.1).

1. Calmodulin (CaM)

CaM is a small, acidic protein whose molecular weight is about 17,000 Da. The complete amino acid sequence of CaM from bovine brain (Watterson et al., 1980), rabbit skeletal muscle (Grand et al., 1981), human brain (Sasagawa et al., 1982), scallop (Toda et al., 1981), *Tetrahymena* (Yazawa et al., 1981), sea anemone (Takagi et al., 1980), spinach (Lukas et al., 1984) and wheat (Toda et al., 1985) have been determined (Figure 1.6). The sequences of CaM from vertebrates are virtually identical except for some discrepancies in amide assignments, indicating that CaM has been highly conserved throughout vertebrate evolution. The amino acid sequences of plant CaMs contain Cys, whereas those of animal CaMs do not. Comparison of the amino acid sequences of CaMs of vertebrates, invertebrates, a protozoan, and plants, showed that the primary structures are not completely conserved among all eukaryotes, but rigid conservations are observed among vertebrates (Toda et al., 1985; Klee and Vanaman, 1982).

Table 1.1 Modulators of the sarcoplasmic reticulum Ca^{2+}-channel protein (adapted from Coronado et al., 1994).

Agent	Effective Concn	Ca^{2+} Release	[^{3}H]ryanodine Binding	Single Channel	Species and Tissue
Anthraquinones					
Daunorubicine	1–100 μM	+	+	ND	Rabbit skeletal, rat cardiac and brain
Digoxin	1–20 nM	+	ND	+	Sheep cardiac
Doxorubicin	1–300 μM	+	+	+	Rabbit skeletal, rat cardiac, dog cardiac
Mitoxantrone	5–10 μM	+	ND	ND	Rabbit skeletal
Rubidazone	15–150 μM	+	ND	ND	Rabbit skeletal
Polyamines					
Gentamicin	1–20 μM	–	ND	ND	Rabbit skeletal
Neomycin	0.01–20 μM	–	–	ND	Rabbit skeletal, dog and rat cardiac
Polylysine	1–10 μg/ml	+	ND	ND	Rabbit skeletal
Protamine	1 μg/ml	+	ND	ND	Rabbit skeletal
Putrescine	1–100 mM	ND	+	ND	Rabbit skeletal
Spermidine	1–100 mM	ND	+	ND	Rabbit skeletal
Spermine	0.4–20 mM	+	+	ND	Rabbit skeletal
Local anesthetics					
Benzocaine	1–10 mM	ND	–	ND	Rabbit skeletal
Chlorpromazine	0.1–1.5 mM	ND	–	ND	Rabbit skeletal
Dibucaine	0.08–1.8 mM	+	–	ND	Rabbit skeletal, sheep brain
Lidocaine	0.1–15 mM	ND	+	ND	Rabbit skeletal, sheep brain
Procaine	1–20 mM	–	–	–	Rabbit skeletal
Tetracaine	0.01–2 mM	–	–	–	Rabbit skeletal, dog cardiac, sheep brain, rat liver
Volatile anesthetics					
Enflurane	2% vol	ND	+	ND	Pig cardiac
Halothane	1.5%, 2% vol	+	+	ND	Dog and pig cardiac
Isoflurane	2%, 2.5% vol	+	NE	ND	Dog and pig skeletal
Fatty acid derivatives					
Long-chain acyl CoA	50 μM	+	ND	ND	Rabbit skeletal
Arachidonic acid	1–50 μM	+	ND	ND	Rabbit skeletal, dog cardiac
Acyl carnitines	50 μM	+	ND	ND	Rabbit skeletal
Palmitoyl carnitine	1–100 μM	+	+	+	Rabbit and pig skeletal
Sphingosine	30–50 μM	+	–	ND	Rabbit skeletal
	0.1–10 μM	–	–	ND	Rabbit skeletal
Stearic acid	16–32 μM	+	ND	ND	Rabbit skeletal
Scorpion toxins					
Buthotus venom	0.1–500 μg/ml	ND	+	+	Rabbit skeletal, bovine cardiac, rat brain
Imperatoxin A	1–1,000 nM	ND	+	+	Rabbit skeletal
Imperatoxin I	1–1,000 nM	ND	–	–	Rabbit skeletal, bovine cardiac
Ryanodine analogues					
Dihydroryanodine	1–1,000 nM	ND	–	ND	Rabbit skeletal, dog cardiac
Ester E	1–1,000 nM	+	–	ND	Rabbit skeletal, dog cardiac
	1–12 μM	–	ND	ND	Rabbit skeletal
Ester F	1–1,000 nM	+	–	ND	Dog cardiac, rabbit skeletal
	1–12 μM	–	ND	ND	Rabbit skeletal
Others					
4-Alkylphenol	10–25 nmol/mg	+	ND	ND	Rat skeletal
BisG10	0.01–10 mM	–	–	ND	Rabbit skeletal
Caffeine	1–100 mM	+	+	+	Rabbit skeletal, dog and rat cardiac
Chlorocresol	0.1–100 mM	+	ND	ND	Rabbit skeletal
Cyclic ADP ribose	1–17 μM	+	NE	NE	Rabbit skeletal
	1–2 μM	NE	–	NE	Skeletal
	1–2 μM	+	+	+	Dog cardiac
Dantrolene	23 nM	ND	–	ND	Rabbit skeletal
DCCD	25–200 μM	–	–	ND	Rabbit skeletal, sheep cardiac
Dithiothreitol	0.5–1 mM	ND	+	ND	Rabbit skeletal
δ-HCH	6–100 μM	+	–	ND	Rat cardiac
FLA365	0.01–20 μM	–	–	ND	Rabbit skeletal, dog and rat cardiac
MBED	0.3–10 μM	+	NE	ND	Rabbit skeletal
Perchlorate	8–100 mM	+	+	+	Rabbit skeletal, rabbit cardiac
Porphyrin	1–60 μM	+	+	+	Rabbit skeletal
Rose bengal	1–200 nM	+	–	+	Rabbit skeletal
Ruthenium red	0.001–20 μM	–	–	–	Rabbit skeletal, dog and rat cardiac
Sulmazole	0.1–10 μM	ND	+	ND	Sheep cardiac
Theophylline	1 mM	ND	ND	ND	Rabbit cardiac
Verapamil	1–1,000 μM	ND	–	–	Rabbit skeletal

+, Stimulation of Ca^{2+}-release, [^{3}H]ryanodine binding, or Ca^{2+}-release channel open probability. -, inhibition of Ca^{2+}-release, [^{3}H]ryanodine binding, or Ca^{2+}-release channel open probability; ND, not determined; NE, no effect. DCCD, N,N'-dicyclohexylcarbodiimide; δ-HCH, δ-hexachloro-cyclohexane;FLA365[2,6-dichloro-4-dimethylaminophenyl] isopropyl-amine; MBED, 9-methyl-7-bromoeudistomin D.

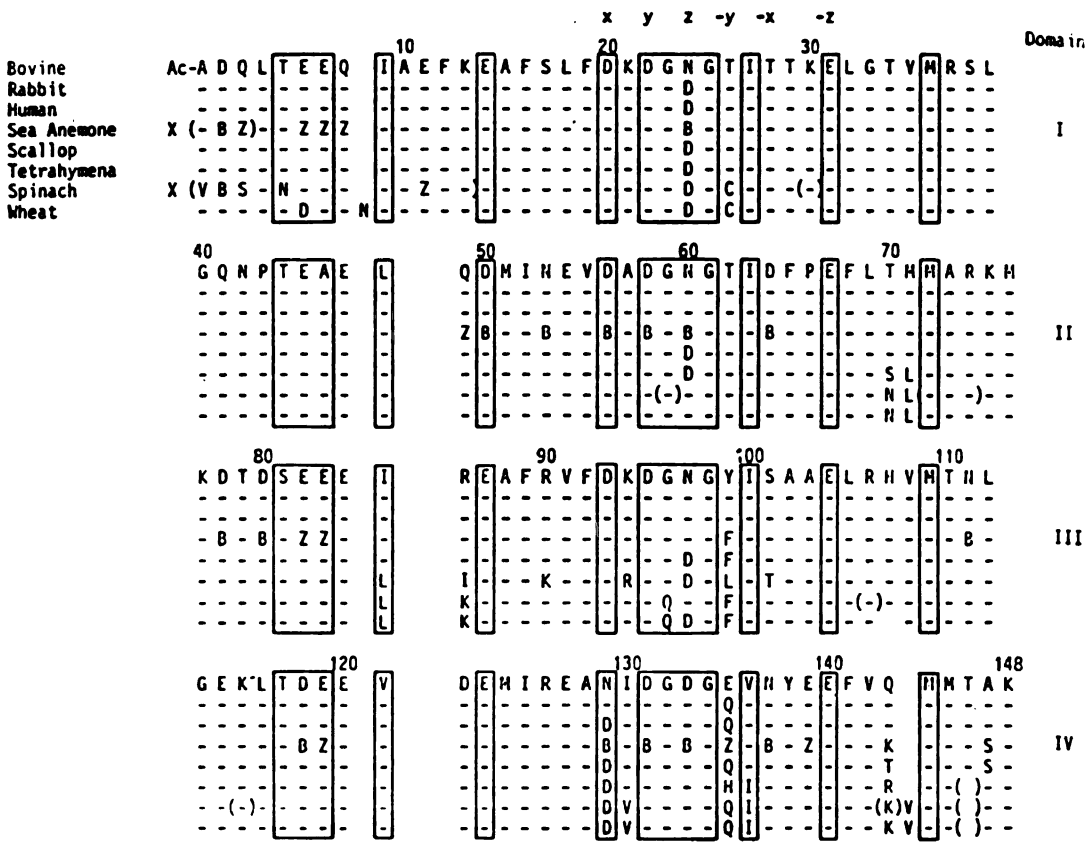


Figure 1.6 Comparison of the amino acid sequences of calmodulins in their four domain structures. Identical or similar residues among eight sequences through the four domains are boxed with solid lines (adapted from Toda et al., 1985).

The CaM sequence, which consists of 148-149 amino acids, can be divided into four domains. Each domain contains one helix-loop-helix structure which provides a Ca^{2+}-binding site. The crystal structure of CaM suggests there are two globular calcium-binding domains, each containing two Ca^{2+}-binding regions with the characteristic helix-loop-helix structure, connected by a long central helix of nearly seven turns (Babu et al., 1988; Taylor et al., 1991; Rao et al., 1993). The four Ca^{2+}-binding sites can be divided into two classes based on affinity: two high affinity sites in the C-domain which bind Ca^{2+}or Mg^{2+} and two low affinity sites in the N-domain which are specific for Ca^{2+} binding (Figure 1.7).

CaM regulates a variety of calcium-dependent intracellular processes including activation of regulatory enzymes such as certain kinases, phosphatases, cyclases, phospodiesterases, and ATPases (see Klee and Vanaman, 1982 for review). An important question regarding the mechanism of action of CaM concerns which portions of this protein participate in the interaction with individual target enzymes. O'Neil and DeGrado (1990) suggest that as a general mechanism CaM will bind in a Ca^{2+}-dependent manner with high affinity and a 1:1 stoichiometry to a variety of peptides known to form amphiphilic helices. The affinity of these peptides for CaM correlates with their propensities for forming positively charged helices suggesting this structural feature might be important for binding. On the other hand, the hydrophobic helices of these peptides may interact with their target substrates or proteins. Flexibility in the helix connecting the globular domains of CaM will clearly facilitate CaM binding to a variety of such sequences because the details of specific interaction in the complex can vary depending on the precise manner in which the two globular domains approach each other. The flexibility thus provides a mechanism by which

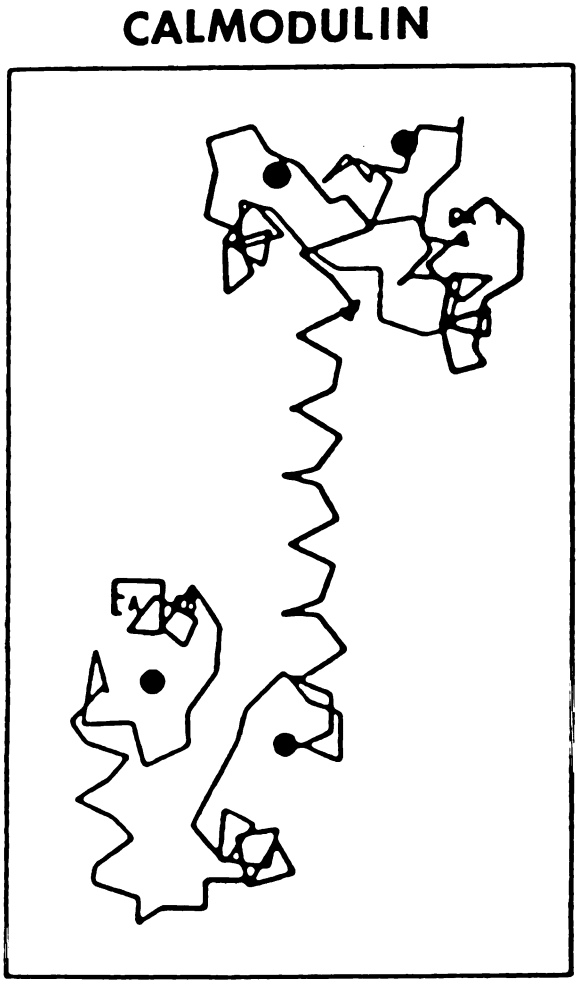


Figure 1.7 The crystal structure of calmodulin (adapted from Babu et al., 1985). The four Ca^{2+} ion are represented by dotted circles.

the highly conserved CaM molecule can bind to a wide variety of target enzymes whose CaM binding sequences display considerable variability.

Previous studies have shown that Ca^{2+} release from the heavy SR vesicles which contain the Ca^{2+}-channel protein, is directly inhibited by CaM both in skeletal muscle (Meissner, 1986a; Plank et al., 1988) and cardiac muscle (Meissner and Henderson, 1987). The SR vesicles were passively loaded with $^{45}Ca^{2+}$ in the presence of CaM and its inhibitors, followed by measurement of the Ca^{2+} release rate. The results showed that CaM at a concentration of 2-10 μM reduces Ca^{2+} efflux rates from SR vesicles by 2-3 fold in skeletal muscle (Meissner, 1986a). In cardiac SR vesicles, up to 6-fold reduction of Ca^{2+} release was observed (Meissner and Henderson, 1987). Subsequently, Smith et al. (1989), using the planar bilayer-vesicle fusion technique, reported that the inhibitory effect of CaM on both the skeletal and cardiac SR Ca^{2+}-channel proteins resulted from reduction of the open state probability via direct binding of CaM to the Ca^{2+}-channel protein rather than via phosphorylation of substrates.

More recently, fluorescence anisotropy was used to characterize CaM interaction with the skeletal muscle Ca^{2+}-channel protein in SR vesicles (Yang et al., 1994). The results showed that at low Ca^{2+} concentrations (in the presence of 1 mM EGTA), the tetrameric Ca^{2+}-channel protein binds 20-24 molecules of CaM with high affinity (K_d=8.6 nM), corresponding to 5-6 molecules of CaM per channel protein subunit. In the presence of 0.1 mM Ca^{2+}, approximately 4 high affinity (K_d=4.3 nM) and 16 low affinity (K_d=239 nM) sites per intact Ca^{2+}-channel protein were observed. In the presence of 0.1 mM Ca^{2+} and 1 mM Mg^{2+}, a single binding site with a very high affinity (K_d=0.1 nM) and around 7 sites with a lower affinity (K_d=70 nM) were obtained.

Similar results were observed by Tripathy et al. (1995), using [^{125}I]CaM and [^{3}H]ryanodine binding to SR vesicles and to the purified Ca^{2+}-channel protein. Their data indicated that the tetrameric Ca^{2+}-channel protein binds CaM with high affinity (K_d= 5-25 nM). The channel tetramer binds 16 CaM at low Ca^{2+} concentration (≤ 0.1 μM) and 4 CaM at 0.1 mM Ca^{2+}. However, their experimental conditions were not able to detect the CaM binding at the low affinity sites (K_d=239 nM) of the Ca^{2+}-channel protein. In addition, SR vesicle-$^{45}Ca^{2+}$ flux and single channel measurements showed that at lower Ca^{2+} concentrations (≤ 0.2 μM) corresponding to the resting muscle state, CaM activates the Ca^{2+} release channel 1.5-5 fold. On the other hand, at micromolar to millimolar Ca^{2+} concentrations, CaM inhibits the Ca^{2+} release channel activity 3-6 fold. These results suggest that the CaM may play dual roles in modulating SR Ca^{2+} release of skeletal muscle at both resting and elevated Ca^{2+} concentrations (Tripathy et al., 1995).

In addition, using fluorescence anisotropy measurements, binding of CaM to porcine cardiac muscle SR vesicles were determined (Strasburg et al., 1993). In the presence of $CaCl_2$ plus $MgCl_2$, binding of CaM to the Ca^{2+}-channel protein in cardiac SR vesicles yielded a K_d of 13 nM and a B_{max} of 55 pmol/mg (Strasburg et al., 1993). The results suggest that there are approximately 3 CaM binding sites per subunit in Ca^{2+}-channel protein (Strasburg et al., 1993).

2. Ryanodine

Ryanodine is a neutral alkaloid isolated from the plant *Ryania speciosa*. The structure of ryanodine is shown in Figure 1.8. Ryanodine first gained attention because of its pharmacological properties. Even at low nanomolar to micromolar concentrations, ryanodine can alter the mechanical performance of vertebrate skeletal muscle and cardiac muscle (Jenden and

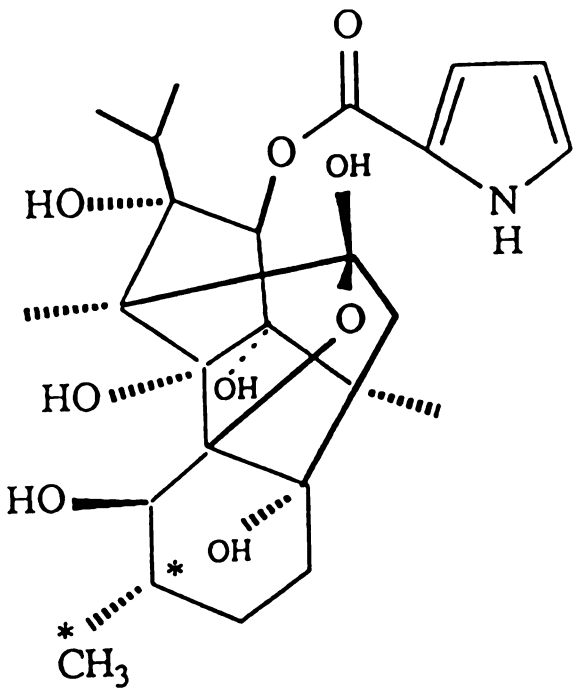


Figure 1.8 Ryanodine structure showing positions of tritium labels (9,21).

Fairhurst, 1969). However, the effects of ryanodine on muscle are complex and depend on muscle type, calcium activity, pattern of muscle stimulation, as well as ryanodine concentration.

In 1986, Meissner reported that ryanodine either stimulates or inhibits Ca^{2+} efflux from skeletal muscle heavy SR vesicles depending on the experimental conditions. At 10 nM ryanodine, the vesicle is rendered permeable to $^{45}Ca^{2+}$ even in the presence of two Ca^{2+}-channel protein inhibitors, Mg^{2+} and ruthenium red. At ryanodine concentrations greater than 10 μM, $^{45}Ca^{2+}$ efflux is inhibited in channel-activating medium (Meissner, 1986b).

The effects of high and low concentrations of ryanodine on the activity of the Ca^{2+}-channel protein were investigated using the channel protein reconstituted from SR into planar lipid bilayers (Bull et al., 1989; Lai et al.,1989; Chu et al., 1990). Upon addition of 10 nM ryanodine at the cis side (SR cytoplasmic side) in the absence of ATP, the open state probability of the Ca^{2+}-channel protein increases from 0.75 to 0.94 without modifying the current amplitude (Bull et al., 1989). However, upon addition of μM ryanodine at the cis side, the Ca^{2+}-channel protein shows long openings (Chu et al., 1990) and enters into a characteristic subconductance state which has ~40% of the normal conductance (Lai et al., 1989). Upon further addition of cis-ryanodine to millimolar concentrations, the subconductance state of the channel protein abruptly disappears; and the channel protein enters into a fully closed state (Smith et al., 1988; Lai et al., 1989). The observation of two distinct effects of low and high ryanodine concentrations on conductance behavior of the channel protein suggested the existence of high and low affinity sites for ryanodine (Lai et al., 1989).

Ryanodine is a good probe for investigating the function of the SR

Ca^{2+}-channel protein because of its specific binding properties for the Ca^{2+}-channel protein. Ryanodine binds specifically to the Ca^{2+}-channel protein in SR vesicles with a stoichiometry of 1 mole of ryanodine per mole of channel protein tetramer (Lai et al., 1988). In addition, ryanodine binds to the Ca^{2+}-channel protein only when the Ca^{2+}-channel protein is in the open state (Michalak et al., 1988; Ogawa and Harafuji, 1990; Inui et al.,1988; Pessah et al.,1986). Therefore, the binding of ^{3}H-ryanodine was used as a probe to characterize the Ca^{2+}-channel protein content in SR, channel structure, and channel activity.

3. Calcium, Magnesium, and Adenine Nucleotide

Both planar lipid bilayer-single channel recording (Smith et al., 1988; Smith et al., 1986b) and ion flux measurements (Meissner and Henderson, 1987; Meissner et al., 1986; Smith et al., 1985) have indicated that the Ca^{2+}-channel protein is activated by micromolar concentrations of cytoplasmic Ca^{2+} and by mM adenine nucleotides, and is inhibited by mM Mg^{2+}. In addition, the results of [^{3}H]ryanodine binding were consistent with the data of the previous studies in SR vesicles (Pessah et al., 1987; Pessah et al., 1986; Pessah et al., 1985; Seifert and Casida, 1986).

Smith et al. (1986b) reported that the open state probability (P_o) of the Ca^{2+}-channel protein is close to zero with nanomolar free Ca^{2+} on the cis side of the bilayer. The addition of micromolar Ca^{2+} to the cis side (cytoplasmic) activates the channel protein and induces rapid channel openings and closings occurring as single events or as bursts that could last from less than one to many milliseconds. The presence of millimolar Ca^{2+} to the cis side results in channel inactivation (Meissner, 1994). Thus, in the absence of other regulatory ligands such as Mg^{2+} and ATP, a bell-shaped Ca^{2+} activation curve of Ca^{2+} efflux from skeletal muscle heavy SR vesicles has been

obtained, with Ca^{2+} efflux being maximal in the 1-10 μM Ca^{2+} concentration range (Fill et al., 1990; Meissner et al., 1986). In addition, the Ca^{2+}-dependence of [^{3}H]ryanodine binding by skeletal SR vesicles also shows a similar bell-shaped binding curve with a Ca^{2+} threshold of 0.1-1 μM, an optimal Ca^{2+} concentration at 10-100 μM, and inhibition at Ca^{2+} concentrations > 1 mM (Imagawa et al., 1987; Mickelson et al., 1988; Chu et al., 1990; Zimanyi and Pessah, 1991). Binding of ryanodine to cardiac SR has a Ca^{2+} threshold of ~1 μM, is optimal at 10 μM to 1 mM, but, in contrast to skeletal Ca-channel proteins, shows little inhibition at high Ca^{2+} concentration (Zimanyi and Pessah, 1991; O'Brien et al., 1995).

Millimolar ATP is found to be as good as or better than Ca^{2+} in activation of Ca^{2+}-channel protein (Smith et al., 1988; Smith et al., 1986b; Meissner et al., 1986; Meissner and Henderson, 1987; Smith et al., 1985; Chu et al., 1990). However, in single channel measurements, both Ca^{2+} and ATP are required to fully activate the channel, i.e., to increase P_o close to 1.0 (Smith et al., 1986b).

Smith et al. (1986b), using the planar lipid bilayer method, indicated that millimolar ATP in the absence of Ca^{2+} increases the frequency of open events and decreases the duration of closed events (P_0=0.6). ATP and Ca^{2+} together produce a synergism of activation that increases the duration of open events and allows the channel protein to remain open nearly 100% of the time (P_o> 0.99).

The addition of 5 mM ATP or of the ATP analogue adenosine 5'-(β,γ-methylenetriphosphate)(AMP-PCP) results in intermediate Ca^{2+} release rates as determined by radioisotope flux-rapid-quench-Millipore filtration method (Meissner et al., 1986). Comparison of the activation of Ca^{2+} release between skeletal and cardiac SR vesicles showed that addition of 5 mM ATP

to 10^{-9} M Ca^{2+} medium increases $^{45}Ca^{2+}$ efflux 100-fold from skeletal SR vesicles, but only 2-fold increase from cardiac SR vesicles (Meissner and Henderson, 1987).

In [^{3}H]ryanodine binding measurements, millimolar ATP has been shown to stimulate ryanodine binding to skeletal Ca^{2+}-channel proteins (Imagawa et al., 1987; Bull et al., 1989; Chu et al., 1990; Ogawa and Harafuji, 1990; Zimanyi and Pessah, 1991), but to have little effect on cardiac Ca^{2+}-channel proteins (Michalak et al., 1988; Zimanyi and Pessah, 1991). In the presence of optimal Ca^{2+} concentration (50 μM), 1 mM AMP-PCP enhances the [^{3}H]ryanodine binding 4-fold in skeletal SR vesicles but only 1.3-fold in cardiac SR vesicles (Zimanyi and Pessah, 1991).

Various other adenine nucleotides (AMP-PCP, ADP, AMP, cAMP, adenosine, adenine) potentiate Ca^{2+} release, which suggests that activation results from binding to an effector site of the Ca^{2+}-channel protein rather than through covalent modification of the Ca^{2+}-channel protein via a phosphorylation reaction (Meissner, 1994).

Mg^{2+} affects the Ca^{2+}-channel protein in the opposite manner. Meissner et al. (1986) indicated that Mg^{2+} partially inhibits Ca^{2+}- and nucleotide-induced $^{45}Ca^{2+}$ efflux of the Ca^{2+}-channel protein in skeletal SR vesicles. Varying concentrations of Mg^{2+} were added to media containing 4 μM free Ca^{2+} and 5 mM AMP-PCP. In the presence of 0.14 mM free Mg^{2+}, the rate constant of $^{45}Ca^{2+}$ efflux was reduced from 56 to 30 s^{-1}. A more dramatic decrease of the rate constant to 1.3 s^{-1} was observed when the free Mg^{2+} concentration was increased to 4 mM. In the absence of AMP-PCP, Ca^{2+} release was fully inhibited at 5 mM Mg^{2+} (Meissner et al., 1986). Likewise, in single channel recordings, cis 4 mM Mg^{2+} partially inhibited the Ca^{2+}-channel activity by decreasing opening probability (Lai et al., 1988).

In addition, when cis 0.12 mM Mg^{2+} (4 μM free Mg^{2+}) was added to the Ca^{2+}-channel protein which had been activated by 1.84 mM ATP and <10 nM cis free Ca^{2+}, the single channel recording produced an unresolvable flickering. However, 30 seconds after Mg^{2+} addition, inhibition was essentially complete, with channel opening occurring infrequently (Smith et al., 1986b).

The cardiac Ca^{2+}-channel protein, like the skeletal channel protein, is inhibited by millimolar Mg^{2+}. Ca^{2+}-induced Ca^{2+} release rates in the presence or absence of 5 mM AMP-PCP were greatly reduced by 3 mM Mg^{2+} (Meissner and Henderson, 1987). Similarly, at the single channel level, 3-5 mM Mg^{2+} quickly blocked the channel, but the block was usually transient, with some activity returning spontaneously (Hymel et al., 1988).

Millimolar Mg^{2+} has been shown to effectively inhibit ryanodine binding to the skeletal Ca^{2+}-channel proteins but has little effect on the binding to cardiac Ca^{2+}-channel proteins (Pessah et al., 1985). The effect of Mg^{2+} has been attributed to a decrease in both the apparent binding capacity, B_{max}, and the binding affinity, K_d, resulting from a slowing of the ryanodine association rate without a change in the dissociation rate (Chu et al., 1990). Mg^{2+} inhibition of ryanodine binding may result from a direct competition between Ca^{2+} and Mg^{2+} for the Ca^{2+} activation site of the Ca^{2+}-channel protein (Pessah et al., 1987; Nagasaki and Kasai, 1983; Meissner and Henderson, 1987).

There are two proposed mechanisms for the inhibition of Ca^{2+} release by Mg^{2+}: (1) Mg^{2+} could competitively bind to low-affinity Ca^{2+} inhibitory sites of the Ca^{2+}-channel protein (Kirino et al., 1983); (2) Mg^{2+} could sterically block the Ca^{2+}-channel protein as it binds to a site near the conduction pathway (Meissner et al., 1986; Smith et al., 1986b). At present

there is insufficient evidence to support or exclude either mechanism.

4. Caffeine

Caffeine enhances the Ca^{2+}-channel protein activity in $^{45}Ca^{2+}$ efflux and in single channel measurements (Kirino et al., 1983; Meissner and Henderson, 1987; Rousseau and Meissner, 1989). Caffeine mimics the effects of cytoplasmic Ca^{2+} in that it is more effective in stimulating Ca^{2+} release from cardiac (500-fold) than skeletal vesicles (20-fold) in low Ca^{2+} medium. At micromolar Ca^{2+}, 10 mM caffeine, like 5 mM nucleotide, optimally stimulates $^{45}Ca^{2+}$ efflux from cardiac vesicles (2500-fold), whereas in skeletal vesicles caffeine has a smaller effect (16-fold) (Meissner and Henderson, 1987). Kirino et al. (1983) also indicated that caffeine enhances $^{45}Ca^{2+}$ efflux about 50% in lower Ca^{2+} concentration (pCa=6-7) but it does not in the higher Ca^{2+} concentration range. In the single-channel measurements (Rousseau and Meissner, 1989), the cardiac Ca^{2+}-channel protein is activated in a steady-state manner by mM caffeine on the cis side. In the presence of cis nM free Ca^{2+} and caffeine, the Ca^{2+} channel protein is activated by increasing the total number of open events, increasing the mean lifetime of the short open state, and causing the appearance of a second longer lasting open state. The caffeine-activated channel is moderately sensitive to the voltage applied across the bilayer, is sensitive to further activation by ATP, and is inhibited by Mg^{2+} and ruthenium red (Rousseau and Meissner, 1989).

In addition, caffeine in the millimolar range has been found to stimulate [^{3}H]ryanodine binding to skeletal Ca^{2+}-channel proteins and, to a lesser extent, to cardiac Ca^{2+}-channel protein. In the absence of Mg^{2+}, the concentration of caffeine resulting in 50% of the maximal activation (EC_{50}) is 0.26±0.03 mM for cardiac SR vesicles, whereas the EC_{50} is 1.8±0.1 mM

for skeletal SR vesicles. The result showed that caffeine is seven times more effective at stimulating [^{3}H]ryanodine binding to Ca^{2+}-channel protein in cardiac than skeletal SR vesicles. In the presence of 1 mM Mg^{2+}, caffeine is 2.5-fold more potent in the [^{3}H]ryanodine binding to cardiac Ca^{2+}-channel protein SR vesicles (EC_{50}=3.52±0.14 vs. 8.55±0.61 mM); but the maximal effect on occupancy is similar in both preparations (Zimanyi and Pessah, 1991). Chu et al. (1990) indicated that caffeine (with or without ATP) enhances the ryanodine association rate of the Ca^{2+}-channel protein without a change in the dissociation rate. However, in the presence of Ca^{2+} and Mg^{2+}, caffeine appears to increase the affinity of the activation site of the Ca^{2+}-channel protein for Ca^{2+} (Pessah et al., 1987; Kirino et al., 1983).

B. Phosphorylation of the Ca^{2+}-channel protein

In cardiac muscle, Ca^{2+} uptake activity by the SR Ca^{2+}-stimulated ATPase is stimulated by CaM- and cAMP-dependent protein kinases, resulting in increased rate of relaxation and increased contractility of the heart. Cardiac contractility may also be enhanced by CaM-dependent phosphorylation of the cardiac channel protein which could result in an increase in the Ca^{2+}-release rate.

Takasago et al. (1991) indicated that the exogenous addition of the cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG) or the endogenous CaM-dependent protein kinase (CaM-kinase) induces rapid phosphorylation of the Ca^{2+}-channel protein in canine cardiac microsomes. Added protein kinase C (PKC) also phosphorylates the cardiac Ca^{2+}-channel protein but at a relatively slow rate. The phosphorylation by PKA, PKG, and PKC increases [^{3}H]ryanodine binding around 20% comparing with control treatment, respectively. In contrast, the endogenous CaM-kinase decreases [^{3}H]ryanodine binding by 38% (added CaM alone)

(0.8 pmol/mg versus 1.2 pmol/mg) or 53% (added CaM and ATP) (0.6 pmol/mg versus 1.2 pmol/mg). The observations of phosphopeptide mapping and phosphoamino acid analysis suggested that PKA, PKG, and PKC predominately phosphorylate serine residue(s) in the same phosphopeptide, whereas the endogenous CaM-kinase phosphorylates serine residue(s) in different phosphopeptide(s). Thus, these results led to the suggestion that the phosphorylation of distinct serine residues in the Ca^{2+}-channel protein up-or-down-regulates its channel activity.

Additionally, the level of ^{32}P incorporation in the presence of PKA, PKC, or PKG was comparable with the maximal level of [^{3}H]ryanodine binding, indicating a stoichiometry of ~1 mole of phosphate per mole of the Ca^{2+}-channel protein tetramer. In contrast, the phosphorylation by endogenous CaM kinase was ~4 times greater than that with PKA, PKG, or PKC (Takasago et al., 1991). However, Witcher et al. (1991) demonstrated that phosphorylation of the channel protein by CaM-dependent kinase occurs at a single serine residue (2809) on each subunit. Phosphorylation of the cardiac channel protein by endogenous CaM kinase was about one-fourth the value achieved with exogenous CaM kinase. These results indicated that the endogenous CaM kinase phosphorylated only one of the available sites, whereas exogenous CaM kinase phosphorylated all four of the channel protein subunits. Phosphorylation of the channel protein, in turn, results in increased Ca^{2+} conductance which could cause increasing muscle contractility.

In preliminary studies, Strasburg et al. (1993) compared binding of CaM to the phosphorylated and unphosphorylated forms of channel in SR vesicles. There are two classes of CaM-binding sites in the Ca^{2+}-channel protein: high affinity and low affinity binding sites. When the cardiac

channel protein was phosphorylated by CaM-dependent kinase, it resulted in elimination of CaM binding to the low affinity CaM-binding sites; but there was no effect on CaM binding to the high affinity sites. The change of CaM binding also could result from small amounts of other proteins in the SR membrane which still bind CaM, or from uncompleted phosphorylation of cardiac channel protein.

Whereas the cardiac Ca^{2+}-channel protein is an excellent substrate for the multifunctional Ca^{2+}/CaM kinase, phosphorylation of skeletal muscle Ca^{2+}-channel protein by endogenous and exogenous CaM kinases was found to be more variable (Seiler et al., 1984; Strand et al., 1993; Wang and Best, 1992; Hain et al., 1994; Mayrleitner et al., 1995). Seiler et al. (1984) found that high molecular mass proteins of junctional SR of both cardiac and skeletal muscle, later identified as the Ca^{2+}-channel protein, were phosphorylated by endogenous CaM kinase or by exogenous PKA. Later, Witcher et al. (1991) indicated that the cardiac Ca^{2+}-channel protein is phosphorylated in junctional SR vesicles by endogenous CaM kinase and that this phosphorylation is stimulated several-fold when exogenous CaM kinase is added. In contrast, the Ca^{2+}-channel protein in canine fast and slow skeletal muscle SR vesicles is not significantly phosphorylated by either endogenous or exogenous CaM kinase (Seiler et al., 1984). Strand et al. (1993) also reported that there is minimal phosphorylation of intact porcine skeletal muscle SR Ca^{2+}-channel protein by either PKA or endogenous CaM kinase. They found that the level of ^{32}P incorporation of the cardiac Ca^{2+}-channel protein in the presence of either PKA (6.4 pmol P_i/mg SR) or CaM (endogenous CaM kinase)(10.6 pmol P_i/mg SR) is approximately equal to or twice the [^{3}H]ryanodine binding activity of this preparation (5.2 pmol/mg). In skeletal muscle, however, the level of PKA or endogenous CaM kinase

catalyzed phosphorylation (0.2 pmol P_i/mg SR for PKA and 2.9 pmol P_i/mg SR for CaM kinase) is much less than the [^{3}H]ryanodine binding activity of this fraction (11.6 pmol/mg). These results indicated that there are one or two phosphorylation sites in the cardiac Ca^{2+}-channel protein tetramer, whereas there is less than one phosphorylation site in the skeletal channel tetramer.

However, more recently, more extensive phosphorylation of the skeletal Ca^{2+}-channel protein by endogenous or exogenous CaM kinase, PKA, PKG, or PKC has been demonstrated (Suko et al., 1993; Mayrleitner et al., 1995). In the skeletal Ca^{2+}-channel protein, Ser 2843 was identified as the major target for PKA, PKG, and CaM kinase (Suko et al., 1993). The site is homologous to Ser 2809 of the cardiac Ca^{2+}-channel protein. Recently, Mayrleitner et al. (1995) found that phosphorylation of skeletal terminal cisternae of SR vesicles with PKA, PKC, or Ca^{2+}/ CaM kinase II reduces the Ca^{2+} loading rate of terminal cisternae of SR vesicles 3-fold, 1.7-fold and 2.1-fold, respectively. Phosphorylation stoichiometries of 1.94 (^{32}P/tetramer) for PKA, 0.89 for Ca^{2+}/CaM kinase II and 0.95 for PKC were obtained.

The effects of protein phosphorylation on skeletal muscle SR Ca^{2+} release showed variable effects. Herrmann-Frank and Varsanyi (1993) reported that endogenous phosphorylation of skeletal muscle SR by CaM kinase resulted in an increase in the open probability and an increase in the Ca^{2+} and ATP sensitivities of skeletal channels incorporated into planar bilayers. Hain et al. (1993) found in single channel studies that the Ca^{2+}-channel protein activity can be made insensitive to block by Mg^{2+} when terminal cisternae of SR, incorporated into planar bilayers, were treated with PKA or Ca^{2+}/ CaM kinase II, and again made sensitive by treatment with

protein phosphatases. In contrast, an inactivation of the Ca^{2+}-channel protein in skeletal muscle SR resulting from CaM kinase phosphorylation was observed (Wang and Best, 1992).

Airey et al. (1993) indicated that the α and β Ca^{2+}-channel protein isoform from chicken skeletal muscle are differentially phosphorylated by CaM kinase II. The α isoform was only slightly phosphorylated relative to that of the β isoform. This is consistent with the limited phosphorylation of mammalian skeletal muscle Ca^{2+}-channel protein which is phosphorylated to an insignificant extent by CaM kinase in comparison to the mammalian cardiac muscle Ca^{2+}-channel protein (Strand et al., 1993; Witcher et al., 1991; Takasago et al., 1991; Seiler et al., 1984). The more extensive phosphorylation of the β isoform than the α isoform in the chicken skeletal muscle SR vesicles is consistent with the similar function of this isoform with the mammalian cardiac muscle Ca^{2+}-channel protein (Airey et al., 1993).

VI. Diseases Related to the Skeletal Ca^{2+}-channel Protein Gene: Porcine Stress Syndrome and Malignant Hyperthermia

Porcine stress syndrome (PSS) has been recognized as a problem in the pork industry since the late 1950s. PSS develops with exposure of animals to cold or high temperatures, crowding, transport, and often results in deleterious pale, soft, and exudative pork (PSE) (Harrison, 1979). In addition, this syndrome is triggered following exposure of animals to volatile, halogenated anesthetics such as halothane (Hall et al., 1980). PSE meat is usually associated with stress-susceptible animals (Cheah et al., 1984). These animals have an abnormal calcium ion homeostasis that causes a flood of calcium into the system upon stress which activates muscle contraction and glycolysis (Mickelson et al., 1988, 1986). PSE meat displays

a rapid initial postmortem pH decline because of increased glycolysis, a reduction in water-holding capacity, altered protein solubility, and a pale, unstable color.

PSS is an inherited skeletal muscle disorder which is controlled by a recessive gene with incomplete penetrance referred to as the halothane (HAL) gene (Reik et al., 1983; Zhang et al., 1992). The presence of this gene is associated with smaller litter size, slower growth rate, shorter carcass length, larger longissimus muscle area, greater lean percentage, and a higher incidence of PSE pork (Simpson and Webb, 1989; Webb and Simpson, 1986). The HAL gene, when homozygous (nn), seems to increase the water content in lean muscle and suppress fat deposition in lean tissues while reducing meat quality, whereas the heterozygous individuals have higher meat quality and grow more quickly (Zhang et al., 1992).

Malignant hyperthermia (MH) is a potentially fatal genetic disease characterized by an accelerated muscle metabolism, contracture development, and rapidly rising temperature in response to certain anesthetics such as halothane and depolarizing muscle relaxants such as succinylcholine (Johnson, 1993). In humans, the trait is usually inherited in an autosomal dominant fashion, but in halothane-sensitive pigs with a similar genotype, inheritance of the disease is autosomal recessive or co-dominant. From studies of an animal model of MH condition, PSS, abnormalities of the Ca^{2+}-channel protein were postulated as the underlying cause of this condition in pigs (Ball and Johnson, 1993).

Biochemical studies involving genetically defined swine first suggested the molecular basis for abnormality in PSS-susceptible animals. SR vesicles isolated from skeletal muscle (Fill et al., 1990; Mickelson et al., 1986, 1988) and skinned muscle fiber preparations (Endo et al., 1983) from PSS-

susceptible animals displayed higher rates of Ca^{2+}-release as triggered by a variety of agents including μM Ca^{2+}, caffeine, and halothane. Other studies indicated that the resting muscle Ca^{2+} concentration in PSS-susceptible animals is elevated (Lopez et al., 1986). These data suggested that there is a defect in the Ca^{2+}-release channel protein of the skeletal muscle SR.

The studies using isolated porcine muscle SR vesicles loaded with $^{45}Ca^{2+}$ demonstrated that the rate constant for calcium release follows a bell-shaped curve with respect to the ionized Ca^{2+} concentration (Fill et al., 1990; Mickelson et al., 1986). This calcium-induced calcium release from isolated SR vesicles of PSS pigs is approximately twice as fast as calcium release from normal SR vesicles (Fill et al., 1990; Mickelson et al., 1988, 1986).

Another approach that has been used to examine the SR Ca^{2+}-channel protein function in PSS-susceptible pigs is [^{3}H] ryanodine binding. When the ryanodine-binding activities of SR vesicles derived from genetically characterized normal Yorkshire and PSS-susceptible Pietrain pigs were compared, it was observed that SR from the PSS-susceptible animals bound ryanodine with significantly higher affinity than normal SR (K_d=92 nM and 265 nM, respectively), whereas the amount of ryanodine bound was not significantly different between breeds (Mickelson et al., 1988). Furthermore, in studies with Yorkshire-Pietrain crosses, it was observed that animals heterozygous for the halothane-sensitivity gene displayed intermediate rates of Ca^{2+}-release and ryanodine affinity; i.e., Ca^{2+}-release and ryanodine affinity correlated precisely with the presence of 0, 1, or 2 copies of the halothane-sensitivity gene (Mickelson et al., 1989). These data suggested that while both normal and PSS-susceptible animals possess equal channel protein content, there could be an abnormality in the channel protein structure. This altered channel protein, in turn, could result in defective

Ca^{2+}-release and Ca^{2+}-regulation in PSS-susceptible animals.

The ultimate cause of PSS in pigs has been identified as a mutation in the skeletal muscle Ca^{2+}-channel protein at residue 615 where an arginine residue has been converted to a cysteine in the stress-susceptible animals (Fujii et al., 1991). The gene for the SR Ca^{2+}-channel protein is now known to be on swine chromosome 6 (segment 6p11-q21) as is the well known HAL linkage group (Harbitz et al., 1990).

Modern turkeys, like swine, have been subjected to intense genetic selection for rapid lean muscle growth which may partially be responsible for increased incidence of such conditions as leg weakness and edema, deep pectoral myopathy (DPM) and focal myopathy (FM) (Sosnicki and Wilson, 1991; Wilson et al., 1990). FM of turkey skeletal muscle was detected as a disorder characterized by segmental necrosis and hyaline degeneration (hypercontraction or "giant" fiber) of the fast-contracting and glycolytic (FG) and slow-contracting oxidative (SO) muscles of the pectoral and cervical regions (Sosnicki, 1993). A FM may be associated with the incidence of PSE-like breast muscle and alteration in the texture, cohesiveness and juiciness of processed turkey breast muscle.

It is striking that the turkey industry, which has intensely bred birds for the same desirable growth traits, has recently been observing severe meat quality problems which closely resemble PSE pork. Necrosis and hypercontraction of muscle fibers are commonly observed in PSE prone pigs and FM is seen in turkey muscle (Sosnicki et al., 1988; 1991a; 1991b). Factors which increase the incidence of the PSE-like condition in turkey include exposure to heat or cold stress, and loading turkeys into trucks and transporting them to the slaughter plant (Froning et al., 1978). Meat from these stress-susceptible birds tends to be very exudative, and shows lower pH

after 25 minutes post-mortem (pH 5.7) (Sosnicki and Wilson, 1992), softer texture, poorer bind and higher cooking losses compared to non-stressed birds (Sosnicki, 1993). Therefore, the similarities between the PSE-like meat from turkey with that of PSE pork (which results from PSS in the pig) strongly suggest that genetic selection for desirable growth traits in both species may have inadvertently selected for a mutated form of the SR channel resulting in undesirable meat quality characteristics.

CHAPTER 2

BIOCHEMICAL PROPERTIES OF THE SR Ca^{2+}-CHANNEL PROTEIN FROM GENETICALLY UNIMPROVED TURKEY SKELETAL MUSCLE

I. Introduction

A key step in excitation-contraction coupling is the transduction of the depolarization signal from the T-tubule to the SR, resulting in calcium release and muscle contraction (Endo, 1977). Two membrane proteins located in the T-tubule and junctional SR membrane, respectively, the DHP receptor and the SR Ca^{2+}-channel protein, regulate the process of signal transduction. However, the precise mechanisms involved in regulation of Ca^{2+} release are poorly understood. Recent studies suggest that mutations in the SR Ca^{2+}-channel protein result in muscle disease such as malignant hyperthermia (MH) (Fujii et al., 1991), which in pork, contributes a significant fraction of pale, soft, exudative (PSE) meat.

The SR Ca^{2+}-channel protein has been isolated and characterized from mammalian skeletal muscle, such as rabbit (Lai et al., 1988; Smith et al., 1988), swine (Yang et al, 1994), and dog (Seiler et al., 1984); nonmammalian vertebrate skeletal muscle, such as toadfish (O'Brien et al., 1993), blue marlin (O'Brien et al., 1995), and frog (Lai et al., 1992); avian skeletal muscle, such as chicken (Olivares et al., 1991; Airey et al., 1993; Airey et al., 1990).

The Ca^{2+}-channel protein activity is stimulated by μM Ca^{2+}, mM

caffeine, nM ryanodine, and mM adenine nucleotides (Meissner, 1986b; Meissner and Henderson, 1987). On the other hand, Ca^{2+}-channel protein activity is inhibited by mM Mg^{2+}, ruthenium red, μM ryanodine and the intracellular Ca^{2+}-binding protein calmodulin (CaM) (Meissner, 1986a, b; Meissner and Henderson, 1987). The inhibitory effect of CaM on both the skeletal and cardiac SR channel proteins results from direct binding of CaM to the channel protein (Meissner and Henderson, 1987). Crosslinking studies suggested that the binding of CaM to the porcine skeletal channel protein is Ca^{2+}-independent (Yang et al., 1994). These crosslinking results were subsequently confirmed by fluorescence studies (Yang et al., 1994).

Unlike mammalian muscle which has one skeletal muscle channel protein isoform, avian skeletal muscle contains two distinct isoforms which are designated α and β. However, the physiological roles and the importance of having two Ca^{2+}-channel protein isoforms are unclear. The different Ca^{2+}-channel protein isoforms reportedly differ with respect to CaM affinity labeling (Airey et al., 1993), phosphorylation (Seiler et al., 1984; Strand et al., 1993), sensitivity to Ca^{2+}-activation (Murayama and Ogawa, 1992), and sensitivity to Ca^{2+} inhibition (O'Brien et al., 1995).

The increased incidence of PSE turkey in recent years has triggered this study to characterize the biochemical defect leading to PSE meat. The central hypothesis upon which this study is based on that an altered Ca^{2+} release channel is present in a fraction of the commercial turkey population which results in production of PSE meat. To address this hypothesis the specific objectives of this study were to develop a purification procedure for turkey skeletal muscle SR highly enriched in Ca^{2+}-channel protein and to characterize a genetically unimproved population of turkeys with respect to skeletal SR Ca^{2+}-channel protein function. The plant alkaloid ryanodine has

proven very useful in the study of the Ca^{2+}-channel protein for two reasons. First, ryanodine binds specifically to the Ca^{2+}-channel protein in SR vesicles with a stoichiometry of 1 mole of ryanodine per mole of channel protein tetramer (Lai et al., 1988). Second, altered affinity of the channel protein for ryanodine has been used to characterize the mutation of the Ca^{2+}-channel protein in porcine MH (Mickelson et al., 1988). Thus, in this study, the binding of [^{3}H]ryanodine was used in this study as a probe to characterize the Ca^{2+}-channel protein content and channel activity in turkey SR. In addition, these turkey skeletal SR preparations were characterized with respect to stoichiometry and affinity of the Ca^{2+}-channel protein for CaM, a regulator of Ca^{2+} release in muscle. The results of this study provide a model by which subsequent studies using preparations from commercial turkeys will be compared.

II. Materials and Methods

A. Materials

1.Turkey

Turkeys belonging to a randomly back-crossed, genetically unimproved line of birds (McCartney, 1964) were obtained from the Ohio Agricultural Experimental Station (Wooster, OH). Experimental turkeys were killed by intravenous injection of sodium pentobarbital (12.5 mg/ml). The breast muscles were then removed, cut into approximately one-inch cubes, and immediately frozen in liquid nitrogen. Samples were stored at -80°C.

2. Chemicals

Benzophenone-4-maleimide and tetramethylrhodamine-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) and Wako Chemical USA, Inc. (Richmond, VA).

B. Methods

1. Preparation of Sarcoplasmic Reticulum Vesicles

Skeletal muscle SR was isolated from the breast muscles by the procedure of Mickelson et al. (1986). Briefly, the frozen muscle cubes were homogenized in a Waring blender for 60 seconds using 5 vols. (w/v) of 0.1 M NaCl, 5 mM Tris-maleate (pH 6.8). The procedure was modified to include three proteinase inhibitors (0.1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) in the homogenization buffer and in each subsequent step of the preparation. After centrifugation of the homogenate for 30 min. at 3500 x g_{max}, the pellet was discarded and the resultant supernatant was centrifuged for 30 min. at 10,000 x g_{max}. This pellet was resuspended with 0.6 M KCl, 5 mM Tris-maleate (pH 6.8) and centrifuged for 40 min. at

180,000 x g_{max} in a Beckman Ti-70 rotor. For preparation of crude total muscle membrane fraction (crude SR), the pellet was resuspended in 10% sucrose and then centrifuged at 180,000 x g_{max} in Ti-70 rotor for 40 min. The pellet was resuspended in 10% sucrose, quick-frozen and stored at -80°C. The yield of crude SR was ~150 mg from 100 g of unimproved turkey breast muscle.

Skeletal muscle SR was also isolated from the turkey breast muscle by the method of Airey et al. (1993). Briefly, muscles were homogenized 3 times for 1 min. in a Waring blender in 5 ml/g wet tissue weight in a solution containing 0.3 M sucrose, 10 mM imidazole, pH 7.4, 1.1 μM leupeptin, and 230 μM PMSF. The homogenate was centrifuged at 8,000 x g_{max} for 14 min. and the pellet was rehomogenized and centrifuged as above. Supernatants from both centrifugations were combined. Crude membranes collected by centrifugation at 130,000 x g_{max} for 90 min. were resuspended in the above solution, rapidly frozen in liquid nitrogen and stored at -80°C.

For purification of the SR fraction enriched in the Ca^{2+}-channel protein (heavy SR), the pellet from the 0.6 M KCl extraction by the Mickelson et al. (1986) procedure or from 130,000 x g_{max} for 90 min by the Airey et al. (1990) procedure was resuspended in 10% sucrose (w/v), 0.4 M KCl, 20 μM $CaCl_2$, 5 mM Tris-maleate (pH 6.8) and placed on discontinuous sucrose gradients (22%, 36%, and 45%) containing 0.4 M KCl, 20 μM $CaCl_2$, 5 mM Tris-maleate (pH 6.8) in all layers. The tubes were centrifuged for 5 hr at 112,000 x g_{max} in a Dupont Sorvall AH-629 rotor, and the material located at the interface between each sucrose layer was removed. Fractions were diluted at least 1:2 with 10% sucrose, and centrifuged at 180,000 x g_{max} in Ti-70 rotor for 40 min. The pellets were resuspended in 10% sucrose, quick-

frozen and stored at -80°C. The yield was typically 17 mg skeletal heavy SR from 100 g of unimproved turkey muscle.

2. Ryanodine Binding Assays

The ryanodine binding assays are based on that of Mickelson et al. (1988). [^{3}H]Ryanodine binding was performed by incubating 0.2 mg of heavy SR protein/ml for 90 min at 37°C in media containing 0.25 M KCl, 25 mM PIPES (pH 7.0), 4 or 10 nM [^{3}H] ryanodine, and a $CaCl_2$-EGTA-nitrilotriacetic acid buffer to give a specific $[Ca^{2+}]_{free}$ ranging from 0.01-1000 μM. Free Ca^{2+} concentrations were calculated using the computer program of Perrin and Sayce (1967), which is based on equilibrium concentrations of metal-ligand mixtrues. This information was used to derive optimal $[Ca^{2+}]_{free}$ for subsequent [^{3}H]ryanodine binding studies designed to obtain the K_d and B_{max} values. The ryanodine concentration was varied by addition of unlabeled ryanodine. Samples were filtered with Whatman GF/B filters and washed three times with 5 ml of ice-cold buffer (0.25 M KCl, 25 mM PIPES, pH 7.0). Specific ryanodine binding was determined by subtracting the nonspecific binding obtained in presence of 100 μM unlabeled ryanodine.

The K_d and B_{max} values for ryanodine binding by the SR preparations were calculated from the fit of bound vs. free ryanodine using Enzfitter computer program (Biosoft, Cambridge, UK) when ryanodine was varied from 1-500 nM.

3. Calmodulin Purification and Derivatization

Wheat germ calmodulin was prepared as described by Strasburg et al. (1988). Chicken CaM, site-specifically mutated at glutamine 143 to a cysteine residue (Q143C), was obtained from Dr. Albert Wang, Boston Biomedical Research Institute. For crosslinking experiments, both wheat germ and Q143C chicken CaM were radiolabeled with ^{125}I and

monofunctionally substituted at Cys 27 of wheat germ CaM or at Cys 143 of chicken CaM with the photo-activatable crosslinker, benzophenone-4-maleimide (Strasburg et al., 1988). For fluorescence experiments, wheat germ CaM was labeled at Cys 27 with tetramethylrhodamine-X-maleimide (Strasburg et al., 1988).

4. Calmodulin Crosslinking

Affinity labeling of CaM-binding proteins in turkey skeletal SR vesicles was performed by incubating in darkness 0.1 μM wheat germ or Q143C [^{125}I]-Bz-CaM with 100 μg of heavy SR vesicles using the buffer conditions of Yang et al. (1994) [20 mM Hepes, pH 7.5, and 0.15 M NaCl], or of Airey et al. (1993) [70 mM MOPS, pH 7.4]. $CaCl_2$, $MgCl_2$, and EGTA were included as indicated in the figure legends.

The mixtures were placed in plastic microcentrifuge tubes, the tubes were covered with a plastic wrap film, and the samples were illuminated for 5 min in a Stratalinker 1800 photoreactor (Stratagene Crop., La Jolla, CA) equipped with lamps of λ_{max} = 254 nm. After photolinking, the samples were centrifuged in Beckman TL-100 centrifuge at 88,000 x g_{max} for 20 min. The membrane pellets were resuspended in 50 μl water. For electrophoresis, 25 μl sample buffer containing 3.7% SDS, 12 mM EGTA, 30% glycerol, ~0.001% bromphenol blue, and 46 mM Hepes, pH 7.5 was added to each sample.

5. Electrophoresis

The electrophoresis procedure employed was that described by Laemmli (1970) with the modification that 4-10% linear acrylamide gradient gels were used. Slab gels were stained with 0.05% Coomassie blue in 50% methanol/10% acetic acid and then destained with 50% methanol/10% acetic acid. The gels were dried and placed with Kodak Omat XAR-5X-ray film

in autoradiography cassettes equipped with Dupont Lightning Plus intensifying screens.

6. Fluorescence Anisotropy Measurements

Fluorescence anisotropy was used to characterize CaM/calcium-channel protein interaction in SR vesicles. The principle underlying fluorescence anisotropy is that the rapid Brownian tumbling of a small molecule is slowed upon binding to a larger molecule to form a complex. If the smaller molecule is fluorescent, the altered mobility of this species resulting from complex formation can be detected by measuring the change in depolarization of fluorescent light emitted upon excitation by polarized light (Lakowicz, 1983).

Fluorescence measurements were performed using an SLM 4800 spectrofluorometer equipped with two detectors in T-format detector. Samples were held in a thermostated cell block maintained at 22°C. The excitation wavelength of Rh-CaM was 532 nm, monochromator slits were set at 4 nm, and emitted light was isolated using Schott OG-570 filters. During titrations, samples were allowed to equilibrate for 4 min after each addition of SR or Rh-CaM. All samples included 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM 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 (Yang, et al., 1994).

The anisotropy of ligand (Rh-CaM) is directly proportional to the

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

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

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 three species. If the change in quantum yield is negligible upon binding of ligand, then the equation (1) reduces to:

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

The fraction of ligand bound, and the concentrations of bound and free Rh-CaM are readily calculated.

The anisotropy of unbound Rh-CaM, A_f, was measured in the absence of SR. The anisotropy of fully bound species, A_b, was obtained by titration of Rh-CaM with SR vesicles, following by curve-fitting using the Enzfitter computer program (Biosoft, Cambridge, UK) for a single class of ligand binding site. Corrections for light scattering and background fluorescence were made by application of the equation:

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

where A, A_1 and A_2 are the anisotropies of 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.

7. Protein Assay

SR protein concentrations were determined by the Lowry method (Lowry et al., 1951). Using bovine serum albumin as a standard.

8. Statistical analysis

Comparisons of the mean K_d and B_{max} values for CaM binding in different conditions were made with Student's t test.

III. Results and Discussions

A. Purification of SR from Turkey Skeletal Muscle

In order to study the biochemical properties of the turkey skeletal Ca^{2+}-channel protein, it was first necessary to optimize the preparation of the SR membrane fraction heavy SR which is highly enriched in the Ca^{2+}-channel protein. Thus, heavy SR from turkey skeletal muscles was purified by two methods to obtain effective yield of the Ca^{2+}-channel protein: (1) the method of Airey et al. (1993) which was developed for chicken SR; (2) the method of Mickelson et al. (1986) which has been used for pig. Since both chicken and turkey are avian, it was expected that the SR purification procedure developed for chicken skeletal muscle should also work for turkey. However, the SDS-polyacrylamide gels of purified light and heavy SR vesicles from turkey skeletal muscle using both purification procedures indicated significant differences in heavy SR quality (Figure 2.1). As with the chicken skeletal muscle SR Ca^{2+}-channel protein (Airey et al., 1993), turkey skeletal muscle SR Ca^{2+}-channel protein has two isoforms, α and β, which were distinguished by differences in mobility on SDS-PAGE (Figure 2.1). Comparison of the Ca^{2+}-channel protein yields from both methods indicates the preparation of SR from turkey skeletal muscle by the procedure of Mickelson et al. (1986) resulted in SR vesicles with greater channel protein content than that obtained by the procedure of Airey et al. (1993) (Figure 2.1, lanes 3, 5, and 7). In addition, the heavy SR preparation using Mickelson et al. procedure without mincing of the muscle before blending showed greater channel protein yield than that with a mincing step (Figure 2.1, lanes 5 and 7). Thus, the method of Mickelson et al. (1986) was adopted for all subsequent SR preparations.

B. Ca^{2+}-dependence of [^{3}H]Ryanodine Binding Assays

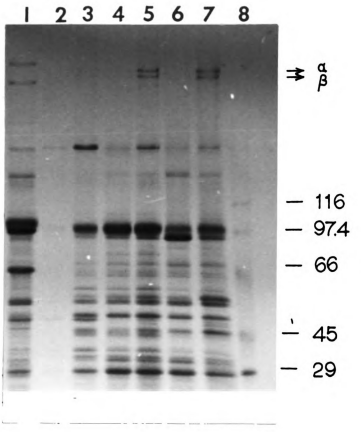


Figure 2.1 SDS-polyacrylamide gel of SR preparations from unimproved turkey and porcine skeletal muscles. Lane 1: porcine skeletal muscle crude SR; lane 2: turkey light SR prepared using Airey et al. (1993) method; lane 3: turkey heavy SR prepared as in lane 2; lane 4: turkey light SR prepared using Mickelson et al. (1986) method with mincing step; lane 5: turkey heavy SR prepared as in lane 4; lane 6: turkey light SR prepared using Mickelson et al. method without mincing step; lane 7: turkey heavy SR as in lane 6; lane 8: molecular weight markers.
Arrows indicate the Ca^{2+}-channel protein subunits which occur as two isoforms in turkey. The lower band of doublet in porcine SR has been idenitified as a proteolytic degradation product of the upper band.

Prior to characterizing the PSE-like meat quality problems of commercial turkeys which are highly selected for particular traits, it is first necessary to establish the basic biochemical properties of SR from turkeys which are derived from a randomly back-crossed, genetically unimproved line. The [^{3}H]ryanodine binding assay is a good approach used to characterize the biochemical properties of the Ca^{2+}-channel protein. Thus, the Ca^{2+}-channel protein activity, inactivation, and sensitivity of the unimproved turkey were studied by using [^{3}H]ryanodine binding assays.

The Ca^{2+} dependence of [^{3}H]ryanodine binding to turkey skeletal muscle heavy SR vesicles from seven individual preparations is indicated in Figure 2.2. [^{3}H]ryanodine binding was activated at a threshold concentration of approximately 0.2 μM Ca^{2+}, and reached a plateau of binding over the range of 3 to 30 μM free Ca^{2+} (Figure 2.2). The mean [^{3}H]ryanodine binding at the plateau between 3 and 30 μM Ca^{2+} concentrations was 0.59 pmol/mg of heavy SR protein. When the free Ca^{2+} concentration reached 1 mM, the average [^{3}H]ryanodine binding decreased only slightly to a mean value of 0.47 pmol/mg of heavy SR protein, corresponding to a 20% inhibition of ryanodine binding. Since ryanodine binding is an indicator of Ca^{2+}-channel protein activity, these results suggest that mM cytoplasmic levels of Ca^{2+} have only a slight effect on reducing the open state of the Ca^{2+}-channel protein. Thus, high cytoplasmic Ca^{2+} levels would not be likely to inhibit Ca^{2+} release in turkey muscle.

These results are similar to those obtained for blue-marlin skeletal swimming muscle (which contains both α and β isoforms) and for marlin cardiac muscle. Ryanodine binding in the fish skeletal SR system was inhibited only about 40% at 1 mM Ca^{2+}; the cardiac SR ryanodine binding activity was inhibited only about 20% (O'Brien et al., 1995). In contrast, the

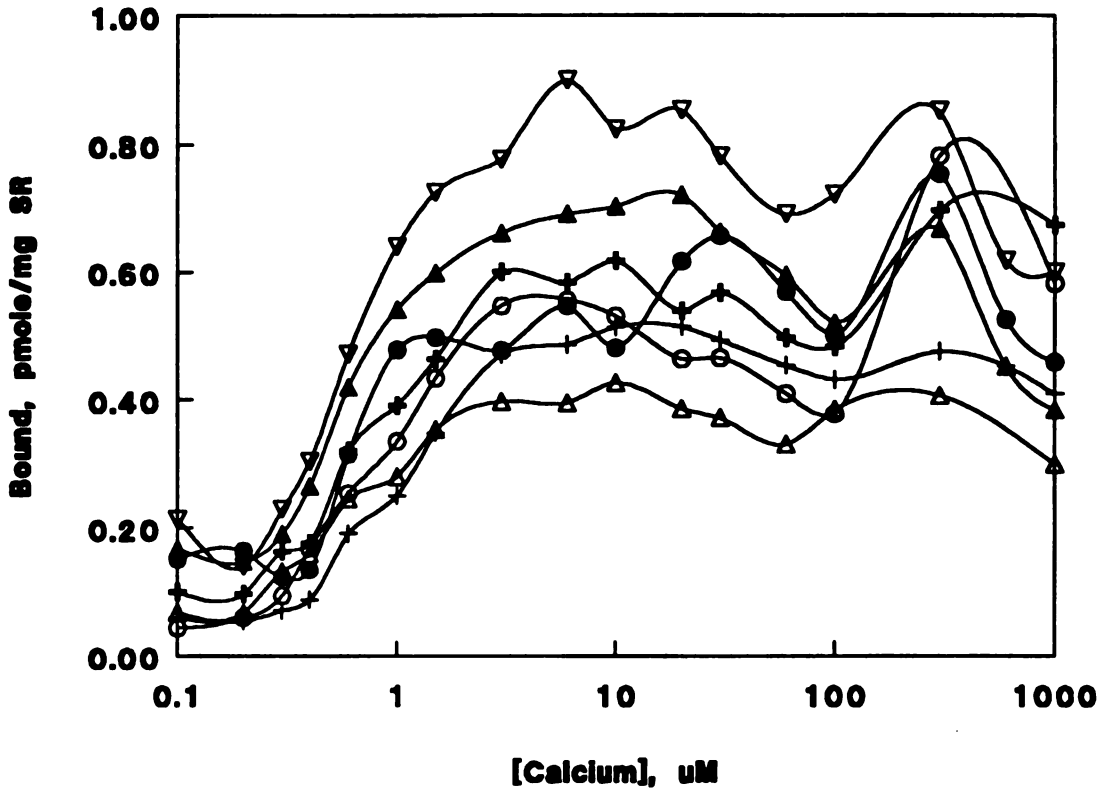


Figure 2.2 Ca^{2+} dependence of [^{3}H]ryanodine binding to genetically unimproved turkey skeletal muscle heavy SR vesicles. Curves represent results from seven individual unimproved turkey SR vesicle preparations.

marlin superior rectus extraocular muscle SR which contains only the α isoform, was virtually complete inactivation of ryanodine binding at 1 mM Ca^{2+} (O'Brien et al., 1995). This response by the α isoform is very similar to that of mammalian skeletal muscle (O'Brien et al., 1995; Mickelson et al., 1988). Thus, the lack of inactivation at high Ca^{2+} concentrations is probably because of the inherent properties of the β isoform which is functionally similar to the cardiac Ca^{2+}-channel protein isoform (Airey et al., 1993; O'Brien et al., 1995).

Five of the seven turkey SR preparations also displayed an unusual [^{3}H]ryanodine binding peak in the range of 300-600 μM Ca^{2+} in addition to the broad peak centered around 10 μM Ca^{2+}. Other muscle preparations which include both α and β isoforms (fish), show a Ca^{2+} dependence of the Ca^{2+}-channel protein activity reaching a single maximum at about 100 μM Ca^{2+}. The unusual [^{3}H]ryanodine binding peak shown at 300 μM Ca^{2+} by some, but not all turkey skeletal Ca^{2+}-channel protein might result from different genetic variants of the β isoform in turkeys.

C. Ryanodine-dependence of [^{3}H]Ryanodine Binding Assays

[^{3}H]ryanodine-dependent ryanodine binding assays were conducted to characterize the Ca^{2+}-channel protein content and channel activity of heavy SR vesicles from turkey. [^{3}H]ryanodine binding to turkey skeletal muscle heavy SR vesicles was measured at various ryanodine concentrations in the presence of 10 μM Ca^{2+}, the optimal [Ca^{2+}] determined from the previous section (Figure 2.3). The binding capacity (B_{max}) and affinity (K_d) of SR preparations were calculated using the Enzfitter program applied for a single-site ligand binding model (Figure 2.3). The heavy SR preparations (n=7) exhibited an average K_d of 20.5±7.6 nM and a B_{max} of 4.01±0.17 pmol/mg of protein (Figure 2.3). The B_{max} is similar with that reported for chicken

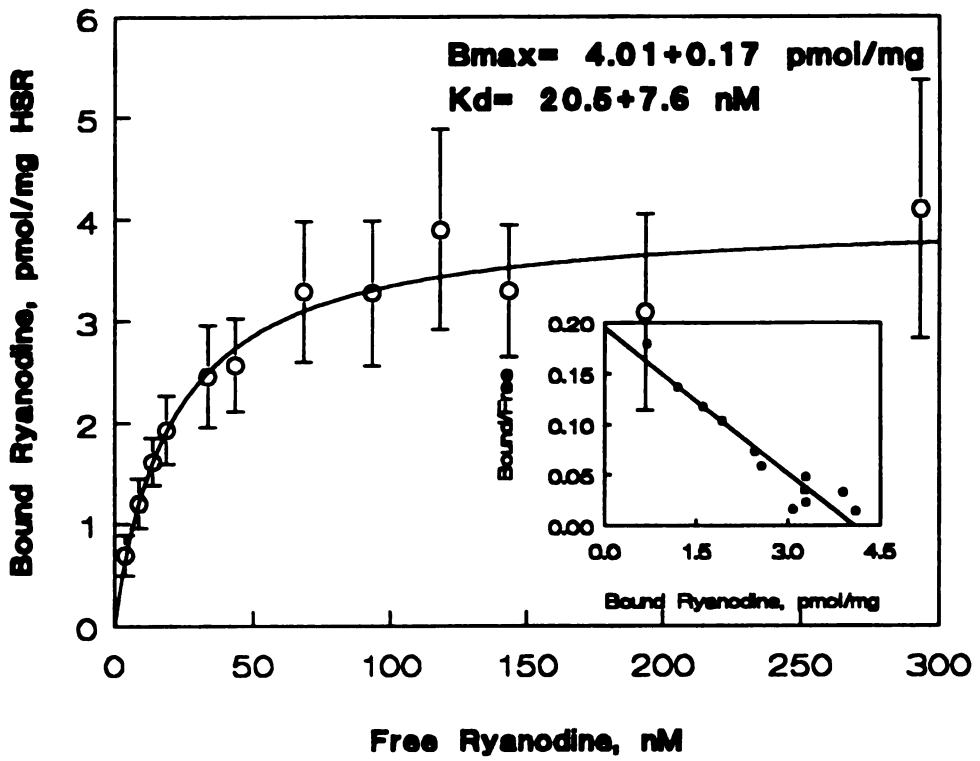


Figure 2.3 Ryanodine dependence of [^{3}H]ryanodine binding to unimproved turkey skeletal muscle heavy SR vesicles at 10 μM Ca^{2+}. The inset is a Scatchard plot of ryanodine binding to SR vesicles. Points represent the means ± SE of duplicates for each of seven different heavy SR preparations. B_{max} and K_d values were calculated using the Enzfitter program as indicated in Methods.

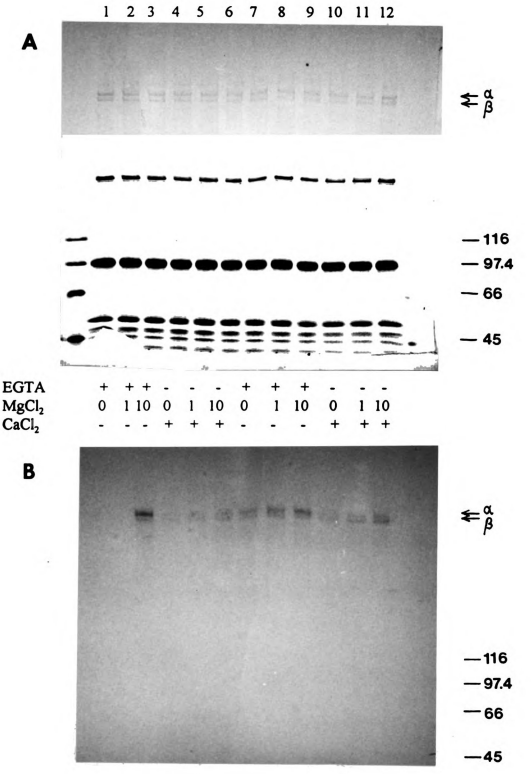
skeletal heavy SR, 3.45 pmol/mg protein (Airey et al., 1990).

D. Calmodulin Binding Activity of Ca^{2+}-channel Protein Isoforms

Airey et al. (1993) suggested that there is differential binding of CaM by the two channel protein isoforms. Using crosslinking methods they showed that the α Ca^{2+}-channel protein isoform from chicken bound CaM to a much greater extent than the β-isoform. In addition, CaM affinity labeling was strongly enhanced by the presence of 0.4 mM Ca^{2+} over that of 1.5 mM EGTA, suggesting that CaM binding is Ca^{2+}-sensitive. The observed differences in affinity labeling patterns between the porcine channel (Yang et al., 1994) and the avian channel proteins (Airey et al., 1993) could result from three possibilities: 1) differing buffer conditions between the two experiments; 2) different calmodulin derivatives employed for crosslinking; and 3) species differences in CaM-binding activity. Thus, this study tried to clarify the reason(s) resulting in these different observations.

The two isoforms of the Ca^{2+}-channel protein in turkey skeletal muscle are evident in the gel electrophoretogram (Figure 2.4A). The autoradiogram of the gel electrophoretogram (Figure 2.4B) shows that the Ca^{2+}-channel protein is the major protein which formed a complex with CaM in turkey SR. The autoradiogram (Figure 2.4B) also indicates that binding activity of both turkey isoforms for the wheat germ [^{125}I]-Bz-CaM is similar in the presence of Ca^{2+}, in contrast to that reported by Airey et al. (1993). Using the crosslinking conditions of Airey et al. (1993) (Figure 2.4, lanes 1-6) in which there was no added NaCl, binding of CaM was Ca^{2+}-dependent, although Mg^{2+} clearly enhanced binding of CaM. In contrast, using the conditions of Yang et al. (1994) in which 0.15 M NaCl was included, CaM binding was Ca^{2+}-independent. This suggests that ionic strength effects rather than species differences account for the previously reported Ca^{2+}-

Figure 2.4 [Mg^{2+}] and [Ca^{2+}] dependence of affinity labeling of turkey skeletal muscle heavy SR with wheat germ [^{125}I]-Bz-CaM under different buffer conditions. These experiments were conducted using wheat germ CaM with the benzophenone-maleimide cross-linker at Cys-27, and using buffer conditions of Yang et al. described previously (1994) [20 mM Hepes, pH 7.5, and 0.15 M NaCl] (A and B, lanes 7-12), or Airey et al. (1993) [70 mM MOPS, pH 7.4] (A and B, lanes 1-6). (A) Coomassie blue stained gel. (B) Autoradiogram of dried gel. 1 mM EGTA (lanes 1-3, and 7-9) or 0.1 mM $CaCl_2$ (lanes 4-6, and 10-12) were included in buffer with 0, 1, or 10 mM $MgCl_2$. Molecular weight markers were indicated as kDa.


1 2 3 4 5 6 7 8 9 10 11 12
A
α
β
116
97.4
66
45
EGTA + + + - - - + + + - - -
MgCl2 0 1 10 0 1 10 0 1 10 0 1 10
CaCl2 - - - + + + - - - + + +
B
α
β
116
97.4
66
45

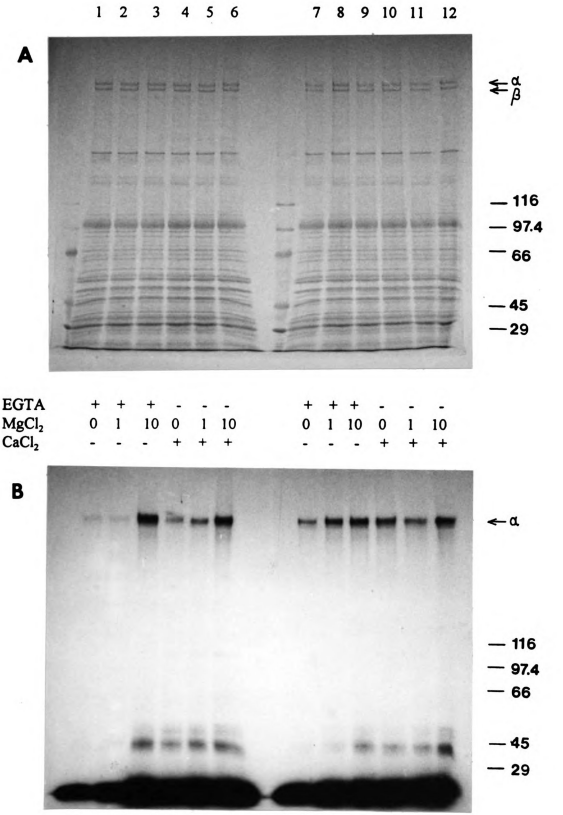
sensitive CaM binding by the avian Ca^{2+}-channel protein.

The CaM derivatives employed by the two laboratories also differed. Airey et al. (1993) used a mammalian CaM with a methyl azido benzimidate crosslinker which attaches at various amino sites on the CaM molecule, mostly near the C-domain. Yang et al. (1994) used wheat germ CaM which has a single site for crosslinking in the N-domain. To determine whether the differing crosslinking results could also be due to differences in crosslinking efficiency between the N- and C-domain of CaM, another CaM derivative, a site-specific mutant of chicken CaM which has a Cys in the C-domain of protein (Q143C) was employed. The benzophenone-4-maleimide crosslinker was conjugated to this location.

The crosslinking results with wheat germ CaM and Q143C CaM show CaM affinity labeling patterns similar to that reported by Airey et al. (1993) results when using their buffer condition (Figure 2.4 and 2.5). However, the autoradiogram (Figure 2.5B) indicates the α-isoform is the predominantly affinity labeled isoform of the Ca^{2+}-channel protein when using (Q143C) [^{125}I]-Bz-CaM. Comparison of autoradiograms of Figure 2.4 and Figure 2.5 indicates that effects of buffer conditions on affinity labeling of the α subunit are the same with both forms of CaM. These results further support the importance of buffer condition on the affinity labeling patterns.

The crosslinking results with Q143C CaM further suggest that the α and ß isoforms of turkey muscle may interact differently with CaM, since the ß isoform is not affinity-labeled by this chicken mutant CaM derivative under any of the buffer condition examined (Figure 2.5B). The lack of crosslinking by the β subunit of the channel protein with Q143C CaM and reduced crosslinking of the β subunit by the CaM derivative employed by Airey et al. (1993) suggest that the binding site of CaM for β subunit of

Figure 2.5 [Mg^{2+}] and [Ca^{2+}] dependence of affinity labeling of turkey skeletal muscle heavy SR with chicken (Q143C) [^{125}I]-Bz-CaM under different buffer conditions. These studies were conducted using the site-specific mutant of chicken CaM which has a Cys in the C-domain of protein (Cys143). Crosslinking experiments were conducted as indicated in Figure 2.4. (A) Coomassie blue stained gel. (B) Autoradiogram of dried gel. 1 mM EGTA (lanes 1-3, and 7-9) or 0.1 mM $CaCl_2$ (lanes 4-6, and 10-12) were included in buffer with 0, 1, or 10 mM $MgCl_2$. Molecular weight markers were indicated as kDa.


1 2 3 4 5 6 7 8 9 10 11 12
A
α
β
116
97.4
66
45
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EGTA + + + - - - + + + - - -
MgCl2 0 1 10 0 1 10 0 1 10 0 1 10
CaCl2 - - - + + + - - - + + +
B
α
116
97.4
66
45
29

channel protein differs from that of the α subunit. The channel protein binding site may be in a different location on the CaM molecule such that the crosslinker is not in the proper orientation for crosslinking, alternatively the crosslinkers in the C-domain may sterically block binding of the β isoform.

These studies indicate that the factors of species and CaM derivative differences did not cause the observed differences between our previous studies with pig (Yang et al., 1994) and those of Airey et al. (1993) with chicken. In addition, in the presence of NaCl at physiological ionic strength, the CaM affinity labeling of the channel protein was Ca^{2+}-independent (Figure 2.4) which is consistent with porcine studies (Yang et al., 1994). These results suggest that differences in affinity labeling patterns between avian and mammalian channel proteins are affected by crosslinking conditions.

E. Quantitation of Calmodulin-binding Activity of Ca^{2+}-channel Protein in Turkey Skeletal Muscle

Crosslinking studies provide valuable information on identification of channel proteins which bind CaM. However, crosslinking studies are not quantitative, and therefore, may yield misleading results on affinity of the channel protein for CaM. In particular, crosslinking of CaM to target proteins in the presence of EGTA may occur if weak complexes are formed. These crosslinking patterns might not be physiologically representative. Fluorescence studies were initiated to determine whether CaM indeed strongly binds under the conditions of low Ca^{2+} concentrations.

Turkey skeletal heavy SR vesicles were titrated into Rh-CaM under two different conditions: 1 mM EGTA which would represent the case of resting muscle, or 0.1 mM $CaCl_2$ which would simulate contracting muscle

[Ca^{2+}] conditions. In both cases, titration of SR vesicles into Rh-CaM resulted in an increase in fluorescence anisotropy which is attributable to the increased molecular mass of the Rh-CaM/Ca^{2+}-channel protein complex (Figure 2.6). These results support the affinity labeling data in Figure 2.4 which indicate that in the presence of 0.15 M NaCl, binding of CaM to the channel protein is Ca^{2+}-insensitive.

Data from these titrations (Figure 2.6) 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) for subsequent experiments to determine stoichiometry of Rh-CaM binding. The average A_b values obtained from three turkey heavy SR vesicle preparations for each metal ion condition are 0.2344 (+0.1 mM $CaCl_2$) and 0.2234 (+1 mM EGTA). The A_f values obtained from 10 nM samples of Rh-CaM, under these two buffer conditions in the absence of added SR, were 0.1653 (+0.1 mM $CaCl_2$) and 0.1656 (+1 mM EGTA). There was no significance in fluorescence intensity upon binding of Rh-CaM to the Ca^{2+}-channel protein indicating that there was no change in quantum yield upon complex formation (q=1.0). Therefore, the fraction of Rh-CaM bound was calculated using equation 2 of Materials and Methods.

CaM crosslinking experiments (Figure 2.4) suggested that ionic strength affects the CaM affinity labeling to the Ca^{2+}-channel protein. To obtain quantitative data on the influence of ionic strength on binding of CaM to the turkey skeletal SR Ca^{2+}-channel protein, a Rh-CaM/SR mixture was titrated with KCl, and binding was monitored by the increase in anisotropy (Figure 2.7). The chosen concentrations of SR vesicles (80 μg for the 0.1 mM $CaCl_2$ condition, 200 μg for the 1 mM EGTA) and Rh-CaM (10 nM) correspond to the mid-points of the titration curves in Figure 2.6. Increased

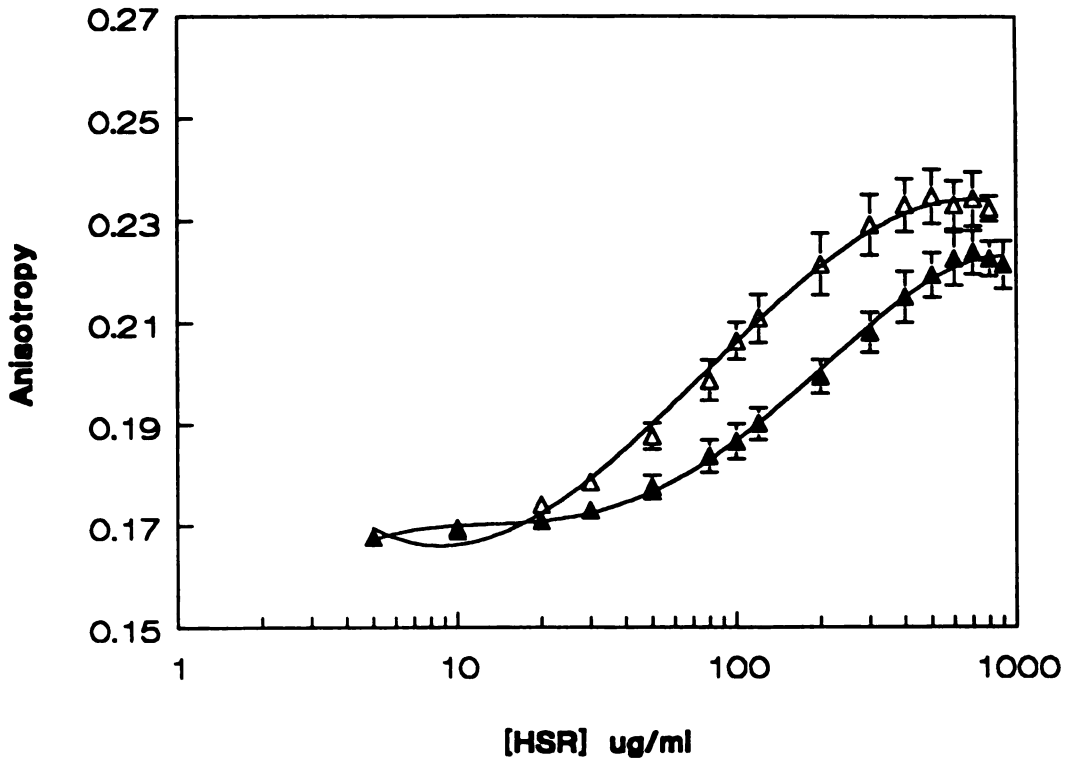


Figure 2.6 Titration of Rh-CaM with turkey skeletal heavy SR vesicles under different conditions. The sample medium contained 10 nM Rh-CaM, 0.3 M sucrose, 0.15 M KCl, and 50 mM Hepes, pH 7.0, and either 1 mM EGTA (▲) or 0.1 mM $CaCl_2$ (△) in a starting volume of 1 mL. The Rh-CaM sample was titrated with SR vesicles in parallel with a buffer blank containing the same media minus Rh-CaM. Corrections were made for light scattering as described under Methods. Points represent the means ± SE of three preparations.

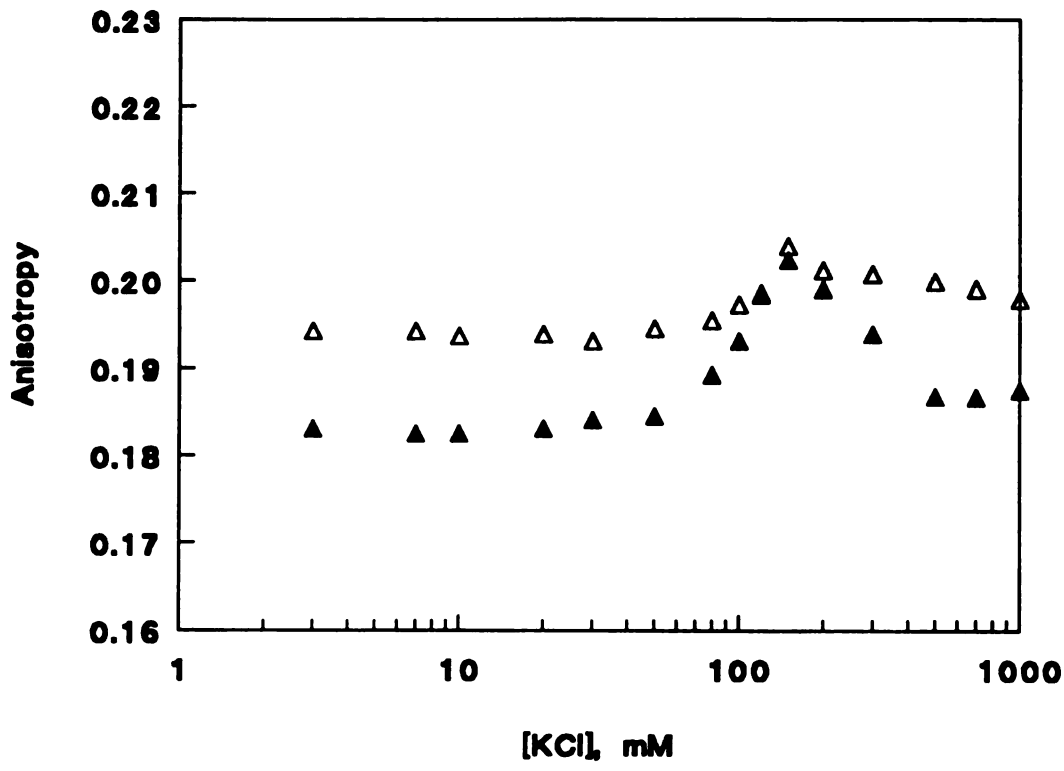


Figure 2.7 [KCl] dependence of binding of Rh-CaM to turkey skeletal heavy SR vesicles under different [Ca^{2+}] conditions. The sample buffer contained heavy SR, 10 nM Rh-CaM, 0.3 M sucrose, 50 mM Hepes, pH 7.0, and either 1 mM EGTA (▲) or 0.1 mM $CaCl_2$ (△) in a starting volume of 1 mL. Points represent the means ± SE of three preparations. CaM bound was calculated from measured anisotropy as described under Methods.

anisotropy as a function of increased [KCl] reflects increased complex formation; decreased anisotropy reflects decreased binding. The KCl titration data clearly show that in the presence of EGTA, the binding of Rh-CaM to the channel protein was highly ionic strength dependent. Binding is strongest over the range of physiological ionic strength, ~100-150 mM. At [KCl]>0.3 M, the anisotropy declined, indicating decreased binding of Rh-CaM to the Ca^{2+}-channel protein. In the presence of 0.1 mM Ca^{2+}, titration of KCl into SR/Rh-CaM mixture also indicated a significantly higher affinity of Rh-CaM for the channel protein. However, in contrast to the EGTA conditions, binding of Rh-CaM to the channel protein did not significantly decrease at higher KCl concentration. These results were similar to that obtained for porcine SR vesicles (Yang et al., 1994), and are consistent with data obtained in crosslinking studies (Figure 2.7).

Taken together, the results of Figures 2.4-2.7 suggest that the observed differences in affinity labeling patterns of the Ca^{2+} channel between Yang et al. (1994) studies with porcine muscle and those of Airey et al. (1993) with avian skeletal muscle result from the different buffer conditions of the experiments.

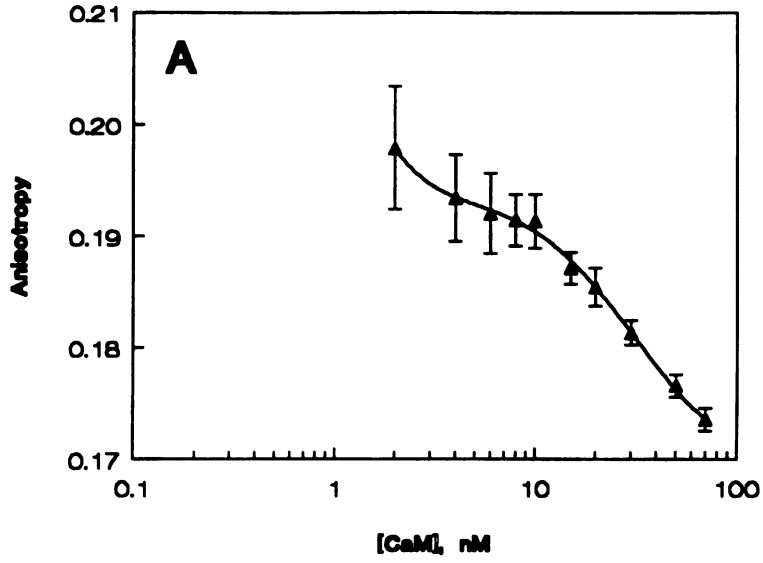
These experimental conditions were subsequently used to determine the binding capacity and affinity of the skeletal Ca^{2+}-channel protein in SR vesicles for Rh-CaM under both Ca^{2+} conditions: 0.1 mM $CaCl_2$, and 1 mM EGTA. Rh-CaM was titrated into a suspension of SR vesicles. A_f and A_b values were used to calculate the fraction of Rh-CaM bound to the Ca^{2+}-channel protein in SR vesicles for each point in the titration. The fraction of Rh-CaM bound was then converted to stoichiometry and affinity.

Results of titrations conducted in the presence of EGTA indicate a single class of binding sites on the turkey SR Ca^{2+}-channel proteins for CaM

(Figure 2.8 and Table 2.1). Analysis of data using the Enzfitter program yields a dissociation constant, K_d, of 9.1±1.7 nM and a binding capacity, B_{max}, of 56.2±4.7 pmol/mg of SR protein. In the presence of 0.1 mM $CaCl_2$, the titration data also suggest a single class of binding site, which has K_d of 7.3±1.4 nM, and B_{max} of 84.4±24.2 pmol/mg of SR protein (Figure 2.9 and Table 2.1). According to statistical analysis, B_{max} values of the Ca^{2+}-channel protein for CaM shows significant difference ($p<0.05$) between these two conditions, whereas K_d values were not significantly different. Ryanodine binding studies on the turkey heavy SR preparations yielded a B_{max} value of 4.01 pmol/mg of SR protein (Figure 2.3). Since one mole of ryanodine binds specifically with high affinity per mole of Ca^{2+} channel protein tetramer, these data suggest that there are approximately 3.5 CaM molecules bound per channel protein subunit (14 CaM molecules per channel protein tetramer) in the presence of EGTA and 5 CaM molecules bound per subunit (21 CaM molecules per channel tetramer) in the presence of 0.1 mM $CaCl_2$. Crosslinking studies in the presence of EGTA indicated substantially greater CaM bound to the α subunit than to β (Figure 2.4, lane 7). In the presence of 0.1 mM Ca^{2+}, the α and β subunits appear to be crosslinked in approximately a 1:1 ratio (Figure 2.4, lane 10), thus suggesting that as Ca^{2+} concentration is increased, the additional CaM binding occurs at β subunit (Figure 2.4). These results suggest that CaM differentially regulates Ca^{2+} release activity by α and β subunits of avian skeletal muscle.

Studies of titrations conducted are indicative of a single class of CaM binding site on the SR Ca^{2+} channel protein for CaM in the presence of EGTA, and two classes of CaM binding sites in the presence of 0.1 mM $CaCl_2$ (Yang et al., 1994). The stoichiometry of CaM binding to the porcine skeletal muscle Ca^{2+}-channel protein is approximately 20 mol/mol of tetramer

Figure 2.8 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 of Rh-CaM binding to SR vesicles. The sample medium contained 200 μg of heavy SR, 0.3 M sucrose, 0.15 M KCl, 50 mM Hepes, pH 7.0, and 1 mM EGTA in a starting volume of 1 mL. Points represent the means ± SE of four preparations. CaM bound was calculated from measured anisotropy as described under Methods.


A
Anisotropy
0.21
0.20
0.19
0.18
0.17
0.1
1
10
100
[CaM], nM

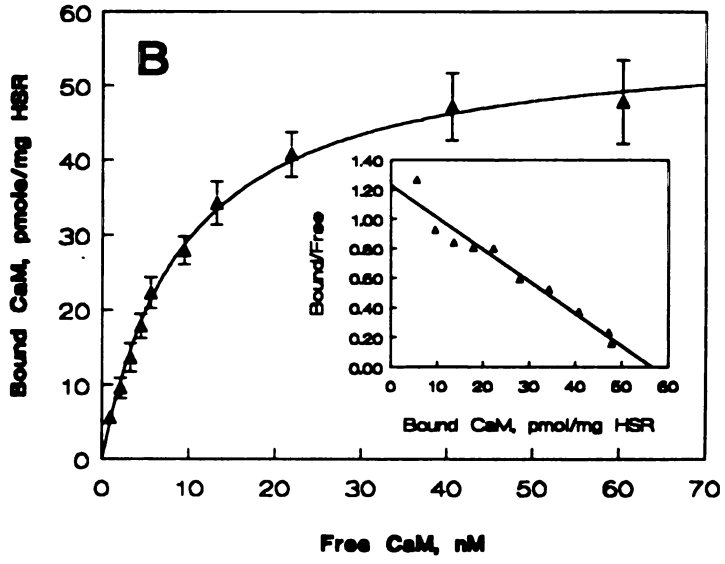
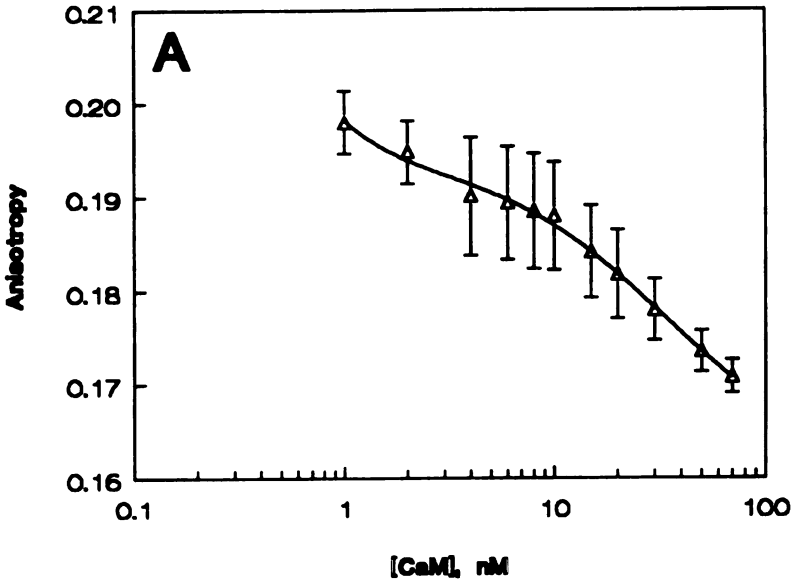

B
Bound CaM, pmole/mg HSR
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Free CaM, nM
Bound/Free
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
10
20
30
40
50
60
Bound CaM, pmol/mg HSR

Figure 2.9 Titration of skeletal heavy SR vesicles 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 Rh-CaM binding to SR vesicles. The sample medium contained 80 μg of heavy SR, 0.3 M sucrose, 0.15 M KCl, 50 mM Hepes, pH 7.0, and 0.1 mM $CaCl_2$ in a starting volume of 1 mL. Points represent the means ± SE of four preparations. CaM bound was calculated from measured anisotropy as described under Methods.


A
Anisotropy
0.21
0.20
0.19
0.18
0.17
0.16
0.1
1
10
100
[CaM], nM

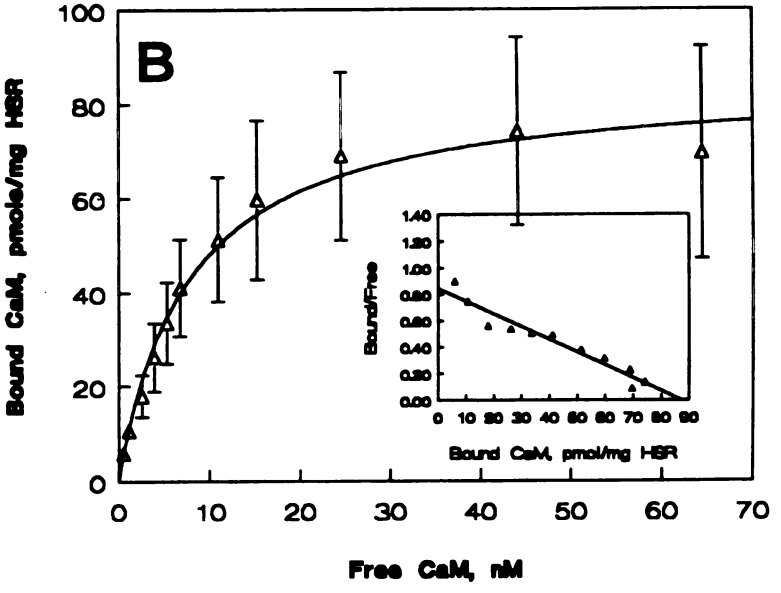

B
Bound CaM, pmole/mg HSR
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Free CaM, nM
Bound/Free
Bound CaM, pmol/mg HSR

Table 2.1 Equilibrium Constants for Rh-CaM Interaction with the Ca^{2+}-channel Protein in Turkey Skeletal Muscle Heavy SR vesicles

Condition	K_d , nM	B_{max}, pmol/mg
.1mM $CaCl_2$	7.3 ± 1.4	84.4 ± 24.2*
1mM EGTA	9.1 ± 1.7	56.2 ± 4.7*

* $p<0.05$.

** Data were obtained from titrations of SR vesicles with Rh-CaM in the presence of 0.3 M sucrose, 0.15 M KCl, 50 mM Hepes, pH 7.0, and metal ion conditions as listed below. Data are ±SE of the means of four preparations each.

(5 mol/mol of subunit) in the presence of 1 mM EGTA. In the presence of 0.1 mM $CaCl_2$, the Ca^{2+}-channel protein binds with 5 CaM at the high affinity site, and 16 CaM at the low affinity site (Yang et al., 1994). In agreement with Yang et al. result, Tripathy et al. (1995) indicated that the stoichiometry of CaM binding to rabbit skeletal muscle Ca^{2+}-channel protein with high affinity is 16 mol/mol at $\leq$0.1 μM Ca^{2+} and 4 mol/mol at 100 μM Ca^{2+}. However, our results of turkey skeletal muscle Ca^{2+} channel protein suggest that there is only one class of CaM binding sites on the α Ca^{2+} channel protein isoform in the presence of both EGTA and 0.1 mM $CaCl_2$ (Figure 2.8 and Figure 2.9). The additional CaM binding in the presence of Ca^{2+} may take place on the β isoform, which is consistent with the "cardiac-like" nature of this isoform.

IV. Conclusions

Like the chicken skeletal muscle Ca^{2+} channel protein, the Ca^{2+} channel protein of turkey skeletal muscle contains two isoforms, α and β. The Ca^{2+} dependence of [^{3}H]ryanodine binding by turkey skeletal muscle heavy SR vesicles shows maximum binding at about 10 μM, and Ca^{2+}-channel protein activity was only partially inhibited at 1 mM Ca^{2+}. This ryanodine binding inactivation phenomena of the Ca^{2+}-channel protein at high Ca^{2+} concentrations were attributed to the properties of the β subunit. In addition, the difference in the K_d values of crude SR at 10 and 300 μM Ca^{2+} suggests different isoform(s) regulate Ca^{2+} release at different Ca^{2+} concentrations. According to the results of O'Brien et al. (1995), the ryanodine binding activity of α isoform is predominant at 10 μM Ca^{2+}, whereas the β isoform has the major channel activity at 100 μM Ca^{2+}. Thus, a ryanodine binding peak shown at 300 μM Ca^{2+} in most turkey heavy SR vesicles could result from the β isoform activity. The result suggested that some turkeys lack this ryanodine binding peak at 300 μM Ca^{2+}, possibly resulting from some defects in β isoform Ca^{2+}-channel protein. Thus, there may be two different genetic groups in the experimental turkey.

The stoichiometries of CaM binding to the turkey skeletal muscle Ca^{2+}-channel protein are approximately 3.5 CaM molecules bound per channel protein subunit in the presence of EGTA and 5 CaM molecules bound per subunit in the presence of 0.1 mM $CaCl_2$. Crosslinking studies in the presence of EGTA indicated substantially greater CaM bound to the α subunit than to β. In the presence of 0.1 mM Ca^{2+}, the α and β subunits appear to be crosslinked in approximately a 1:1 ratio, thus suggesting that as Ca^{2+} concentration is increased, the additional CaM binding occurs at β subunit. These results suggest that CaM differentially regulates Ca^{2+} release

activity by α and β subunits of avian skeletal muscle.

The affinity labeling pattern of both turkey skeletal muscle Ca^{2+} - channel protein isoforms with CaM was dependent on buffer conditions and different kind of CaM. In the presence of the NaCl, the affinity labeling of the channel with CaM was Ca^{2+}-independent. On the other hand, in the absence of NaCl, the affinity labeling was Ca^{2+}-dependent. In addition, the α and β isoforms of turkey skeletal muscle may interact differently with the C-domain of CaM (chicken mutant CaM: Q143C), since the β isoform was not affinity-labeled by this CaM derivative.

This study shows that the characteristics of the turkey skeletal Ca^{2+} - channel protein are different from mammalian, fish and even chicken Ca^{2+} - channel proteins. The unusual properties of turkey Ca^{2+}-channel protein may result from that two isoforms played different regulation roles in different conditions.

CHAPTER 3
THE BIOCHEMICAL BASIS FOR PSE QUALITY PROBLEMS ASSOCIATED WITH TURKEY

I. Introduction

The modern turkey industry has grown rapidly over the past twenty years to meet consumer demand for lean, inexpensive and convenient meat products. To meet this demand, the industry has intensely bred for the efficient growth and heavy muscling. Over the past few years the turkey processing industry has been experiencing severe meat quality problems which closely resemble pale, soft, and exudative (PSE) pork. Meat from these stress-susceptible birds shows a very rapid pH decline resulting in product which is very exudative, and has softer texture, poorer bind and higher cooking losses compared to non-stressed birds (Sosnicki, 1993). Stress factors which increase the incidence of the PSE condition in turkey include exposure to heat or cold, and loading turkeys into trucks and transporting them to the slaughter plant (Froning et al., 1978). The striking similarity of the factors leading to development of PSE meat in turkey to that of swine suggests that there may be a genetic basis for this syndrome in turkeys.

Porcine stress syndrome (PSS) is an inherited skeletal muscle disorder which affects 10-20% of the swine population in the U.S. (Vansickle, 1989). This syndrome results in substantial economic losses to farmers, owing to death of animals from stresses of transportation, heat, crowding, etc., before

reaching market (Louis, 1993). A substantial fraction of PSE pork is directly attributable to PSS (Pommier and Houde, 1993). While PSS has been observed in many swine breeds, the prevalence of this disorder correlates strongly with genetic selection of leaner, more heavily muscled and faster growing animals (Zhang et al., 1992).

Biochemical studies indicated that skeletal muscle SR vesicles or skinned muscle fibers from the PSS-susceptible animal have abnormal Ca^{2+} regulation (Mickelson et al., 1986, 1988; Endo et al., 1983). These data suggested that there is a defect in the Ca^{2+}-release channel protein of the skeletal muscle SR. The ultimate cause of PSS in pigs has been identified as a point mutation in the skeletal muscle Ca^{2+}-channel protein (Arg^{615} to Cys) (Fujii et al., 1991).

The similarities in factors which influence formation of PSE meat from turkey with those of PSE pork strongly suggests that genetic selection for desirable growth traits in both species may have resulted in inadvertent selection for a mutated form of the SR channel which is associated with development of undesirable meat quality characteristics.

Our hypothesis is that a subpopulation of commercial turkeys have an increased frequency of an altered SR Ca^{2+}-channel protein, resulting in the abnormal Ca^{2+}-channel protein activity which is responsible for development of PSE meat. To test this hypothesis, the biochemical approaches used to characterize the defect in PSS swine were employed to determine whether a similar abnormality might exist in turkeys. Ryanodine binding assays were conducted on SR vesicles prepared from commercial turkeys (as a stress-susceptible group) and from a genetically unimproved (as a control group) turkeys to determine whether the Ca^{2+}-channel protein is altered in the population of commercial turkeys. SDS-PAGE and CaM affinity labeling

experiments were also used to investigate the basis for differences between commercial and unimproved turkeys.

II. Materials and Methods

A. Materials

1.Turkeys

Two different populations of turkeys were utilized in this study. A random-bred genetically unimproved group of turkeys (McCartney, 1964) was used as a control population. These turkeys were obtained from the Ohio Agricultural Experiment Station, Wooster, Ohio. Commercial turkeys were obtained with the cooperation of BilMar Foods (Zeeland, MI). Turkeys were killed by intravenous injection of sodium pentobarbital (12.5 mg/ml). The breast muscles were removed, cut into one-inch cubes, and immediately frozen in liquid nitrogen. Samples were stored at -80°C.

2. Chemicals

Benzophenone-4-maleimide and tetramethylrhodamine-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) or Wako Chemical USA, Inc. (Richmond, VA). Stains-all dye was obtained from Sigma (St. Louis, MO).

B. Methods

1. Preparation of Sarcoplasmic Reticulum Vesicles

Skeletal muscle SR was isolated from the breast muscles by the procedure of Mickelson et al. (1986). Briefly, the frozen muscle cubes were homogenized in a Waring blender for 60 seconds using 5 vols. (w/v) of 0.1 M NaCl, 5 mM Tris-maleate (pH 6.8). The procedure was modified to include three proteinase inhibitors (0.1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) in the homogenization buffer and in each subsequent step of the preparation. After centrifugation of the homogenate for 30 min. at

3500 x g_{max}, the pellet was discarded, and the resultant supernatant was centrifuged for 30 min. at 10,000 x g_{max}. This pellet was resuspended with 0.6 M KCl, 5 mM Tris-maleate (pH 6.8) and centrifuged for 40 min. at 180,000 x g_{max} in a Beckman Ti-70 rotor. For preparation of crude total membrane fraction (crude SR), the pellet was resuspended in 10% sucrose and then centrifuged at 180,000 x g_{max} in Ti-70 rotor for 40 min. The pellet was resuspended to 10% sucrose, quick-frozen and stored at -80°C. The yield of crude SR was ~150 mg from 100 g of unimproved or commercial turkey breast muscles.

For purification of the SR fraction enriched in the Ca^{2+}-channel protein (heavy SR), the pellet from the 0.6 M KCl extraction was resuspended in 10% sucrose (w/v), 0.4 M KCl, 20 μM $CaCl_2$, 5 mM Tris-maleate (pH 6.8) and placed on discontinuous sucrose gradients (22%, 36%, and 45%) containing 0.4 M KCl, 20 μM $CaCl_2$, 5 mM Tris-maleate (pH 6.8) in all layers. The tubes were centrifuged for 5 hr at 112,000 x g_{max} in a Dupont Sorvall AH-629 rotor, and the material located at the interface between each sucrose layer was removed. Fractions were diluted at least 1:2 with 10% sucrose, and centrifuged at 180,000 x g_{max} in Ti-70 rotor for 40 min. The SR fraction collected between 22% and 36% sucrose gradient layers was light SR; the SR fraction obtained between 36% and 45% sucrose gradient layers was heavy SR. The pellets were resuspended in 10% sucrose, quick-frozen and stored at -80°C. The yields were typically 17 mg skeletal heavy SR from 100 g of unimproved turkey muscle and 30 mg skeletal heavy SR from 100 g of commercial turkey muscle.

2. Ryanodine Binding Assays

The ryanodine binding assays are based on that of Mickelson et al. (1988). [^{3}H]Ryanodine binding was performed by incubating 0.2 mg of

heavy SR protein/ml or 1 mg of crude SR protein/ml for 90 min at 37°C in media containing 0.25 M KCl, 25 mM PIPES (pH 7.0), 4 or 10 nM [^{3}H] ryanodine, and a $CaCl_2$-EGTA-nitrilotriacetic acid buffer to give a specific $[Ca^{2+}]_{free}$ ranging from 0.01-1000 μM. Free Ca^{2+} concentrations were calculated using the computer program of Perrin and Sayce (1967), which is based on equilibrium concentrations of metal-ligand mixtrues. This information was used to derive optimal $[Ca^{2+}]_{free}$ for subsequent [^{3}H]ryanodine binding studies designed to obtain the K_d and B_{max} values. The ryanodine concentration was varied by addition of unlabeled ryanodine. Samples were filtered with Whatman GF/B filters and washed three times with 5 ml of ice-cold buffer (0.25 M KCl, 25 mM PIPES, pH 7.0). Specific ryanodine binding was determined by subtracting the nonspecific binding obtained in the presence of 100 μM unlabeled ryanodine.

The K_d and B_{max} values for ryanodine binding by the SR preparations were calculated from the fit of bound vs. free ryanodine using Enzfitter computer program (Biosoft, Cambridge, UK) when ryanodine was varied from 1-500 nM.

3. Calmodulin Purification and Derivatization

Wheat germ calmodulin was prepared as described by Strasburg et al. (1988). Chicken CaM, site-specifically mutated at glutamine 143 to a cysteine residue (Q143C), was obtained from Dr. Albert Wang, Boston Biomedical Research Institute. For crosslinking experiments, both wheat germ and Q143C chicken CaM were radiolabeled with ^{125}I and monofunctionally substituted at Cys 27 of wheat germ CaM or at Cys 143 of chicken CaM with the photo-activatable crosslinker, benzophenone-4-maleimide (Strasburg et al., 1988). For fluorescence experiments, wheat germ CaM was labeled at Cys 27 with tetramethylrhodamine-X-maleimide

(Strasburg et al., 1988).

4. Calmodulin Crosslinking

Affinity labeling of CaM-binding proteins in turkey skeletal SR vesicles was performed by incubating in darkness 0.1 μM wheat germ or Q143C [^{125}I]-Bz-CaM with 100 μg of heavy SR vesicles using the buffer conditions of Yang et al. (1994) [20 mM Hepes, pH 7.5, and 0.15 M NaCl]. $CaCl_2$, $MgCl_2$, and EGTA were included as indicated in the figure legends.

The mixtures were placed in plastic microcentrifuge tubes, the tubes were covered with plastic wrap, and the samples were illuminated for 5 min in a Stratalinker 1800 photoreactor (Stratagene Crop., La Jolla, CA) equipped with lamps of λ_{max} = 254 nm. After photolinking, the samples were centrifuged in Beckman TL-100 centrifuged at 88,000 x g_{max} for 20 min. The membrane pellets were resuspended in 50 μl water. For electrophoresis, 25 μl dye buffer containing 3.7% SDS, 12 mM EGTA, 30% glycerol, ~0.001% bromphenol blue, and 46 mM Hepes, pH 7.5 was added to each sample.

5. Electrophoresis

The electrophoresis procedure employed was that described by Laemmli (1970) with the modification that 4-10% linear acrylamide gradient gels were used. Slab gels were stained with 0.05% Coomassie blue in 50% methanol/10% acetic acid and then destained with 50% methanol/10% acetic acid. The gels were scanned using GS 300 Transmittance Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA). The gels were dried and placed with Kodak Omat XAR-5X-ray film in autoradiography cassettes equipped with Dupont Lightning Plus intensifying screens.

6. Stains-all Staining

Staining with the cationic carbocyanine dye Stains-all (1-ethyl-2-[3-(1-ethyl-naphtho [1,2d] thiazolin-2-ylidene)-2-methylpropenyl] naptho [1,2d] thiazolium bromide) was carried out as described by Campbell et al. (1983). Slab gel was fixed overnight with 25% isopropyl alcohol and washed exhaustively in 25% isopropyl alcohol to remove SDS. The gel was then stained in the dark for at least 48 hours with 0.0025% Stains-all, 25% isopropyl alcohol, 7.5% formamide, and 30 mM Tris-base, pH 8.8. This staining procedure resulted in blue staining of Ca^{2+}-binding proteins (such as calsequestrin) but other proteins stained pink. The slab gel was destained completely in 25% isopropyl alcohol, and then stained with Coomassie blue.

7. Amino Acid Sequencing

The 75 kDa protein identified by 4-10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was analyzed at the MSU Macromolecular Structure Facility. The 75 kDa band was removed from the gel, cut to ~1 mm^2 pieces, and equilibrated with 100 mM ammonium bicarbonate buffer, pH 8.0 at 37°C at least 24 hours and adjusted pH to 8.0 with NaOH. After the final pH reached 8.0, the trypsin was added to reach final concentration to 4% and incubated at 37°C for 24 hours (Fernandez et al., 1992). The mixture was fractionated by HPLC using a C_{18} column (0.8 μM x 250 mm), and buffers (0.1% trifluoroacetic acid and 90% CH_3CN/10% H_2O/0.085% trifluoroacetic acid) to elute the peptides. The fractions were sequenced by Sequencer 494 (Perkin Elmer Applied Biosystems Divison, Foster City, CA) for seven to eleven cycles to obtain partial amino acid sequence compared with the database sequence (GeneBank, EMBL, and Swiss-pro) to look for possible candidate proteins to identify the unknown protein.

8. Protein Assay

SR protein concentrations were determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

9. Statistical analysis

Comparisons of the mean K_d and B_{max} values for the unimproved and commercial turkey, for the crude and heavy SR, for the crude SR at two different Ca^{2+} concentrations were made with Student's t test.

III. Results and Discussions

A. Electrophoretic Analysis of Skeletal Muscle Heavy SR Proteins from Unimproved and Commercial Turkeys

The electrophoretic analysis of heavy SR preparations purified from seven commercial and seven unimproved turkeys is shown in Figure 3.1. The arrows shown near the top of the gel indicate the two isoforms (α and β) of the Ca^{2+}-channel protein from both types of turkey skeletal muscles. In addition, one major band of 75 kDa was observed in five of seven commercial turkey samples. This protein was apparently present in low abundance in each sample of unimproved turkey. When present in commercial turkey samples, this protein was usually the most abundant protein in the SR preparations (Figure 3.1). The densitometric analysis of the gel shown in Table 3.1 indicated that the relative amounts of the 75 kDa protein range were from 19.9-26.1% of the total protein in the five commercial turkey preparations showing high abundance of this protein. In the other two preparations the 75 kDa protein comprised 4.5-6.1% of the total protein. The seven genetically unimproved turkey preparations showed the 75 kDa protein present in the range of only 0.9-2.4% of the total protein (Table 3.1). The results suggest that there may be two different phenotypes or groups in commercial turkeys based on the variable amount of the 75 kDa protein found in these samples.

The relative amounts of both Ca^{2+}-channel protein isoforms (α and β) in unimproved turkey heavy SR preparations were at least three times as great as those in the commercial turkey heavy SR (Table 3.1). However, the ratio of the amounts of α isoform to the β isoform was consistently around 1:1 in both kind turkey heavy SR preparations (Table 3.1). One explanation for the decreased relative abundance of Ca^{2+}-channel protein isoforms in

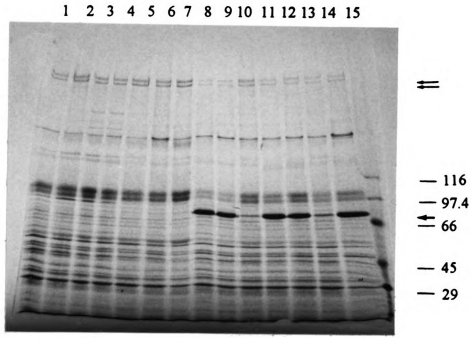


Figure 3.1 SDS-polyacrylamide gel of heavy SR preparations from unimproved and commercial skeletal muscles. Lanes 1-7: skeletal muscle heavy SR from seven individual genetically unimproved turkeys; lanes 8-14: skeletal muscle heavy SR from seven individual commercial turkeys; lane 15: molecular weight markers. The upper two arrows indicate the Ca^{2+}-channel protein subunits which occur as two isoforms in turkey. The lower arrow indicates the 75 kDa band. The molecular weight markers were indicated as kDa.

Table 3.1 Densitometric Analysis of SDS-PAGE of Unimproved and Commercial Turkey Heavy SR

Individual Unimproved Turkey*	1	2	3	4	5	6	7	Mean
Protein	% Total Protein							
α isoform	1.6	0.8	1.3	1.3	1.8	1.6	3.2	1.7
β isoform	1.3	0.8	1.0	1.1	1.4	1.3	3.8	1.5
75 kDa	2.4	1.1	1.1	1.2	0.9	1.6	1.8	1.4
Individual Commercial Turkey*	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Mean**
Protein	% Total Protein							
α isoform	0.5	0.6	0.4	0.4	0.5	0.6	0.7	0.53
β isoform	0.4	0.5	1.0	0.4	0.4	0.4	0.7	0.54
75 kDa	22.5	22.8	4.5	23.4	19.9	6.1	26.1	17.9

* Individual turkey numbers correspond to lanes from gel electrophoretogram in Figure 3.1.

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commercial turkey preparations could be an artifact of the high abundance of the 75 kDa protein. However, this was not the case, as indicated by ryanodine binding analysis (see below).

Mickelson et al. (1988) used SDS-PAGE to compare malignant hyperthermia-susceptible (MHS) and normal porcine heavy SR preparations. Unlike turkey SR preparations, the SDS-PAGE patterns of the heavy SR prepared from normal and MHS porcine skeletal muscles were not different. In addition, densitometric analysis of the gel demonstrated no significant difference in the content of the Ca^{2+}-channel protein between MHS and normal SR (Mickelson et al., 1986, 1988).

B. <u>Ca^{2+}-dependence of Ryanodine Binding to the Ca^{2+}-channel Protein from Unimproved and Commercial Turkeys</u>

[^{3}H]ryanodine binding assays were applied to determine whether there are differences in SR Ca^{2+}-channel activity between genetically unimproved and commercial turkeys. If a defect in function is present in the Ca^{2+}-channel of SR from commercial turkeys, the results will probably be manifested as an altered affinity for ryanodine, as an altered Ca^{2+}-dependence for ryanodine binding, or both.

The Ca^{2+} dependence of [^{3}H]ryanodine binding of commercial turkey skeletal muscle heavy SR vesicles is shown in Figure 3.2. Seven turkey skeletal muscle SR vesicle preparations were examined. Two SR Ca^{2+}-channel protein preparations have higher [^{3}H]ryanodine binding because both SR preparations contained less 75 kDa protein (Figure 3.1, lanes 10 and 13). [^{3}H]ryanodine binding was activated with a threshold of approximately 0.2 μM Ca^{2+} and was maximal between 3 and 30 μM free Ca^{2+} (Figure 3.2). The mean [^{3}H]ryanodine binding at the plateau was 0.24 pmol/mg of heavy SR protein (Figure 3.3). As with the unimproved turkey SR samples, when

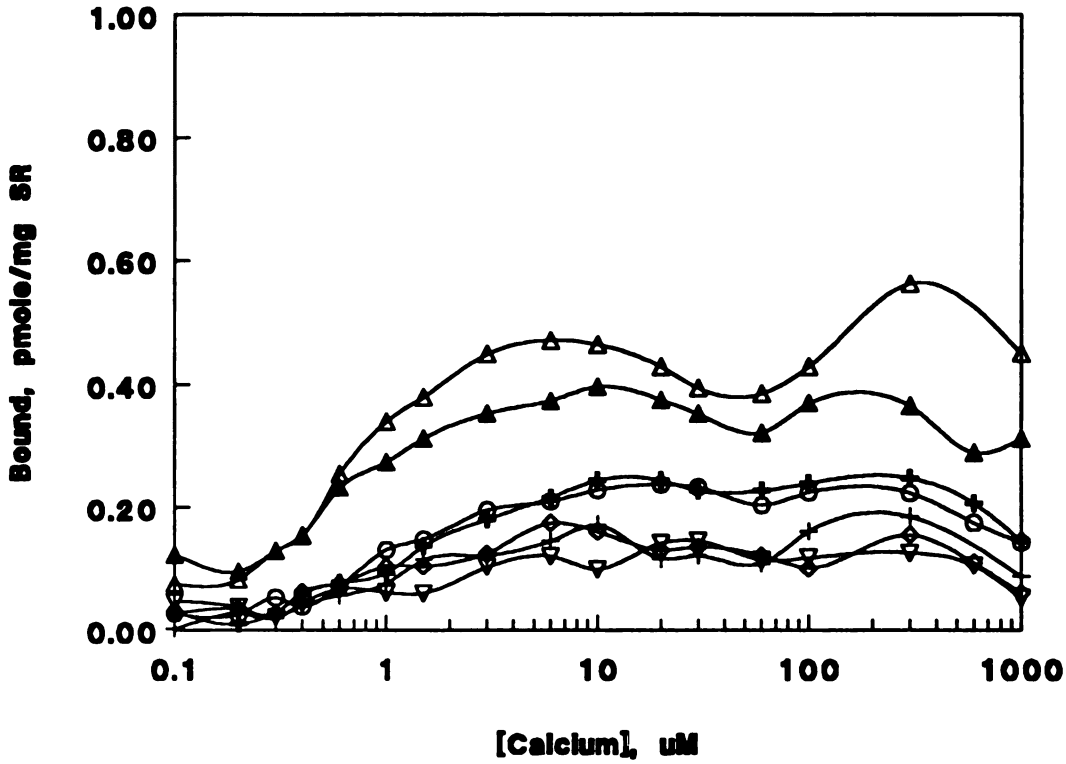

Figure 3.2 Ca^{2+} dependence of [^{3}H]ryanodine binding to commercial turkey skeletal muscle heavy SR vesicles. Curves represent results from seven individual commercial turkey SR vesicle preparations.

Bound, pmole/mg SR

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re
(C

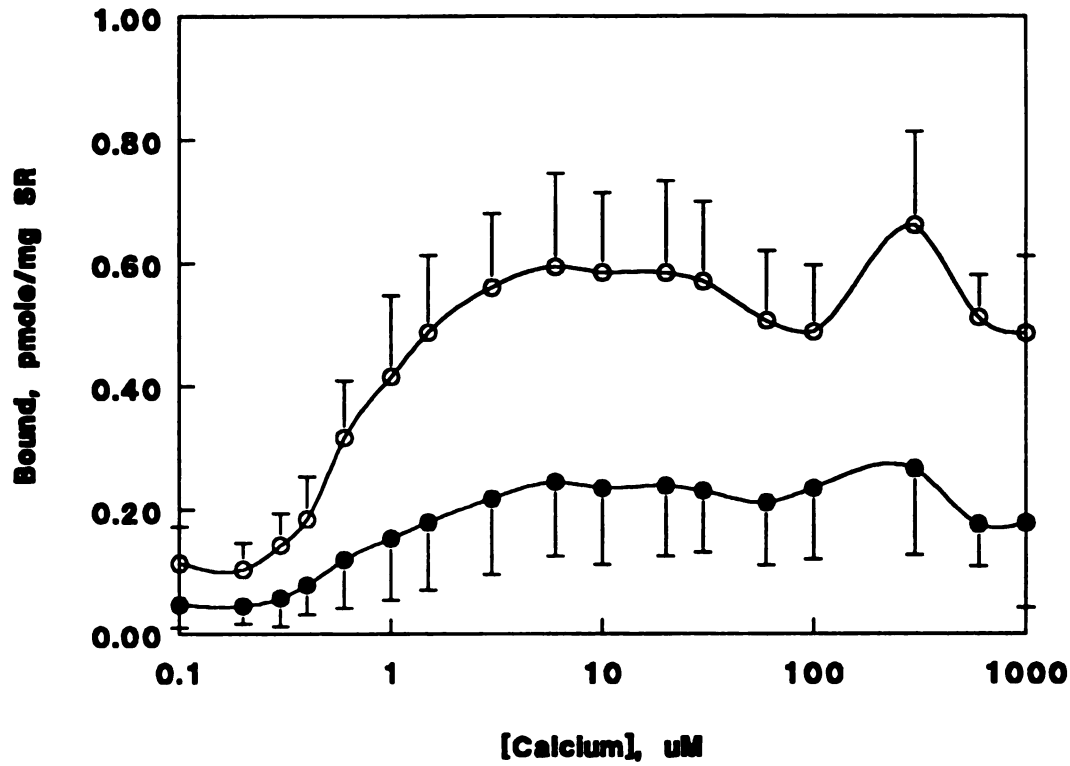


Figure 3.3 Ca^{2+} dependence of [^{3}H]ryanodine binding to unimproved and commercial turkey skeletal muscle heavy SR vesicles. Points represent the means ± SE of duplicates for each of seven individual unimproved (○) and commercial (●) heavy SR preparations.

free Ca^{2+} concentration reached 1 mM, the [^{3}H]ryanodine binding of the commercial turkey preparations was not inactivated. The mean value of [^{3}H]ryanodine binding at 1 mM Ca^{2+} was 0.17 pmol/mg of heavy SR protein, corresponding to a 25% inhibition compared to 0.22 pmol/mg at 10 μM free Ca^{2+} (Figure 3.3). Thus, the Ca^{2+} dependence of [^{3}H]ryanodine binding showed the similar tendency in both unimproved and commercial turkey skeletal muscle heavy SR (Figure 2.2, Figure 3.2, and Figure 3.3). At the same time, the maximal [^{3}H]ryanodine binding of the commercial turkey heavy SR was about 36% of that of the unimproved turkey (0.22 pmol/mg vs. 0.60 pmol/mg)(Figure 3.3). According to densitometric analysis (Table 3.1), the total amount of the Ca^{2+}-channel protein in unimproved turkey heavy SR preparations was ~3-fold greater than that from commercial turkey. Therefore, after normalizing the ryanodine binding values of commercial turkey, the Ca^{2+} dependence of [^{3}H]ryanodine binding curves were not significantly different between commercial and unimproved turkey based on the [^{3}H]ryanodine binding values.

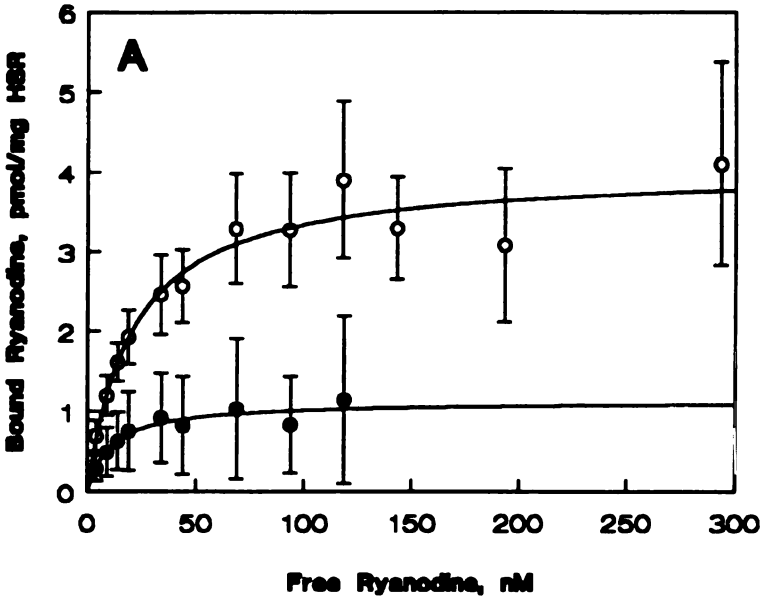
The Ca^{2+} dependence of [^{3}H]ryanodine binding to the isolated heavy SR from turkeys are in sharp contrast to that observed for porcine SR. The Ca^{2+}-dependence of [^{3}H]ryanodine binding from both normal and MHS pigs follows a bell-shaped curve, but [^{3}H]ryanodine binding by MHS SR is consistently greater than that of normal SR at various ryanodine concentrations (Mickelson et al., 1988). The [^{3}H]ryanodine binding of rabbit skeletal muscle heavy SR is also a broad bell-shaped curve, which shows complete inactivation at 1 mM Ca^{2+} (O'Brien et al., 1995). These results with turkey SR preparations showed Ca^{2+} dependence of [^{3}H]ryanodine binding curve which extends over a greater range of [Ca^{2+}] and shows limited inactivation property at high Ca^{2+} concentration (1 mM)

in both commercial and unimproved turkey heavy SR (Figure 2.2 and Figure 3.2). O'Brien et al. (1995) reported that the β isoform of the Ca^{2+}-channel protein from fish is more insensitive to inactivation by millimolar Ca^{2+} than the α isoform. Thus, the difference in Ca^{2+} dependence of [^{3}H]ryanodine binding between porcine and turkey SR probably results from the fact that the Ca^{2+}-channel protein of turkey skeletal muscle consists of two isoforms (both α and β), whereas porcine skeletal muscle only one isoform which functionally resembles the α Ca^{2+}-channel protein isoform. In addition, our data showed no difference in [^{3}H]ryanodine binding to commercial and unimproved turkey SR at 4 nM ryanodine after normalizing the data, except for a more pronounced ryanodine binding peak at 300 μM Ca^{2+} in most samples of unimproved turkey SR (Figure 3.3). The physiological significance of this peak is as yet unclear.

C. Ryanodine-dependence of Ryanodine Binding to SR Vesicles from Unimproved and Commercial Turkeys

As noted in the previous section, functional differences in channel protein activity may be reflected by altered channel protein affinity for ryanodine. [^{3}H]ryanodine binding activity of commercial turkey skeletal heavy (Figure 3.4A) and crude (Figure 3.5A) SR vesicles was measured in the presence of 10 μM Ca^{2+} over a range of ryanodine concentrations from 0-200 nM, the ryanodine binding parameters were calculated using the Enzfitter program, and Scatchard plots were constructed (Figure 3.4B). For the seven commercial turkey preparations, an average K_d of 12.2±5.9 nM and a B_{max} of 1.10±0.06 pmol/mg of heavy SR protein were obtained (Table 3.2). Comparing [^{3}H]ryanodine binding parameters of the unimproved and commercial turkey SR (Table 3.2), both K_d and B_{max} of the commercial turkey heavy SR were significantly different from those of the unimproved

Figure 3.4 Ryanodine dependence of [^{3}H]ryanodine binding to unimproved and commercial turkey skeletal muscle heavy SR vesicles at 10 μM Ca^{2+}. (A) Specific [^{3}H]ryanodine binding by unimproved (○) and commercial (●) turkey heavy SR. (B) Scatchard plot of [^{3}H]ryanodine binding by unimproved (○) and commercial (●) turkey heavy SR. Points represent the means ± SE of duplicates for each of seven different heavy SR preparations.


A
Bound Ryanodine, pmol/mg HSR
Free Ryanodine, nM
0
1
2
3
4
5
6
0
50
100
150
200
250
300

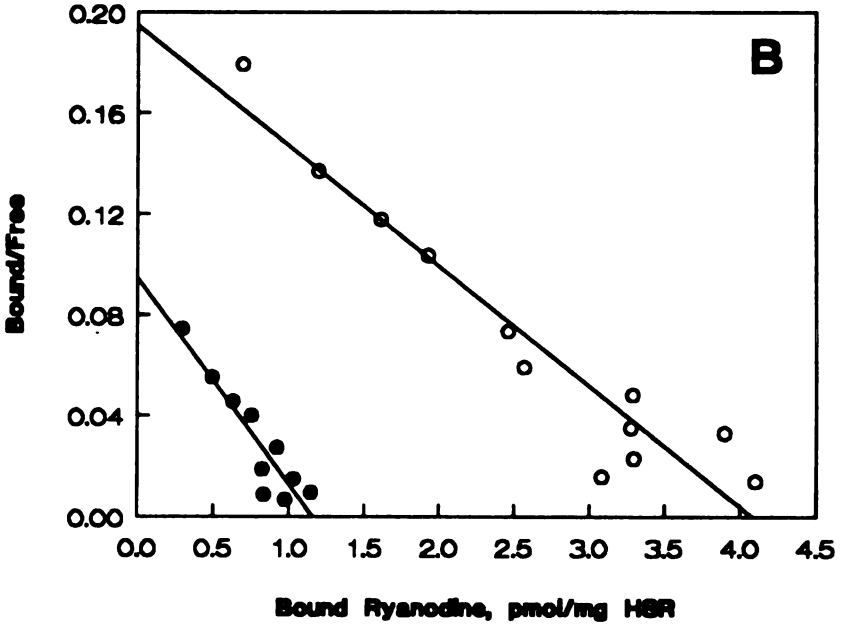
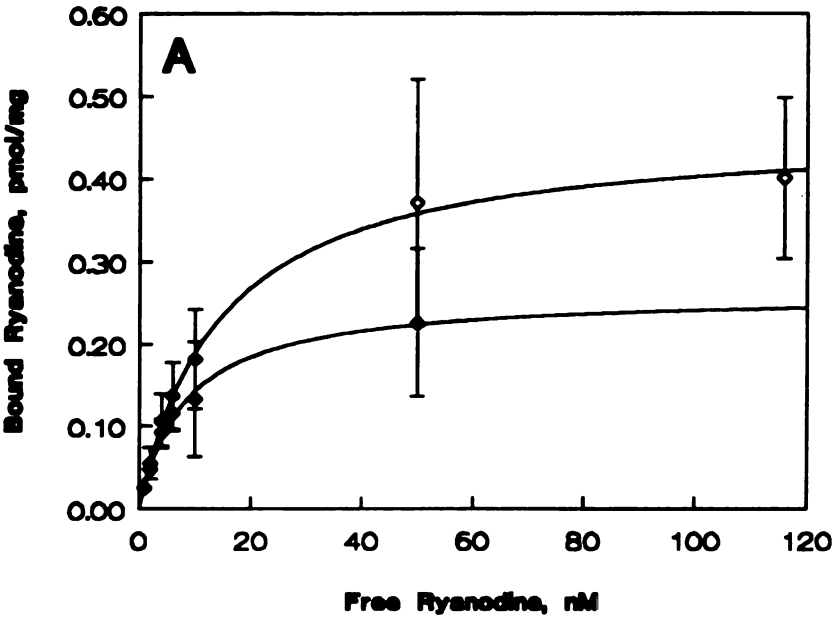

B
Bound/Free
Bound Ryanodine, pmol/mg HSR
0.00
0.04
0.08
0.12
0.16
0.20
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

Figure 3.5 Ryanodine dependence of [^{3}H]ryanodine binding to unimproved and commercial turkey skeletal muscle crude SR vesicles at 10 μM Ca^{2+}. (A) Specific [^{3}H]ryanodine binding by unimproved (◊) and commercial (♦) turkey crude SR. (B) Scatchard plot of [^{3}H]ryanodine binding by unimproved (◊) and commercial (♦) turkey crude SR. Points represent the means ± SE of duplicates for each of four different crude SR preparations.


A
Bound Ryanodine, pmol/mg
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
20
40
60
80
100
120
Free Ryanodine, nM

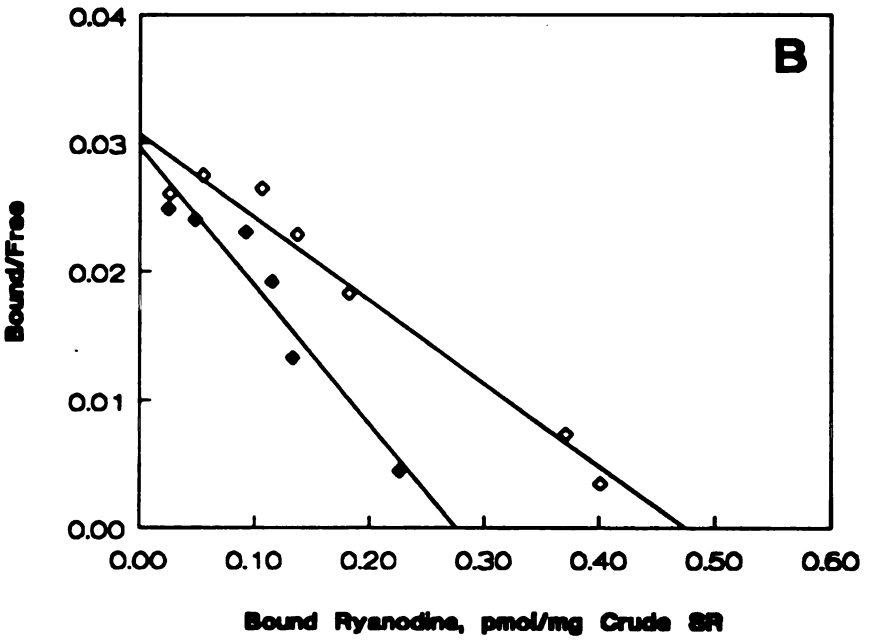

B
Bound/Free
0.04
0.03
0.02
0.01
0.00
0.00
0.10
0.20
0.30
0.40
0.50
0.60
Bound Ryanodine, pmol/mg Crude SR

Table 3.2 Equilibrium Constants of [^{3}H]Ryanodine Binding of Crude and Heavy SR from Unimproved and Commercial Turkeys at 10 μM or 300 μM [Ca^{2+}]

	K_d , nM	B_{max}, pmol/mg
Unimproved		
10 μM [Ca^{2+}]		
Heavy SR*	20.5 ± 7.6[a]	4.01 ± 0.17[e]
Crude SR**	19.0 ± 4.3[a]	0.52 ± 0.13[f]
300 μM [Ca^{2+}]		
Crude SR**	25.1 ± 2.6[b]	0.58 ± 0.22[f]
Commercial		
10 μM [Ca^{2+}]		
Heavy SR*	12.2 ± 5.9[c]	1.10 ± 0.06[g]
Crude SR**	8.9 ± 5.1[c]	0.29 ± 0.12[h]
300 μM [Ca^{2+}]		
Crude SR**	15.7 ± 3.7[d]	0.34 ± 0.06[h]

[a,b,c,d] Different letters in column indicate significant differences ($p<0.05$).
[e,f,g,h] Different letters in column indicate significant differences ($p<0.05$).
* n=7.
** n=4.

turkey heavy SR. For commercial turkey heavy SR the K_d=12.2±5.9 compared to 20.5±7.6 nM for unimproved turkey ($p<0.05$). The Ca^{2+}-channel protein content or B_{max}=1.10±0.06 for commercial turkey heavy SR vs. 4.01±0.17 pmol/mg for unimproved turkey ($p<0.0005$). The differences in affinity of channel proteins of populations of unimproved vs. commercial turkeys suggest that there are functional differences in one or both channel isoforms. The differences in channel protein content further suggest functional differences in Ca^{2+} regulation in the muscle cell.

The difference in B_{max} between commercial and unimproved turkey heavy SR could result from artifactual differences in distributions of the Ca^{2+}-channel protein in SR membrane. To address this question, [^{3}H]ryanodine binding from total SR membrane preparations (crude SR) between commercial and unimproved turkeys were compared.

The [^{3}H]ryanodine binding to commercial turkey skeletal muscle crude SR vesicles yielded a K_d of 8.9±5.1 nM and a B_{max} of 0.29±0.12 pmol/mg at 10 μM Ca^{2+} (Figure 3.5 and Table 3.2). The results indicated that the overall Ca^{2+}-channel protein content of commercial turkey crude SR was still significantly lower than that of the unimproved turkey crude SR (0.29±0.12 vs. 0.52±0.13 pmol/mg for crude SR, $p<0.05$). These results suggest the difference in B_{max} between commercial and unimproved turkey heavy SR does not result from the different distribution, but rather different amounts of the Ca^{2+}-channel protein in SR membranes. At the same time, even after normalizing the B_{max} value of commercial turkey heavy SR (3 times), the binding capacity of commercial turkey heavy SR is still lower than that of unimproved turkey heavy SR.

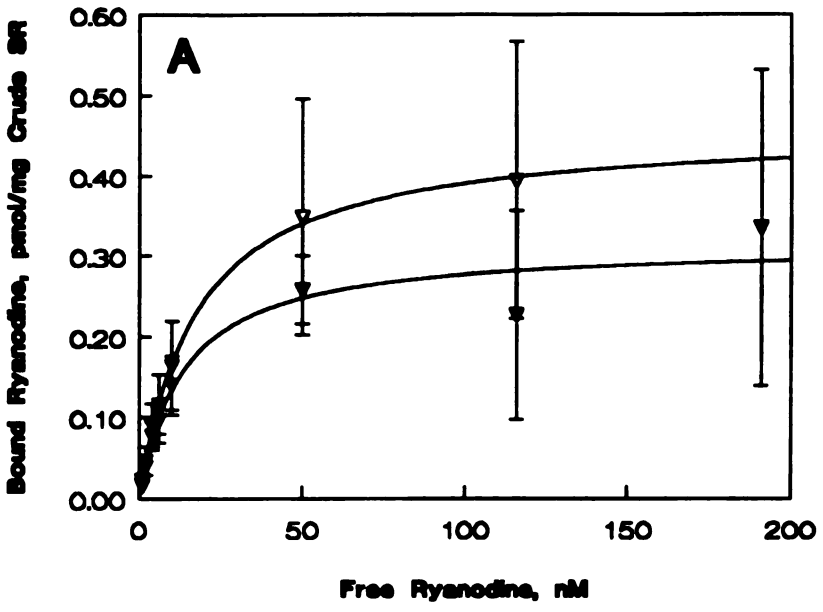
The consistent K_d values between crude and heavy SR in both turkey indicate that the affinity of Ca^{2+}-channel protein for ryanodine is not affected

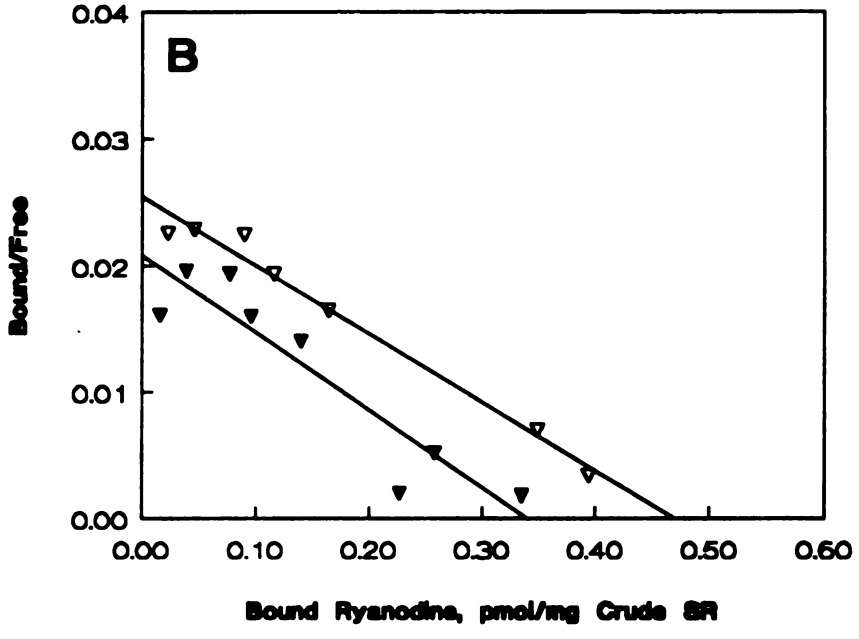
by other SR membrane proteins which might be lost upon fractionation of heavy SR (Table 3.2). In addition, the Ca^{2+}-channel protein binding affinities of the commercial turkey heavy and crude SR were significantly higher than those of unimproved turkey heavy and crude SR (K_d=12.2±5.9 vs. 20.5±7.6 nM for heavy SR; 8.9±5.1 vs. 19.0±4.3 nM for crude SR)(Table 3.2). Therefore, the result indicates that there could be alterations of the commercial turkey Ca^{2+}-channel protein activity and quantity.

Studies on the porcine MH defect suggest that alterations in Ca^{2+}-channel protein content as well as activity may play a role in the defect. Mickelson et al. (1994) indicated that crude membrane preparations from 28-day-old homozygous MHS pigs exhibited only 75% of the [^{3}H]ryanodine binding capacity of preparations of crude SR isolated from normal pigs. Likewise, our results showed that crude SR from a commercial turkey preparation displayed only 56% of [^{3}H]ryanodine binding of crude SR isolated from unimproved turkeys. However, in contrast to our observations with heavy SR, [^{3}H]ryanodine binding by porcine heavy SR is not different between MHS and normal pigs (Mickelson et al., 1994). Since heavy SR preparations are highly enriched in junctional membranes, these results suggest that there may be differences in the localization of Ca^{2+}-channel proteins between the two populations.

Because of the presence of the additional ryanodine binding peak at 300 μM Ca^{2+}, ryanodine binding assays were conducted at this [Ca^{2+}] to determine whether functional differences in channel activity might emerge at Ca^{2+} concentrations corresponding to that of contracting muscle. At 300 μM Ca^{2+}, the [^{3}H]ryanodine binding by commercial turkey crude SR vesicles has a K_d of 15.7±3.7 nM and B_{max} of 0.34±0.06 pmol/mg (Figure 3.6 and Table 3.2). As with unimproved turkey, the B_{max} values of the commercial

Figure 3.6 Ryanodine dependence of [^{3}H]ryanodine binding to unimproved and commercial turkey skeletal muscle crude SR vesicles at 300 μM Ca^{2+}. (A) Specific [^{3}H]ryanodine binding by unimproved (∇) and commercial (▾) turkey crude SR. (B) Scatchard plot of [^{3}H]ryanodine binding by unimproved (▽) and commercial (▾) turkey crude SR. Points represent the means ± SE of duplicates for each of four different crude SR preparations.


A
Bound Ryanodine, pmol/mg Crude SR
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
50
100
150
200
Free Ryanodine, nM

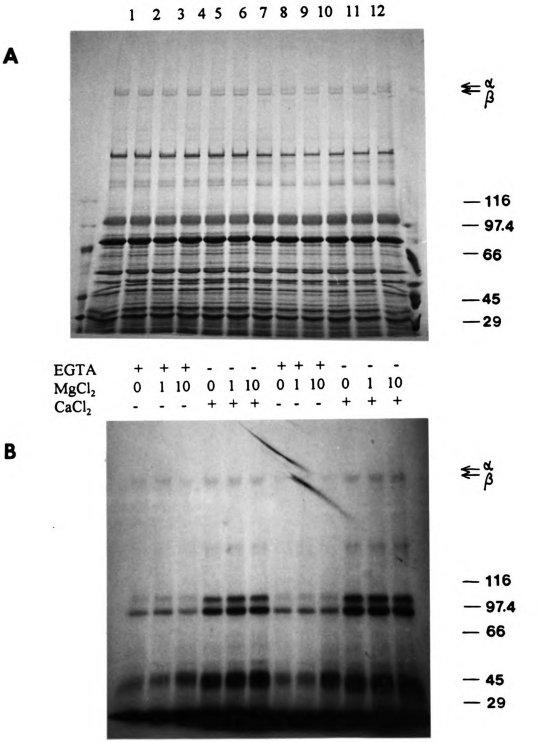

B
Bound/Free
0.04
0.03
0.02
0.01
0.00
0.00
0.10
0.20
0.30
0.40
0.50
0.60
Bound Ryanodine, pmol/mg Crude SR

turkey SR were not significantly different between 10 μM and 300 μM Ca^{2+} treatments in crude SR vesicles. However, at 300 μM Ca^{2+}, the binding affinity of the Ca^{2+}-channel protein of the unimproved turkey for ryanodine was significantly lower than that at 10 μM Ca^{2+} concentrations ($p<0.05$). The data showed the relationships among those parameters of the commercial turkey SR are similar with those of the unimproved turkey SR (Table 3.2). The difference in ryanodine binding affinity between 10 μM and 300 μM Ca^{2+} concentrations in both turkeys indicates that different Ca^{2+}-channel protein isoforms are responsible for ryanodine binding at different Ca^{2+} concentrations. At 10 μM Ca^{2+}, both α and β isoforms are likely activated in different ratios; at 300 μM Ca^{2+}, only β isoform is likely activated because α isoform is inhibited by high Ca^{2+} concentrations (O'Brien et al., 1995). These results suggest that there may be functional differences between β isoforms Ca^{2+}-channel protein of the commercial and unimproved turkeys.

D. Calmodulin Affinity Labeling of SR Vesicles from Unimproved and Commercial Turkeys

Previous results using CaM derivatized with the affinity label in the C-domain (Q143C CaM) indicated affinity labeling of only the α-isoform of the channel protein (Figure 2.5B). As a beginning step to determine whether CaM regulation of the Ca^{2+}-channel protein isoforms might be altered in a population of commercial turkeys, the question was asked "do the two commercial turkey Ca^{2+}-channel protein isoforms have the same CaM affinity labeling pattern as the unimproved turkey Ca^{2+}-channel protein isoforms?" Results of Q143C CaM affinity labeling of commercial turkey SR are indicated in the autoradiogram (Figure 3.7B) of the gel electrophoretogram (Figure 3.7A). In contrast to the results from unimproved turkey SR, the commercial turkey SR shows binding activity of both commercial turkey

Figure 3.7 [Mg^{2+}] and [Ca^{2+}] dependence of affinity labeling of commercial turkey skeletal muscle heavy SR with chicken (Q143C) [^{125}I]-Bz-CaM. These studies were conducted using the site-specific mutant of chicken CaM which has a Cys in the C-domain of protein (Cys143). [^{125}I]-Bz-CaM was incubated with commercial skeletal muscle heavy SR vesicles (lanes 1-6 and 7-12 represent two different commercial turkey heavy SR preparations) in 20 mM Hepes (pH 7.5)/0.15 M NaCl plus following components: lanes 1 and 7, 2 mM EGTA; lanes 2 and 8, 2 mM EGTA and 1 mM $MgCl_2$; lanes 3 and 9, 2 mM EGTA and 10 mM $MgCl_2$; lanes 4 and 10, 0.1 mM $CaCl_2$; lanes 5 and 11, 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$; lanes 6 and 12, 0.1 mM $CaCl_2$ and 10 mM $MgCl_2$. Molecular weight markers were indicated as kDa. (A) Coomassie blue stained gel. (B) Autoradiogram of dried gel.


1 2 3 4 5 6 7 8 9 10 11 12
A
α
β
116
97.4
66
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29
EGTA + + + - - - + + + - - -
MgCl2 0 1 10 0 1 10 0 1 10 0 1 10
CaCl2 - - - + + + - - - + + +
B
α
β
116
97.4
66
45
29

isoforms for the Q143C [^{125}I]-Bz-CaM. These results suggested that the C-domain of CaM may interact differently with the β isoform compared to the unimproved turkey skeletal muscle Ca^{2+}-channel protein. Thus, the results imply that there are some functional differences associated with CaM regulation of the β isoform between unimproved and commercial turkeys.

The overall affinity labeling pattern of commercial turkey preparations using both wheat germ and Q143C CaM showed additional proteins binding CaM. The autoradiogram showed the Ca^{2+}-channel protein of the commercial turkey was no longer the main CaM receptor (Figure 3.7B and Figure 3.8). Intense crosslinking bands were identified in the autoradiograms (Figure 3.7B and Figure 3.8), whereas molecular weights were calculated as 111 kDa and 94 kDa. After subtracting the molecular weight of CaM (17 kDa), these CaM receptors in commercial turkey heavy SR preparations are approximately 94 kDa and 77 kDa proteins. The latter protein could be the 75 kDa protein which has been found in high abundance in the commercial turkey heavy SR preparations previously (Figure 3.1). The 111 kDa complex could be derived from the 75 kDa protein crosslinked with two CaM molecules. In support of this argument, these intense CaM crosslinking bands of 111 kDa and 77 kDa did not appear in the commercial turkey SR preparation which contains low 75 kDa protein abundance (data not shown). Alternatively this protein could be triadin, a 95 kDa SR protein (Kim et al., 1990); or glycogen phosphorylase, a 97 kDa protein which can be phosphorylated in the presence of Ca^{2+} and CaM (Molla et al., 1985). These results suggest that the differences in composition distribution of heavy SR vesicles between commercial and unimproved turkey could change the CaM regulation for the Ca^{2+}-channel protein.

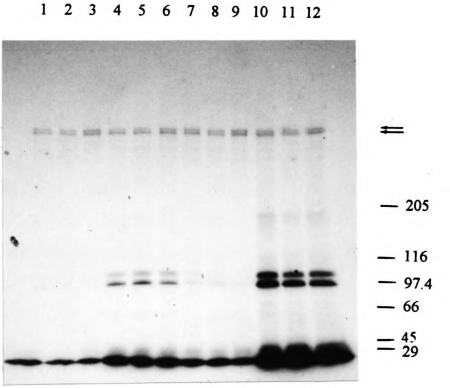


Figure 3.8 [Mg^{2+}] and [Ca^{2+}] dependence of affinity labeling autoradiogram of commercial turkey skeletal muscle heavy SR with wheat germ [^{125}I]-Bz-CaM under different buffer conditions. These experiments were conducted using wheat germ CaM with the benzophenone-maleimide cross-linker at Cys-27. 0.1 μM (lanes 1-6) and 0.5 μM [^{125}I]-Bz-CaM (lanes 7-12) were incubated with commercial skeletal muscle heavy SR vesicles in 20 mM Hepes (pH 7.5)/0.15 M NaCl plus following components: lanes 1 and 7, 2 mM EGTA; lanes 2 and 8, 2 mM EGTA and 1 mM $MgCl_2$; lanes 3 and 9, 2 mM EGTA and 10 mM $MgCl_2$; lanes 4 and 10, 0.1 mM $CaCl_2$; lanes 5 and 11, 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$; lanes 6 and 12, 0.1 mM $CaCl_2$ and 10 mM $MgCl_2$. Molecular weight markers were indicated as kDa.

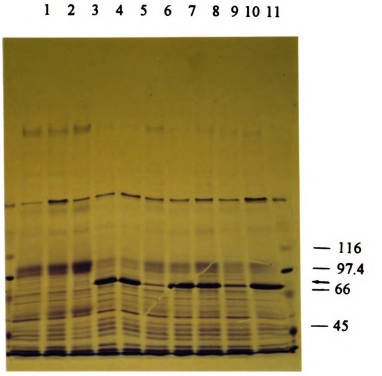
E. Partial Characterization of 75 kDa Protein of Commercial Turkey Skeletal Muscle Heavy SR

The presence of the 75 kDa protein in such great abundance in most commercial turkey SR preparations could be an important indicator associated with the meat quality problems. The identity of this protein on the basis of molecular weight alone is unknown. Chadwick et al. (1988) showed that a 71 kDa protein crosslinked with the Ca^{2+}-channel protein of terminal cisternae in rabbit skeletal muscle. However, they did not identify this protein which was present in low abundance. Another candidate to be considered for analysis was calsequestrin, a Ca^{2+}-binding protein in normally present high abundance in SR preparations. Calsequestrin exhibits varying mobility on SDS-PAGE (44,000-63,500) depending on the type of gel used, and the tissue source. Because of its abundance and variable mobility on gels, the cationic carbocyanin dye "Stains-all" was used to determine whether the 75 kDa protein could be calsequestrin which, because of its very acidic amino acid composition, stains dark blue or purple. In addition, Stains-all dye will stain the other acidic Ca^{2+}-binding proteins, such as calmodulin, troponin C, and S-100, dark blue or purple, while most other proteins with red or pink (Campbell et al., 1983).

1. Stains-all Staining Gel

Ten heavy SR preparations, purified from three unimproved and seven commercial turkeys, were analyzed by SDS-PAGE and staining with Coomassie blue (Figure 3.9A) and Stains-all (Figure 3.9B). In Figure 3.9B (lanes 4-10), the 75 kDa protein is clearly stained pink rather than blue. However, most proteins were hard to see in the Stains-all gel, because of the pink background (Figure 3.9B). Further destaining tended to decolorize protein as well as background. There was one blue band clearly observed

Figure 3.9 Coomassie blue and Stains-all stained SDS-polyacrylamide gel of heavy SR preparations from unimproved and commercial skeletal muscles. Lanes 1-3: 3 unimproved turkey skeletal muscle heavy SR; lanes 4-10: 7 commercial turkey skeletal muscle heavy SR; lane 11: molecular weight markers. (A) Coomassie blue stained gel. (B) Stains-all stained gel. The upper two arrows indicate the Ca^{2+}-channel protein subunits which occur as two isoforms in turkey. The lower arrow indicates the 75 kDa band.


1 2 3 4 5 6 7 8 9 10 11
116
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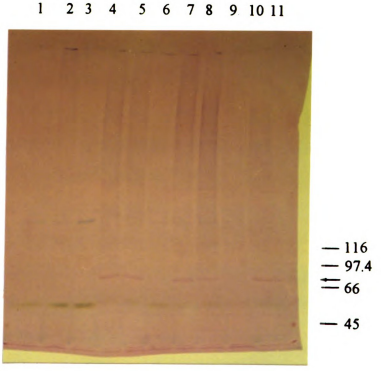
1 2 3 4 5 6 7 8 9 10 11
116
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45

at 157 kDa and one dark blue band at 45 kDa in unimproved and commercial turkey heavy SR preparations (Figure 3.9B). The former could be the 160-kDa glycoprotein associated with the DHP receptor (Campbell et al., 1983). The 45 kDa band is probably calsequestrin because its apparent molecular weight (45,000) is consistent with that reported for mammalian skeletal muscle (Campbell et al., 1983). These results indicate that the 75 kDa protein in commercial turkey heavy SR preparations is not calsequestrin, nor is it an acidic Ca^{2+}-binding protein, or glycoprotein.

2. Amino Acid Sequencing Analysis

Amino acid sequence analysis was used in an effort to identify the 75 kDa protein. Attempts to determine the N-terminal sequence of the 75 kDa protein were unsuccessful because the N-terminus of the 75 kDa is blocked as is the case with most muscle proteins. To obtain partial sequence information, the 75 kDa protein was subjected to limited digestion with trypsin, and then peptide fragments were purified by capillary HPLC. The amino acid sequence of one purified trypsin-digested fragment was identified as "Asp-Phe-Glu-Ile-Val-Pro-Gly-Ser-Gly-Lys".

After matching the peptide using computer database sequences and matching candidates to a molecular size of approximately 75 kDa, the candidates which emerged were guanylate cyclase (83.3% identify in 6 amino acids overlap), and anthranilate synthase (75.0% identify in 8 amino acids overlap).

Guanylate cyclase is a 73 kDa enzyme which catalyzes the generation of cyclic 3',5'-guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP serves as a second messenger which activates a cGMP-dependent protein kinase. Recently, Galione et al. (1993) proposed cyclic ADP-ribose (cADPR), which is an endogenous activator of Ca^{2+}-

induced Ca^{2+} release, was enhanced by cGMP. However, cADPR has no effect on the type 1 Ca^{2+}-channel protein of skeletal muscle (α isoform) so its messenger function is restricted to type 2 cardiac Ca^{2+}-channel protein (β isoform) (Berridge, 1993). It seems unlikely that there would be so much guanylate cyclase in SR.

Anthranilate synthase is a ~65 kDa enzyme which involved with the biosynthesis of tryptophan. It converts chorismate to anthranilate, the first step in the reaction sequence leading on to tryptophan production. However, the tryptophan synthesis pathway is only present in microorganisms and plants, the enzymes that synthesize essential amino acids have apparently been lost early in animal evolution. Thus, the identity and function of the 75 kDa protein are unknown.

IV. Conclusions

The commercial turkey population which likely includes a stress-susceptible sub-population and the genetically unimproved control turkey skeletal muscle SR Ca^{2+}-channel activities were very different in ryanodine dependence of [^{3}H]ryanodine binding. The commercial turkey heavy SR had higher binding affinity (K_d=12.2 versus 20.5 nM) and lower binding capacity (B_{max}=1.10 versus 4.01 pmol/mg) than unimproved turkey. The differences in binding affinity and capacity were also observed in crude SR from both turkeys. The results suggest that there is an altered Ca^{2+}-channel protein content and altered activity of the Ca^{2+}-channel protein in at least some of the commercial turkey population, which may be related to the incidence of PSE meat.

The contents of the SR Ca^{2+}-channel protein in heavy SR preparations between commercial and unimproved turkey were different. The content of both α and β isoforms in the commercial turkey skeletal muscle heavy SR (0.8-1.4% total protein) was lower than that of the unimproved turkey heavy SR (1.6-7.0% total protein). In addition, the SDS-PAGE indicated that there was a 75 kDa protein which is the predominant SR protein in most but not all commercial turkey SR samples examined. This protein was in very low abundance in unimproved turkey SR preparations. The identity and function of this 75 kDa protein, which binds CaM, is still unknown.

The Ca^{2+} dependence of [^{3}H]ryanodine binding in both commercial and unimproved turkey heavy SR displayed no characteristic differences. Avian Ca^{2+}-channel protein has both α and β isoforms, whereas mammalian only has α isoform. Therefore, unlike mammalian muscle, the avian Ca^{2+}-channel protein activity was not inhibited very much at high Ca^{2+} concentration. This behavior is attributable to the β isoform of the Ca^{2+}-channel protein (O'Brien

et al., 1995). However, both the Ca^{2+} dependence and ryanodine dependence of the [^{3}H]ryanodine binding of the Ca^{2+}-channel protein SR indicated the Ca^{2+}-channel protein from commercial turkey have different channel activity and content compared to unimproved turkey when measured at 300 μM Ca^{2+} concentration.

The crosslinking studies showed differences in the CaM affinity labeling of the Ca^{2+}-channel protein β isoform between commercial and unimproved turkeys. Q143C [^{125}I]-Bz-CaM binds to the β isoform of the commercial turkey Ca^{2+}-channel protein, but the β isoform of unimproved turkey does not show crosslinking with this CaM derivative. Results with wheat germ CaM indicate that the β subunit does bind CaM. These results suggest that there could be a defect in the commercial turkey SR Ca^{2+}-channel protein β isoform which is associated with the meat quality problems with commercial turkeys.

CHAPTER 4

CALMODULIN INTERACTION WITH THE PURIFIED CARDIAC Ca^{2+}-CHANNEL PROTEIN (β-LIKE ISOFORM)

I. Introduction

The contraction and relaxation of cardiac muscle depends on the cytosolic level of Ca^{2+} ions. Ca^{2+} release from the sarcoplasmic reticulum (SR) via the Ca^{2+}-channel protein contributes to muscle contraction; the Ca^{2+} uptake into the SR gives rise to muscle relaxation via the Ca^{2+}-ATPase. Although the Ca^{2+} uptake process has been described in considerable detail, the molecular mechanisms and regulations involved in Ca^{2+} release are, by comparison, poorly understood.

In the inotropic effect in the heart, myocardial contractility is enhanced though accelerated Ca^{2+} uptake via phosphorylation of phospholamban, decreased affinity of troponin C for Ca^{2+} resulting from phosphorylation of troponin I, and enhanced Ca^{2+} activation of Ca^{2+}-release via phosphorylation of the DHP receptor. The precise role of the SR Ca^{2+}-channel protein in the inotropic effect of the heart, and the precise mechanisms governing its regulation are still unclear.

Numerous molecular mechanisms modulate the SR Ca^{2+}-channel protein activity. Ca^{2+}-release is enhanced by micromolar concentrations of cytoplasmic Ca^{2+} (Ca^{2+}-induced Ca^{2+}-release), and by adenine nucleotides, caffeine, and nanomolar concentrations of ryanodine (Meissner et al., 1986). In contrast, the Ca^{2+} channel protein activity is inhibited by Mg^{2+}, calmodulin

(CaM), ruthenium red and micromolar concentrations of ryanodine (Smith et al., 1985; Meissner and Henderson, 1987). CaM may modulate the cardiac Ca^{2+}-channel protein activity through two different ways: by directly binding to the Ca^{2+}-channel protein and inhibiting Ca^{2+}-release (Meissner, 1986; Smith et al., 1989) or by CaM-dependent phosphorylation of the Ca^{2+}-channel protein which enhances channel activity by increasing the open probability of the channel (Witcher et al., 1991).

The studies of Chapters 2 and 3 suggest that the β isoform of the Ca^{2+}-channel protein in turkey skeletal muscle could play an important role in determining the PSE-like meat quality problem in commercial turkeys. Therefore, a better understanding of the biochemical properties of the β isoform may help solve the PSE problems in turkeys. Immunological techniques identified epitopes in the amphibian α and β skeletal isoforms common to the mammalian skeletal and cardiac forms of the Ca^{2+}-channel protein, respectively (Lai et al., 1992). Thus, the interaction of the cardiac Ca^{2+}-channel protein with CaM is an indirect way to understand the biochemical properties of the β isoform from skeletal muscle. The objective of this study is to define the stoichiometry and affinity of binding of CaM to the cardiac Ca^{2+}-channel protein under different physiological conditions.

II. Materials and Methods

A. Materials:

1. Purified Canine Cardiac Ca^{2+} -channel Proteins

Purified cardiac Ca^{2+}-channel protein preparations from canine were provided by Dr. Jones at the Department of Pharmacology and Toxicology, and the Department of Medicine at Indiana University. Purified cardiac Ca^{2+}-channel protein was in medium containing 1 M NaCl, 0.2 mM $CaCl_2$, 20 mM MOPS, pH 7.2, 0.5% CHAPS, 50 μg/mL Pefabloc, and 10-25% sucrose.

2. Chemicals

Benzophenone-4-maleimide, tetramethylrhodamine-x-maleimide, and CaM-dependent protein kinase II 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).

B. Methods

1. Calmodulin Purification and Derivatization

Wheat germ calmodulin was prepared as described by Strasburg et al. (1988). For crosslinking experiments, wheat germ CaM were radiolabeled with ^{125}I and monofunctionally substituted at Cys 27 of wheat germ CaM with the photo-activatable crosslinker, benzophenone-4-maleimide (Strasburg et al., 1988). For fluorescence experiments, wheat germ CaM was labeled at Cys 27 with tetramethylrhodamine-X-maleimide (Strasburg et al., 1988).

2. Calmodulin Crosslinking

Affinity labeling of CaM-binding proteins in purified canine cardiac Ca^{2+}-channel proteins was performed by incubating in darkness 0.1 μM wheat germ [^{125}I]-Bz-CaM with 80 μl (~0.9 μg) of purified cardiac Ca^{2+}-

channel proteins using the buffer conditions of Yang et al. (1994) [20 mM Hepes, pH 7.0, and 0.15 M NaCl] (final volume=140 µl). $CaCl_2$, $MgCl_2$, and EGTA were included as indicated in the figure legends.

The mixtures were placed in plastic microcentrifuge tubes, the tubes were covered with a plastic wrap film, and the samples were illuminated for 5 min in a Stratalinker 1800 photoreactor (Stratagene Crop., La Jolla, CA) equipped with lamps of λ_{max} = 254 nm. For electrophoresis, 25 µl sample buffer containing 3.7% SDS, 12 mM EGTA, 30% glycerol, ~0.001% bromphenol blue, and 46 mM Hepes, pH 7.5 was added to each sample.

3. Electrophoresis

The electrophoresis procedure employed was that described by Laemmli (1970) with the modification that 4-10% linear acrylamide gradient gels or 5% mini-gels were used. The gels were stained with 0.05% Coomassie blue in 50% methanol/10% acetic acid and then destained with 50% methanol/10% acetic acid. The gels were dried and placed with Kodak Omat XAR-5X-ray film in autoradiography cassettes equipped with Dupont Lightning Plus intensifying screens.

4. Ryanodine Binding Assay

The ryanodine binding assays are based on that of Mickelson et al. (1988). [^{3}H]ryanodine binding was performed for 90 min at 37°C in media containing 25 µl purified canine cardiac Ca^{2+}-channel protein, 1 M KCl, 100 mM PIPES (pH 7.0), 10 nM [^{3}H]ryanodine, 65 nM unlabeled ryanodine and 20 µM $CaCl_2$. This information was used to determine maximal ryanodine binding activity of the purified Ca^{2+}-channel protein. Subsequently, samples were filtered with Whatman GF/B filters and washed three times with 5 ml of ice-cold buffer (0.25 M KCl, 25 mM PIPES, pH 7.0). Specific ryanodine binding was determined by subtracting the nonspecific binding obtained in

presence of 100 μM unlabeled ryanodine.

5. Fluorescence Anisotropy Measurements

Fluorescence anisotropy was used to characterize CaM/calcium-channel protein interaction in SR vesicles. The principle underlying fluorescence anisotropy is that the rapid Brownian tumbling of a small molecule is slowed upon binding to a larger molecule to form a complex. If the smaller molecule is fluorescent, the altered mobility of this species resulting from complex formation can be detected by measuring the change in depolarization of fluorescent light emitted upon excitation by polarized light (Lakowicz, 1983).

Fluorescence measurements were performed using an SLM 4800 spectrofluorometer equipped with two detectors in T-format detector. Samples were held in a thermostated cell block maintained at 22°C. The excitation wavelength of Rh-CaM was 532 nm, monochromator slits were set at 4 nm, and emitted light was isolated using Schott OG-570 filters. During titrations, samples were allowed to equilibrate for 4 min after each addition of SR or Rh-CaM. All samples were measured in buffer containing 10% sucrose, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM NaF, 50 mM Hepes, pH 7.0, and 0.15 M NaCl and proteinase inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM PMSF).

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 (Yang et al., 1994).

The anisotropy of ligand (Rh-CaM) is directly proportional to the fraction of ligand bound to receptor (Ca^{2+}-channel protein). Thus, if A_f is the anisotropy of free Rh-CaM and A_b is the anisotropy of 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 three species. If the change in quantum yield is negligible upon binding of ligand, then the 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.

The anisotropy of unbound Rh-CaM, A_f, was measured in the absence of SR or purified Ca^{2+}-channel protein. The anisotropy of fully bound species, A_b, was obtained by titration of Rh-CaM with SR vesicles or purified Ca^{2+}-channel proteins, following by curve-fitting using the Enzfitter computer program (Biosoft, Cambridge, UK) for a single class of ligand binding site. 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 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.

III. Results and Discussions

A. Calmodulin Binding Activity of the Purified Cardiac Ca^{2+}-channel Protein

Ryanodine-activity was measured to determine biological activity of purified Ca^{2+}-channel protein and to determine channel protein content in the preparations. The SDS-PAGE of the cardiac purified Ca^{2+}-channel protein preparation (Figure 4.1A) showed high purity of the Ca^{2+}-channel protein. There are two major bands: the upper one is the Ca^{2+}-channel protein and the lower one has been identified as the degradation fragment from the Ca^{2+}-channel protein. In addition, there is a faint band at ~100 kDa which is probably Ca^{2+}-ATPase (Figure 4.1A).

Purified Ca^{2+}-channel protein from canine cardiac muscle preparations was incubated with the affinity-labeling derivative [^{125}I]-Bz-CaM to confirm binding activity of the channel protein for CaM and to demonstrate that no other CaM-binding protein were present in the purified preparations. The autoradiogram (Figure 4.1B) of the gel electrophoretogram (Figure 4.1A) indicated that the major complex formed was a doublet of Mr > 450,000 which corresponds to CaM plus the channel protein subunit (Seiler et al., 1984). The amount of complex obtained was much greater in the presence of Ca^{2+} than in the presence of EGTA at each Mg^{2+} concentrations examined (Figure 4.1B), suggesting that CaM-binding to cardiac Ca^{2+}-channel protein is Ca^{2+}-dependent.

These results are similar to those obtained by Seiler et al. (1984) and showed that the azido-[^{125}I]CaM labeling of the canine cardiac Ca^{2+}-channel protein in SR vesicles was largely dependent upon the presence of Ca^{2+}. In contrast, Takasago et al. (1991) using [^{125}I] Denny-Jaffe-labeled-CaM with similar buffer conditions showed that [^{125}I]CaM bound to the canine cardiac Ca^{2+}-channel protein preferentially in the absence of Ca^{2+}. The differences

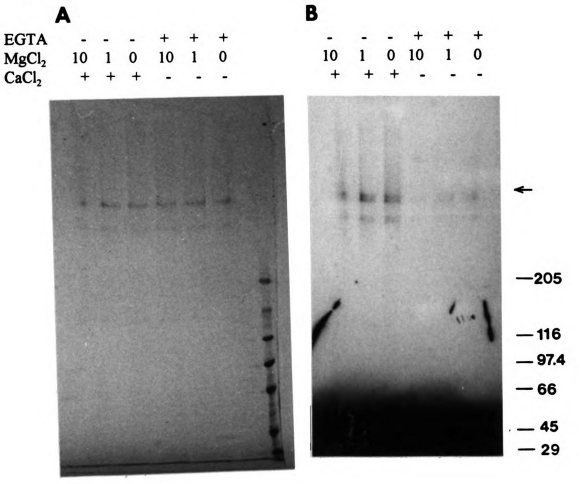


Figure 4.1 [Mg^{2+}] and [Ca^{2+}] dependence of affinity labeling of the purified cardiac Ca^{2+}-channel protein with wheat germ [^{125}I]-Bz-CaM under different buffer conditions. These experiments were conducted using wheat germ CaM with the benzophenone-maleimide cross-linker at Cys-27, and using buffer conditions: 20 mM Hepes, pH 7.0, and 0.15 M NaCl. (A) Coomassie blue stained gel. (B) Autoradiogram of dried gel. The lower band has been idenitified as a proteolytic degradation product of the upper band.

in the CaM affinity labeling patterns between Takasago et al. (1991) results and ours could result from differences in the CaM derivative employed.

The CaM affinity labeling pattern of the cardiac Ca^{2+}-channel protein differs from that of the skeletal muscle Ca^{2+}-channel protein isoform. Crosslinking studies using porcine skeletal muscle SR vesicles suggested that CaM-binding is Ca^{2+}-independent (Yang et al., 1994). Those results were confirmed by fluorescence analysis of binding (Figure 4.1). The result indicates that CaM affinity labeling binding with α isoform of Ca^{2+}-channel protein is more insensitive to Ca^{2+}; the CaM affinity labeling binding of α isoform is greater in the presence of EGTA than that in the presence of $CaCl_2$ (Yang et al., 1994). These results suggest that CaM differentially regulates Ca^{2+} release activity by α and β isoforms.

B. Stoichiometry and Affinity of Rh-CaM with the Purified Cardiac Ca^{2+}-channel Protein

Previous results of SR cardiac Ca^{2+}-channel protein showed that there was not CaM binding activity at all in the presence of EGTA by fluorescence anisotropy measurement (Strasburg et al., 1993). In agreement with previous results, the purified cardiac Ca^{2+}-channel protein showed little affinity labeling with CaM in the presence of EGTA in both crosslinking experiments (Figure 4.1) and fluorescence measurement (Figure 4.2). Therefore, to quantify CaM binding activity under conditions, the simulating contracting muscle buffer condition were chosen which included $CaCl_2$ plus $MgCl_2$ for the fluorescence anisotropy study.

Purified cardiac Ca^{2+}-channel proteins were titrated into Rh-CaM under buffer condition containing 0.1 mM $CaCl_2$ plus 2 mM $MgCl_2$. In each preparation, titration of the purified channel protein into Rh-CaM resulted in a large increase in fluorescence anisotropy attributable to the increased

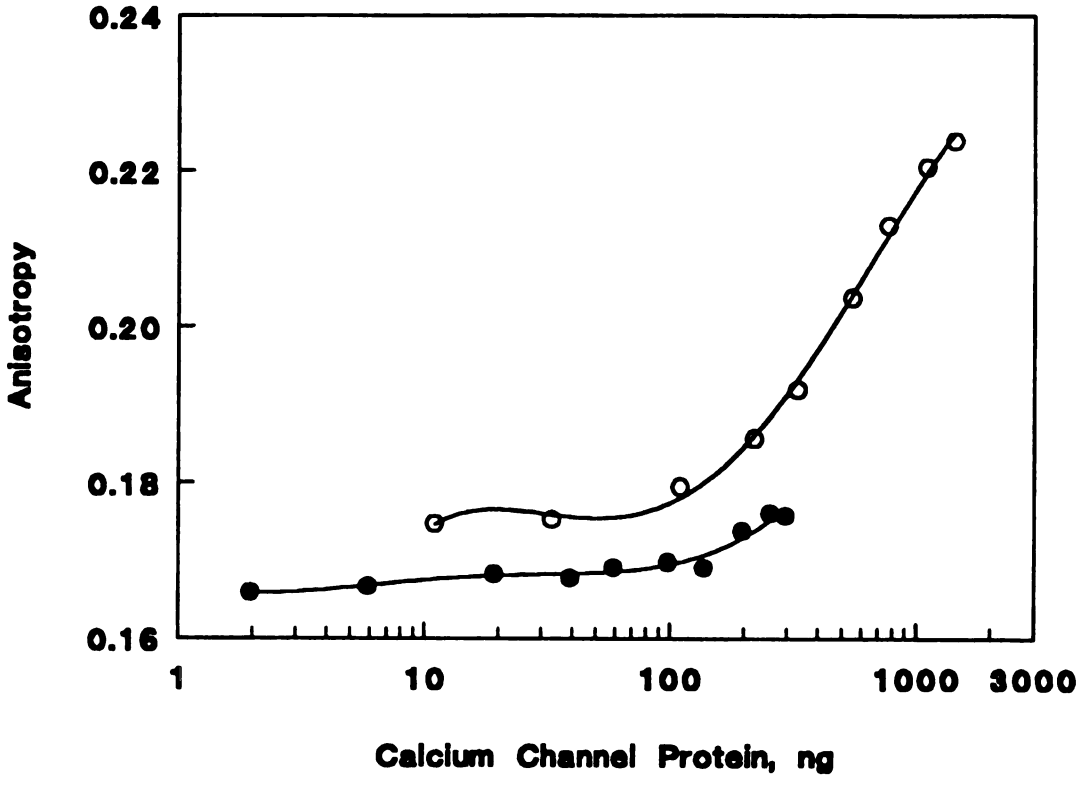
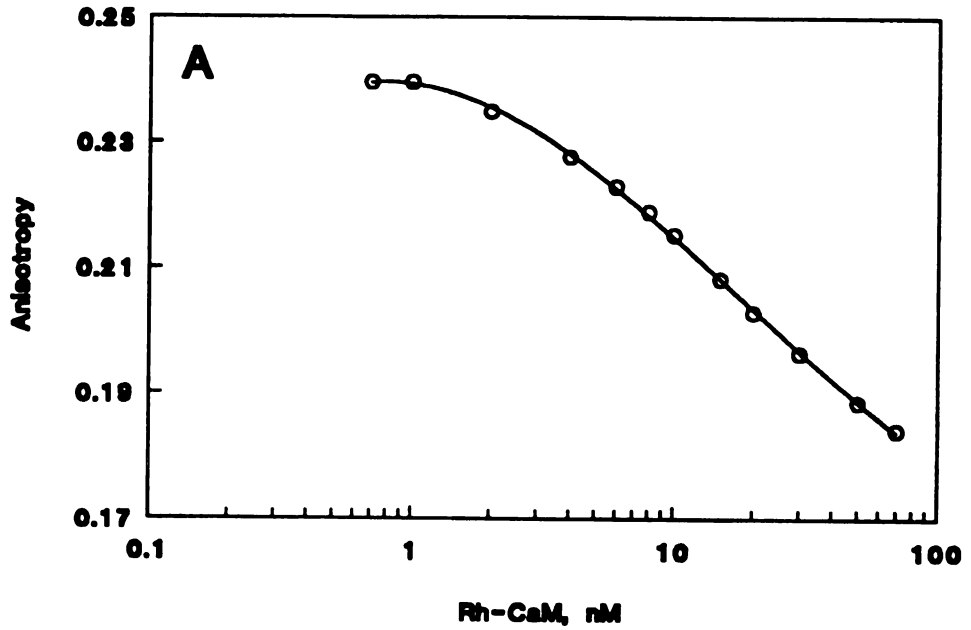


Figure 4.2 Titration of Rh-CaM with the purified cardiac Ca^{2+}-channel protein. The sample medium contained 10 nM Rh-CaM, 0.3 M sucrose, 0.15 M NaCl, and 50 mM Hepes, pH 7.0, plus either 0.1 mM $CaCl_2$ and 0.2 mM $MgCl_2$ (○) or 2 mM EGTA (●) in a starting volume of 1 mL. The Rh-CaM sample was titrated with the purified cardiac Ca^{2+}-channel protein in parallel with a buffer blank containing the same media minus Rh-CaM. Corrections were made for light scattering as described under Methods.

molecular mass of the Rh-CaM/Ca^{2+}-channel protein complex (Figure 4.2). Data from these titrations (Figure 4.2) were used to determine the anisotropy values for free Rh-CaM (A_f) and for the full 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 underestimated in these experiments because the binding curves somewhat decreased, probably because of light scattering, after adding certain amount purified Ca^{2+}-channel protein instead of maintain a saturation state. Therefore, 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 for two preparation averaged 0.2561. The mean value of the A_f from two preparations was 0.1727. 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.

The A_f and A_b values form the basis for subsequent study to estimate the affinity constant and stoichiometry of CaM/Cardiac Ca^{2+}-channel protein interaction. Purified cardiac Ca^{2+}-channel protein preparations were titrated with Rh-CaM, fluorescence anisotropy was measured (Figure 4.3A), and the data were analyzed using the non-linear regression program Enzfitter (Figure 4.3B). Scatchard plot of the data showed two classes of ligand-binding sites on the cardiac Ca^{2+}-channel protein for CaM in the presence of $CaCl_2$ plus $MgCl_2$ (Figure 4.3B). The high-affinity class of sites has K_{d1}=0.61±0.36 nM and B_{max1}=831±293 pmol/mg, and the results of low-affinity binding class site show K_{d2}=7.19±0.95 nM and B_{max2}=5135±246 pmol/mg (Table 4.1). The data suggest that there are ~2 CaM high-affinity binding sites per tetramer Ca^{2+}-channel protein, and ~12 CaM low-affinity binding sites per tetramer in cardiac Ca^{2+}-channel protein.

Figure 4.3 Titration of the purified cardiac Ca^{2+}-channel protein with Rh-CaM in the presence of $CaCl_2$ and $MgCl_2$. (A) Anisotropy plot of titrations of Ca^{2+}-channel proteins with Rh-CaM . (B) Rh-CaM/Ca^{2+}-channel protein saturation binding curve. The inset is a Scatchard plot of Rh-CaM binding to Ca^{2+}-channel proteins. The sample medium contained 1.8 ng (average) of the Ca^{2+}-channel protein, 0.3 M sucrose, 0.15 M NaCl, 50 mM Hepes, pH 7.0, 0.1 mM $CaCl_2$ and .0.2 mM $MgCl_2$ in a starting volume of 1.2 mL. Points represent the means of two preparations. CaM bound was calculated from measured anisotropy as described under Methods.


A
0.25
0.23
0.21
0.19
0.17
Anisotropy
0.1
1
10
100
Rh-CaM, nM

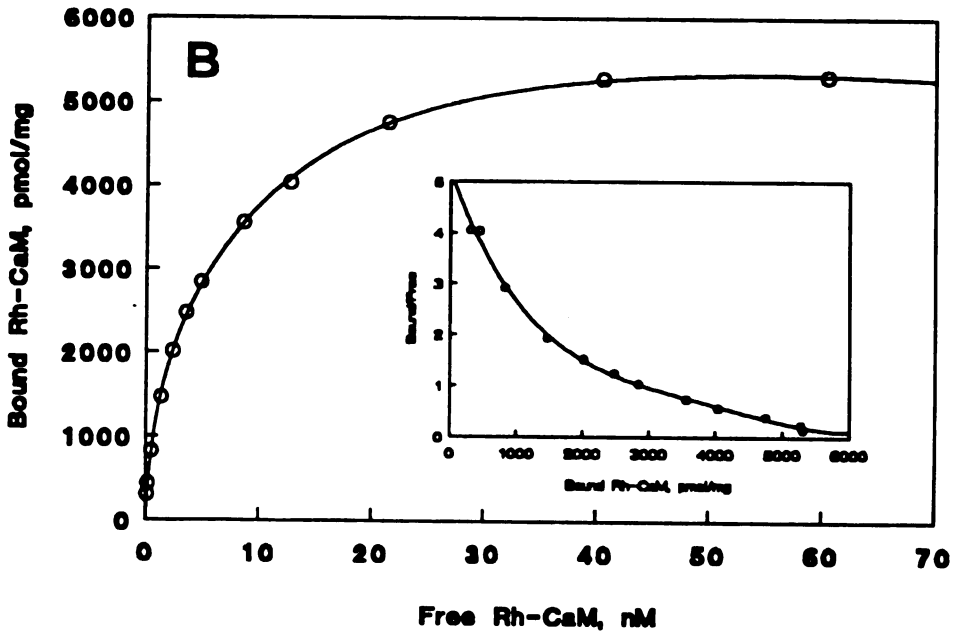

B
6000
5000
4000
3000
2000
1000
0
Bound Rh-CaM, pmol/mg
0
10
20
30
40
50
60
70
Free Rh-CaM, nM

Table 4.1 Equilibrium Constants for Rh-CaM Interaction with the Purified Cardiac Ca^{2+}-channel Protein in the presence of .1 mM $CaCl_2$ + 2 mM $MgCl_2$*

	K_d, nM	B_{max}, pmol/mg	CaM/tetramer
High-affinity site (K_{d1})	0.61 ± 0.36	831 ± 293	~2
Low-affinity site (K_{d2})	7.19 ± 0.95	5135 ± 246	~12

* Data were obtained from titrations of purified cardiac Ca^{2+}-channel proteins with Rh-CaM in the presence of 0.3 M sucrose, 1 mM NaF, 0.15 M NaCl, 50 mM Hepes, pH 7.0, 0.1 mM $CaCl_2$ and 0.2 mM $MgCl_2$. Data are the means of two preparations.

Our previous studies on CaM-binding to Sr vesicles, however, only showed a single class of binding sites on the SR Ca^{2+}-channel protein for CaM with a K_d of 13 nM and a B_{max} of 55 pmol/mg (Strasburg et al., 1993). The data indicate that there are 3 CaM binding sites per channel protein subunit (12 CaM binding sites per tetramer) (Strasburg et al., 1993). Thus, the results with purified cardiac Ca^{2+}-channel protein were consistent with the previous data for the SR Ca^{2+}-channel protein in vesicles. Differences in affinity with that observed previously may result from differences in use of SR vesicles vs. solubilized protein.

Porcine skeletal muscle SR Ca^{2+}-channel protein (α-like isoform) also has two classes of CaM binding sites (Yang et al., 1994). In the presence of $CaCl_2$ and $MgCl_2$, there are 1 CaM high affinity (K_{d1}=0.1 nM) and 7 CaM low affinity binding sites (K_{d2}=17 nM) in Ca^{2+}-channel protein tetramer (Yang et al., 1994). However, in contrast to cardiac muscle Ca^{2+}-channel protein, the skeletal isoform binds CaM even at low Ca^{2+} (Tripathy et al., 1995). Under these conditions CaM activates the skeletal Ca^{2+}-channel protein for Ca^{2+}-release, whereas CaM could not execute this function with the cardiac isoform since there is no binding at low Ca^{2+}. Under contractile conditions, however, CaM inactivates both isoforms.

IV. Conclusions

Physiological differences between the α and β Ca^{2+}-channel proteins have different Ca^{2+}-sensitivity to Ca^{2+}-induced Ca^{2+} release, and to Ca^{2+}-inhibition (O'Brien et al., 1995). Due to the differences in biochemical properties between α and β Ca^{2+}-channel proteins, O'Brien et al. (1995) proposed that α isoforms are directly coupled with the T-tubule DHP receptor. Activation of α isoform channels by voltage changes would result in the release of Ca^{2+} into the sarcoplasm and thereby activate a neighboring "slave" channel, the β isoform Ca^{2+}-channel protein. These results show that CaM interaction with the mammalian skeletal (α-like isoform) and cardiac (β-like isoform) Ca^{2+}-channel protein differs in Ca^{2+}-sensitivity. CaM only bound to the cardiac muscle Ca^{2+}-channel protein in the presence of Ca^{2+}, whereas CaM bound to the mammalian skeletal muscle Ca^{2+}-channel protein in the presence or absence of Ca^{2+}. These results suggest that Ca^{2+}-induced Ca^{2+}-release of the β isoform may be inhibited at a rate limited by direct binding of CaM. Compared with regulation of the α isoform, the CaM inhibitory action in the presence of Ca^{2+} may be more important for the β Ca^{2+}-channel protein because it is directly inhibited by high Ca^{2+} concentrations, while the β isoform remains.

In mammalian skeletal muscle, CaM bound to the Ca^{2+}-channel protein in both muscle resting and contractile states (Yang et al., 1994). Binding of CaM in the presence of low Ca^{2+} may activate the channel protein (Tripathy et al., 1995), whereas in the presence of Ca^{2+}, CaM inhibits channel activity. However, in the presence of EGTA, the cardiac muscle Ca^{2+}-channel protein did not interact with CaM molecules, suggesting increasing cytoplasmic Ca^{2+} is the only way to activate the cardiac Ca^{2+}-channel protein, and that the inhibitory function of CaM for the cardiac Ca^{2+}-channel protein is its primary

function in the heart.

CONCLUSIONS

In muscle excitation-contraction coupling, depolarization of the cell triggers release of Ca^{2+} from the terminal cisternal SR, that fraction of SR which is coupled to the T-tubule via the Ca^{2+}-channel protein. Although this protein plays an important role in this process, the mechanisms involved in regulation of Ca^{2+} release and the relationship of abnormal Ca^{2+} regulation to meat quality are still poorly understood.

PSE meat quality problems, which have caused substantial economic losses to the swine industry for many years, have become increasingly prevalent and severe in the turkey industry. Porcine stress syndrome (PSS) is responsible for a significant fraction of PSE pork; the basis of this inheritable disorder has been identified as a mutation in the gene for the Ca^{2+}-channel protein in pigs. The similarity of the PSE meat quality problems and of factors which influence the prevalence of PSE meat from pigs and turkeys suggests that a subpopulation of commercial turkeys could have a defect in the Ca^{2+}-channel protein resulting in abnormal Ca^{2+} regulation.

In first study, turkey SR preparations were optimized for subsequent studies to define Ca^{2+}-dependence of ryanodine binding, ryanodine binding affinity, channel protein content and calmodulin (CaM) binding properties of the Ca^{2+}-channel protein from a control population of turkeys. Avian skeletal muscle has two Ca^{2+}-channel protein isoforms (α and β) in contrast to mammalian skeletal muscle which only has one isoform. The Ca^{2+}-

dependence of ryanodine binding in turkey SR reflects differences from mammalian muscle in Ca^{2+} regulation, including a broad Ca^{2+}-dependence of Ca^{2+}-induced Ca^{2+} release and lack of Ca^{2+}-inhibition at Ca^{2+} concentrations of muscle contraction. CaM binding to avian α and β Ca^{2+}-channel protein isoforms resembles the binding to the mammalian skeletal and cardiac muscle Ca^{2+}-channel protein, respectively.

The second study indicated that the biochemical properties of the Ca^{2+}-channel protein from commercial turkey SR vesicles show differences compared to those of genetically unimproved turkey. SR preparations from seven commercial turkey show an increased affinity of the Ca^{2+}-channel protein for ryanodine and a decreased channel protein content compared to SR from seven unimproved turkeys. The altered affinity of the Ca^{2+}-channel protein for ryanodine suggests that there is a defect in the SR Ca^{2+}-channel protein in some commercial turkeys. Differences in channel protein content suggest that may be some factors which down-regulate gene expression of the Ca^{2+}-channel protein. The differences in CaM affinity labeling to the β Ca^{2+}-channel protein isoform from commercial and unimproved turkeys suggest that the defect may be specific to the β isoform of commercial turkey.

The presence of the 75 kDa protein in most commercial turkey SR preparations but not genetically unimproved turkey suggests that this protein could be associated with the PSE meat quality in commercial turkeys. Our results suggest that there is a genetic defect in the Ca^{2+}-channel protein in a significant fraction of the commercial turkey population. However, definitive information awaits correlation of changes in channel protein structure with incidence of PSE meat.

In third study, the CaM binding properties of the purified mammalian

cardiac Ca^{2+}-channel protein which resemble the avian β isoform were investigated. Affinity labeling and fluorescence experiments indicate that the cardiac isoform binds four CaM per subunit, but does so only in the presence of Ca^{2+}. This is in contrast to the skeletal muscle isoform which binds five CaM per subunit, independently of $[Ca^{2+}]$. Results from these studies lend support to previous observations that the α subunit of the avian channel protein is regulated in a similar fashion to mammalian skeletal muscle, whereas the β subunit is regulated in similar fashion to mammalian cardiac muscle. The result provides further evidence that α and β isoforms are regulated differently by CaM in different muscle conditions.

FUTURE RESEARCH

In this study, the SR Ca^{2+}-channel protein from commercial turkeys showed an increased binding affinity for ryanodine and a decreased channel protein content in SR membranes. These results suggest that there is a defect in SR Ca^{2+}-channel protein in some commercial turkeys. However, the results do not directly identify the specific defect which occurs in the gene or at the protein level, nor which isoform(s) are specifically responsible for these biochemical property changes. In order to answer these questions, the Ca^{2+}-channel protein will need to be purified from commercial and genetically unimproved turkeys and the biochemical properties (especially ryanodine binding properties) of the Ca^{2+}-channel proteins will need to be compared. If the ryanodine binding properties of the purified Ca^{2+}-channel protein isoforms are different between commercial and unimproved turkeys, these differences would offer more direct evidence of a defect in the Ca^{2+}-channel protein of a subpopulation of the commercial turkey. Otherwise, there could be an unidentified component associated the Ca^{2+}-channel protein in commercial turkey which is responsible for the defect. If the ryanodine binding activities of the purified Ca^{2+}-channel protein are not different between two kinds of turkeys, this could be because an unidentified component is removed by purification.

Secondly, if a defect is present in the Ca^{2+}-channel protein in commercial turkey skeletal muscle, which Ca^{2+}-channel protein isoform(s) are

responsible for the defect? Our results suggest that the β isoform may be a possible candidate. To clarify this question, both α and β isoforms will need to be purified from commercial and unimproved turkey and the biochemical properties will be compared. If a biochemical defect is identified in the channel protein isoform, sequencing of cDNA should indentify the specific defect. A simple genetic test could be developed to screen breeding stock for the presence of the defect gene.

The identity and function of the 75 kDa protein in some commercial turkeys are unknown and could be associated with the formation of PSE meat. Thus, this protein must be purified and characterized with respect to amino acid composition and sequence to identify this protein. If this protein cannot be identified as a known SR protein, then antibodies should be raised against this protein for localization and characterization of function.

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