This is to certify that the

thesis entitled

Sarcoplasmic Reticulum Ca2+-channel Protein Function and Regulation Differences in Random-Bred and Commercial Turkey Populations

presented by

Haiyan Zhang

has been accepted towards fulfillment
of the requirements for

Master degree in Food Science

Dale Strasburg
Major professor

Date 11/28/2000

SARCOPLASMIC RETICULUM Ca^{2+}-CHANNEL PROTEIN FUNCTION AND REGULATION DIFFERENCES IN RANDOM-BRED AND COMMERCIAL TURKEY POPULATIONS

By

Haiyan Zhang

A THESIS

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

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

East Lansing, Michigan
2000

ABSTRACT

SARCOPLASMIC RETICULUM Ca^{2+}-CHANNEL PROTEIN FUNCTION AND REGULATION DIFFERENCES IN RANDOME-BRED AND COMMERCIAL TURKEY POPULATIONS

BY

Haiyan Zhang

Pale, soft and exudative (PSE) meat is a major meat quality problem in the turkey processing industry. Its causes are likely rooted in both genetics and management. The purpose of this study is to test the hypothesis that a defect in one or both sarcoplasmic reticulum Ca^{2+} channel isoforms and/or altered Ca^{2+} channel regulation in a commercial turkey subpopulation contributes to the incidence of PSE meat. [^{3}H]Ryanodine binding assays were optimized for turkey skeletal muscle, and were used to compare two groups of commercial turkeys with random-bred control turkeys. Group I commercial turkeys (n=17) exhibited a higher ($p < 0.05$) affinity for ryanodine and lower ($p < 0.05$) Ca^{2+} channel protein content compared to that from random-bred turkeys (n =8) (K_d=8.4 vs 16.0 nM, B_{max}=5.9 vs 12.5 pmol/mg), suggesting altered function of Ca^{2+} channel in this commercial group. Group II commercial turkeys exhibited similar ryanodine binding constants to that from random-bred and significantly different from that of group I. These results suggest that there is one commercial subpopulation that possesses similar Ca^{2+} channel function to random-bred control turkeys. [^{3}H]PN200-110 binding assays yielded a coupling ratio of one dihydropyridine receptor molecule per ryanodine receptor. There was no significant difference in coupling ratio between commercial and random-bred turkeys.

ACKNOWLEDGEMENTS

I would like to give my special thanks to my major academic advisor, Dr. Gale M. Strasburg, for his knowledge, support and understanding during my study. He is an excellent teacher, good friend.

I am grateful to my committee members: Dr. Alden M. Booren, Matthew E Doumit, for their assistance and guidance. Thanks are also due to Dr. Peter Saama, for all the statistical consultant work. My appreciation extended to the friends and graduate students that I have been working with, for their help and encouragement.

I am extremely grateful for the love and support from my dear parents, Chunxu Zhang, Shuxia Zhao, and my sister, Hongyan Zhang.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The modern turkey industry has grown rapidly during the last 30 years to meet consumers' demand for lean and inexpensive turkey meat products. To meet this demand, breeders have intensively selected for heavy muscling and rapid growth of turkeys. Over the past three decades, the market age of turkeys has shortened from 26-28 weeks to 14-16 weeks, while the market weight has increased from about 20 lb to about 35 lb. However, the improvement in size and growth efficiency has come with a cost: increased incidence of meat quality defects including pale, soft, exudative (PSE) meat. This meat has soft texture, lighter color, decreased water holding capacity and poor cooking yield. Stresses like heat or cold, transportation, crowding, etc., could increase the incidence of the PSE in turkey (Froning et al., 1978).

The PSE problem in turkey seems to be associated with a rapid onset of rigor mortis that is similar to the development of the PSE condition that occurs in pig muscle after death (Greaser, 1986; Sosnicki, 1995). Pigs with the inherited disorder called Porcine Stress Syndrome (PSS) are more likely than non-stress-susceptible pigs to yield PSE pork. Porcine Stress Syndrome (PSS) is associated with the intensive selection for leanness and rapid growth. This syndrome causes substantial economic losses to farmers, owing to death of animals from stresses of transportation, heat, crowding, etc., before reaching market (Louis, 1993). After slaughter, PSS-susceptible pigs exhibit increased glycolytic rates during the conversion of muscle to meat, leading to higher carcass temperatures in combination with acidic pH that favors protein denaturation. This results in decreased protein solubility and poor water holding capacity. Fujii et al., (1991) reported that a single point mutation in the porcine gene for the skeletal muscle

sarcoplasmic reticulum Ca^{2+} channel was correlated with PSS in five major breeds of lean, heavily muscled swine. This Ca^{2+}-release channel protein regulates Ca^{2+} release from sarcoplasmic reticulum, thus triggering muscle contraction and glycolysis. The mutation in PSS-susceptible pigs results in higher channel protein activity, which is responsible for the accelerated glycolysis and heat production.

Based on the numerous similarities between development of PSE pork and PSE turkey, we hypothesize that there is a Ca^{2+}-channel defect in function and/or regulation existing in a subpopulation of commercial turkeys, which is responsible for the PSE meat quality problem. Preliminary studies by Wang et al. (1999) suggested that there could be genetic differences in ryanodine receptor between commercial turkeys and a genetically unimproved, random-bred control population. However, their assay conditions, which were adapted from procedures developed for porcine muscle, yielded significance only when comparing a large number of animals from the two turkey lines. Variation of assay results for individual birds made it impossible to use this assay for identification of differences in channel protein activity among individual birds. Therefore, the first aim of this study was to optimize ryanodine binding procedures for turkey skeletal muscle SR in order to do individual comparisons. The second aim was to use this assay to determine whether there exists a subpopulation of commercial turkeys with altered channel protein activity which could predispose these turkeys to the production of PSE meat. Finally, analysis of the coupling ratio of the voltage sensor (dihydropyridine receptor protein) to Ca^{2+}-channel was conducted to determine whether difference in coupling ratio could account for the increased incidence of PSE meat in commercial turkeys.

Chapter 1

LITERATURE REVIEW

I. Skeletal Muscle Structure

Skeletal muscle is made up of a group of muscle fibers surrounded and supported by the connective tissue proteins, collagen and elastin. Skeletal muscle fibers vary from 1 to 40 mm in length and from 10 to 100 μm in diameter (Leeson and Leeson, 1970). The muscle fiber or cell is the fundamental unit of muscle and in addition to the typical cellular organelles - nulei, mitochondria, etc. - a substantial volume of the cell consists of specialized contractile organelles called myofibrils. The membrane surrounding the myofiber is called the sarcolemma (Figure 1.1). The sarcolemma penetrates the myofiber, forming a network of transverse tubules (T-tubules). Together, the sarcolemma and the T-tubules play a key role in muscle contraction by transmitting the motor nerve impulse as a membrane depolarization from the neuromuscular junction along the length of the fiber. The T-tubules transmit the depolarization signal into the interior of the fiber to the sarcoplasmic reticulum (SR).

The SR is a specialized form of endoplasmic reticulum which consists of a series of enclosed membrane vesicles lying adjacent to both the T-tubules and the myofibrils. The SR serves as a reservoir of Ca^{2+}, which is released into the sarcoplasm when a muscle contraction stimulus is received. When the stimulus stops, Ca^{2+} is pumped back into SR, and the muscle relaxes.

SR can be separated into two fractions, based on differences in density, using sucrose gradient centrifugation. Heavy SR (HSR) is enriched in junctional membrane

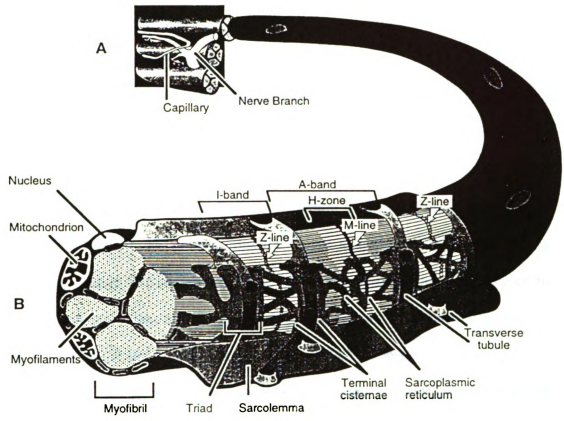


Figure 1.1 Diagrammatic representation of mammalian myofibers freed of their endomysium, demonstrating both A) the arrangement of their blood and nervous supply and B) the arrangement of the specialized membranous systems in relation to the protein scaffold (adapted from Strasburg and Byrem, 2000).

vesicles (including the Ca^{2+}-channel protein and calsequestrin), triads, terminal cisternae, etc. The light SR fraction (LSR) is mainly longitudinal tubule membrane vesicles enriched in the Ca^{2+} pump protein (Meissner, 1975; Franzini-Armstrong, 1980).

II. Excitation-Contraction Coupling

When an electrochemical signal from the motor nerve stimulates the plasma membrane of the skeletal muscle, the voltage sensor of the T-tubule membrane is activated and the signal is transmitted to the SR. Ca^{2+} is released from the SR to the sarcoplasm, causing the sarcoplasmic Ca^{2+} concentration to rise from less than 10^{-7} M at the rest to more than 10^{-5} M. Some of this Ca^{2+} binds to troponin C (TnC), causing a conformational change that alters the position of the troponin-tropomyosin complex in the thin filament, permitting actin and myosin to interact, and thus, leading to muscle contraction (Zot and Potter, 1987). The sarcoplasmic Ca^{2+} concentration is lowered by action of the CaATPase pump (Martonosi 1984). The process whereby depolarization of the sarcolemma is translated into muscle contraction is called Excitation-Contraction coupling (E-C coupling) (Franzini-Armstrong and Protasi, 1997).

There are three distinct E-C Coupling mechanisms found in muscle: 1) Ca^{2+}-induced Ca^{2+} release, i.e., the influx of Ca^{2+} through channels in the T-tubular membrane induces the release of Ca^{2+} from the SR. 2) Second messenger mechanism. The inositol 1,4,5-trisphosphate ($InsP_3$) released from the T-tubular membrane acts as a second messenger to induce the release of Ca^{2+} from the SR. 3) Mechanical coupling induced Ca^{2+} release. The dihydropyridine receptor (DHPR), which serves as the voltage sensor on the T-tubule membrane, physically transmits the signal to the Ca^{2+}-release channel or ryanodine receptor (RyR) in the SR through protein-protein interaction.

The various muscle types exhibit different mechanisms of E-C coupling. It is generally accepted that the mechanism of cardiac E-C coupling is Calcium-Induced Calcium Release (CICR). The first step in the flow of information following electrical excitation in heart muscle is Ca^{2+} influx through voltage-dependent Ca^{2+} channels in the T-tubule. Ryanodine receptor (RyR) Ca^{2+} channels are positioned in junctional SR elements within short distances (~20 nm) of voltage-dependent Ca^{2+} channels (DHPR) in the T-tubules of ventricular heart cells. Ryanodine receptors (RyRs) are activated by local Ca^{2+} which enters the cell through voltage-dependent Ca^{2+} channels ("local control") (Cannell et al., 1995; Cheng et al., 1996; Lopez-Lopez et al., 1995). Local Ca^{2+} in the subsarcolemmal junctional space is likely to reach relatively high concentrations (> 10 μM), corresponding to levels required for significant RyR channel activation. Ca^{2+} released through these activated RyR channels acts as an amplifier for further SR Ca^{2+} release. The SR ultimately contributes 90% of the Ca^{2+} needed for contraction of heart muscle (Jaggar et al., 2000). Thus, local Ca^{2+} control of Ca^{2+} release can explain the graded increases in Ca^{2+} release through RyR channels in response to Ca^{2+} influx in cardiac muscle (Cannell et al., 1995; Lopez-Lopez et al., 1995; Soeller and Cannell, 1997).

The mechanism of E-C coupling in skeletal muscle, however, was believed to differ from cardiac muscle because most DHPRs are non-functional as Ca^{2+} channels in skeletal muscle (Schwartz et al., 1985). Vergara et al. (1985) and Volpe et al. (1985) observed an activation of force after the application of $InsP_3$ to skinned skeletal muscle fibers. Both groups regarded this substance as a second messenger in the coupling process. They suggested that the action potential initiates $InsP_3$'s release from the

sarcoplasmic face of the sarcolemma. $InsP_3$ would diffuse across the gap and bind to a receptor at the SR, causing the release of Ca^{2+} from the terminal cisternae. However, a skeletal muscle $InsP_3$ receptor has not yet been identified unambiguously (Melzer et al., 1995). Also the Ca^{2+} release initiated by $InsP_3$ is hormonally activated while Ca^{2+} release from skeletal muscle is activated by depolarization (Caswell and Brandt, 1989).

Since the influx of Ca^{2+} and the release of $InsP_3$ apparently play no direct role in the E-C coupling process in skeletal muscle, the third possible mechanism, i.e., the voltage-dependent Ca^{2+} release, becomes the center of interest. This model involves protein-protein interactions between the Ca^{2+} release channel of the skeletal muscle SR or ryanodine receptor (RyR1) and the DHPR which leads to Ca^{2+} release from the SR (Meissner and Lu, 1995).

DHPR-mediated opening of Ca^{2+} release channels and Ca^{2+}-induced Ca^{2+} release channel opening have both been proposed to result in elementary Ca^{2+}-release events (sparks) that originate at the frog skeletal muscle triad and lead to further Ca^{2+} release (Klein et al., 1996). Tetracaine, which inhibits Ca^{2+}-induced Ca^{2+} release, eliminated all elementary Ca^{2+} release events, suggesting that Ca^{2+}-induced Ca^{2+} release, and not depolarization, is the mechanism for elementary Ca^{2+} release events. The phenylalkylamine D600, a well-known inhibitor of the membrane voltage sensor, suppressed elementary Ca^{2+} release events from SR and the peak of depolarization-induced Ca^{2+} release (Shirokova and Rios, 1997). The restoration of elementary Ca^{2+} release events and the peak of release by high voltage suggest that, in skeletal muscle, depolarization-induced Ca^{2+} release is the trigger for Ca^{2+}-induced Ca^{2+} release (Leong and MacLennan, 1998). Recent studies of rat muscle fibers suggest that localized Ca^{2+}

release does not exist in mammalian muscle, so that elementary events of Ca^{2+}-induced Ca^{2+} release cannot be a trigger for E-C coupling in mammalian muscle (Shirokova and Rios, 1998).

Upon repolarization, the DHPR undergoes a conformational change which is believed to be transferred to RyR1 to terminate Ca^{2+} release (Suda 1995). RyR1 has also been shown to enhance DHPR channel activity during E-C coupling (Nakai et al, 1996; Yamazawa et al, 1996) through a process called retrograde signaling. This leads further support to the mechanical coupling model of skeletal muscle E-C coupling.

III. Ca^{2+}-Channel Protein and Isoforms

A. SR Ca^{2+} Channel or Ryanodine Receptor

The SR Ca^{2+}-channel protein is an oligomeric protein complex located in the SR junction with the T-tubule, and constitutes about 2-3% of the total SR protein. This channel protein is also called the ryanodine receptor (RyR), because it binds the neutral plant alkaloid ryanodine with high affinity. The RyR complex includes four identical monomers with a calculated molecular mass of 565 kDa (Takeshima, et al. 1989). In addition, each RyR tetramer binds four molecules of calmodulin, four molecules of FK-506 binding protein and perhaps others. There are three isoforms of RyR. RyR1 is mainly found in skeletal muscle, RyR2 is present in cardiac muscle, and RyR1, RyR2 and a third isoform, RyR3, are found in brain as well as other non-muscle tissue. The three isoforms of RyR are remarkably similar in primary structure, consisting of ~ 5000 amino acids with a membrane-spanning hydrophobic domain at the carboxyl-terminal end and a large hydrophilic domain at the amino-terminal end (Takeshima, 1993).

Electron microscopic images of purified RyRs show a quatrefoil structure of 22-28 nm × 22-28 nm × 8-12 nm as is true of the junctional foot proteins seen in micrographs of muscle triads (Franzini-Armstrong and Jorgensen, 1994; Franzini-Armstrong and Protasi, 1997; Wagenknecht and Radermacher, 1997; Jeyakumar et al., 1998; Saito et al., 1988; Orlova et al., 1996). In the RyR1 structure, the hydrophobic domains of the four subunits comprise the baseplate which spans the SR membranes and forms the channel (Lai, et al. 1988, Wagenknecht, et al. 1989). The hydrophilic domains form the cytoplasmic foot structure which bridges the gap between T-tubule and the terminal cisterna of the SR membrane. Each of the four subunits apparently contributes equally to the formation of the foot and the channel region, and the complete tetramer is necessary for ryanodine binding and channel activity (Lai et al., 1988). There are no striking differences in general appearance among image reconstructions of the RyR isoforms (Ogawa et al., 1999).

The protein-staining pattern of mammalian skeletal muscle SR on SDS-PAGE shows a single band of RyR in contrast to closely spaced RyR double bands in gels of SR from frog skeletal muscle and chicken (Olivares et al., 1991; Murayama and Ogawa, 1992). Now it is generally accepted that there is only one ryanodine receptor isoform (RyR1) expressed in mammalian skeletal muscle, whereas there are two isoforms (α- and β-RyR) in most non-mammalian skeletal muscles, e.g. those of fish, chicken, bullfrog, and turkey. Airey et al. (1990) first identified the existence of two isoforms (α and β) in chicken pectoral muscles. In the chicken breast muscle cells, equal amounts of both isoforms are found in microsomes, but the α-isoform is more abundant in microsomes from chicken thigh muscle. Therefore, the two isoforms do not necessarily exist in equal

amounts (Ogawa, 1994). Lizard and snake skeletal muscles show a single band of α-RyR, while turtle, crocodile, fish, and avian show α- and β-RyRs (O'Brien et al., 1993). Certain specialized muscles such as extraocular and swimbladder muscles from fishes, which contract very rapidly, show only α-RyR while their body skeletal muscles have both α- and β-RyR (Ogawa et al., 1999).

Initial studies on comparisons of α and β isoforms of non-mammalian skeletal muscle with the RyR isoforms of mammals suggested that the α isoform was most homologous to RyR1 and that the β-RyR might correspond to RyR2 (Lai et al., 1992; Bull and Marengo, 1993). Airey et al. (1993a) identified a third distinct isoform in avian cardiac muscle, and cross reactivity was observed on Western blot analysis with anti-β-RyR antibody of microsomes from bullfrog cardiac muscle. Sequences of the cloned cDNAs showed that α- and β-RyR of frog skeletal muscle are most homologous to mammalian RyR1 and RyR3, respectively (Oyamada et al., 1994).

Despite the structural similarity and amino acid homology, the α and β isoforms exhibit significant functional differences. Both [^{3}H]ryanodine binding and single channel conductance studies indicate that the α isoform has a biphasic response to Ca^{2+} (O'Brien et al., 1995). The channel is activated at micromolar Ca^{2+}, with peak activation in the 1-10 μM range, and is subsequently inhibited by millimolar Ca^{2+}. This is strikingly similar to RyR1 from mammalian skeletal muscle. In addition to these physiological similarities, the α isoform of fish and other vertebrates is the specific target of Imperatoxin A, a scorpion toxin that selectively activates [^{3}H]ryanodine binding and channel activity of the mammalian skeletal muscle RyR isoform (Valdivia et al., 1994). O'Brien et al. (1995) also indicated the similarities between the fish skeletal muscle β isoform and the cardiac

muscle isoform from both mammals and fish. They are all relatively insensitive to inactivation by millimolar Ca^{2+} when studied either by [^{3}H]ryanodine binding (Pessah et al., 1985; Michalak et al., 1988; Chu et al., 1993) or single-channel recording (Rousseau et al., 1986; Chu et al., 1993). Furthermore, both the β isoform and the mammalian cardiac RyR are insensitive to Imperatoxin A (Valdivia et al., 1994).

There appears to be a physiological distinction in the role of α and β isoforms in muscle. A lethal mutation, crooked neck dwarf mutant, exists in chicks in which skeletal muscle cells lack α-RyR, whereas the β isoform is expressed normally. There is no response to electrical stimulation of the muscle, nor is Ca^{2+}-induced Ca^{2+} release observed, but Ca^{2+} is released with the application of caffeine (Airey et al., 1993b, c; Ivanenko et al., 1995). Likewise in the RyR1-knockout dyspedic mice, myotubes do not respond to electrical stimulation (Takeshima et al., 1994). These studies suggest that α-RyR is critically involved in the E-C coupling of the skeletal muscle, as is the case of RyR1 in mammalian skeletal muscle (Ogawa et al., 1999).

O'Brien et al. (1995) proposed a two component model for the calcium release process in non-mammalian vertebrate skeletal muscle. In this model, voltage-sensitive channels are gated by voltage change across the T-tubular membrane, presumably via the DHPR, whereas the other SR Ca^{2+} release channels are presumed to operate by Ca^{2+}-induced Ca^{2+} release. Activation of one channel by voltage would result in the release of Ca^{2+} and thereby activate a neighboring "slave" channel (Figure 1.2). The α RyR isoform is likely to be in RyR isoform directly coupled to DHPR, and the β isoform is a logical candidate to be the calcium-coupled RyR isoform because it has sequence and

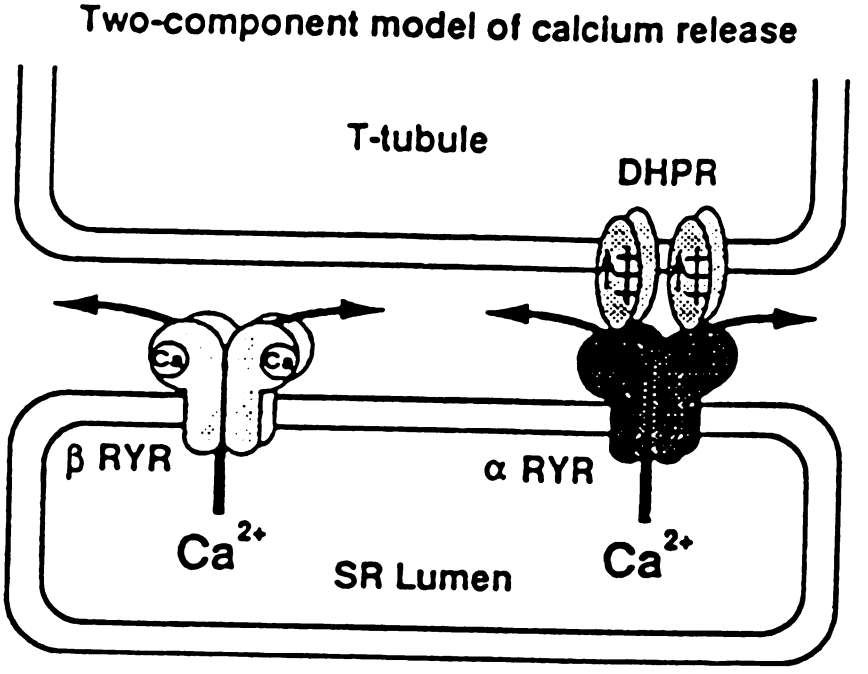


Figure 1.2 A two component model for the calcium release process in non-mammalian vertebrate skeletal muscle (adapted from O'Brien et al., 1995)

functional similarity to the cardiac and nonmuscle RyR, which operate by Ca^{2+}-induced Ca^{2+} release.

The cytosolic domain of RyR1 contains binding sites for various activating ligands including calcium (μM), ATP, calmodulin (which binds both in the absence and presence of calcium), caffeine and ryanodine (nM), and inactivating ligands such as calcium (> 100 μM) and magnesium in mM concentrations (Meissner, 1994, Coronado et al. 1994). Ryanodine modulates RyR1 in a biphasic manner; nM concentrations can activate while mM concentrations can inactivate the channel. RyRs in the open state can bind [^{3}H]ryanodine specifically and with high affinity (Bull et al., 1989; Lai et al., 1989; Chu et al., 1990).

B. Modulator Regulation of the Ca^{2+}-release Channel Protein (RyR)

1. Calcium (Ca^{2+})

Physiologically, one of the most important substances that modulates the gating of the RyR is Ca^{2+} (Ebashi, 1991; Fleischer and Inui, 1989; Meissner, 1994; Coronado et al., 1994). Ca^{2+} has a two-fold action on the RyR; it can activate the receptor at concentrations lower than 0.1 mM and inhibits the receptor at concentrations above 0.1 mM. In the absence of other regulatory ligands like Mg^{2+} and ATP, a bell-shaped Ca^{2+} activation curve of Ca^{2+} efflux from heavy SR vesicles is observed, with Ca^{2+} efflux being maximal in the micromolar Ca^{2+} concentration range (Fill et al., 1990; Kirino et al., 1983; Meissner et al., 1986a; Moutin and Dupont, 1988; Nagasaki and Kasai, 1983). Such a curve suggests that the Ca^{2+}-release channel possesses high-affinity activating and low-affinity inhibitory Ca^{2+} binding sites ($K_{0.5}$ values of ~ 1 μM and ~1 mM, respectively; Pessah et al., 1985; Michalak et al., 1988).

Many studies of RyR functional activity have been performed using the single channel, planar lipid bilayer technique. A single RyR channel is transferred into a planar lipid bilayer and ion conductance across the bilayer, via the channel, is recorded as a change in current. Smith et al. (1986b) reported that the channel's fraction of open time (P_o) is close to zero with nanomolar free Ca^{2+} on the *cis* (SR cytoplasmic) side of the bilayer. The addition of micromolar Ca^{2+} to the *cis* chamber activates the channel and induces rapid channel openings and closings occurring as single events and bursts that could last from less than one to many milliseconds.

[^{3}H]ryanodine binding measurements also suggest a bimodal regulation of RyR1 by Ca^{2+}, and further indicate that the affinity and cooperativity of interaction between high- and low- affinity [^{3}H]ryanodine binding sites are dependent on Ca^{2+} concentration and ionic strength (Chu et al., 1990; Lai et al., 1989; McGrew et al., 1989; Meissner and El-Hashem, 1992; Mickelson et al., 1990; Pessah et al., 1987).

2. Magnesium (Mg^{2+})

By measuring the $^{45}Ca^{2+}$ efflux from SR vesicles under various conditions, it was demonstrated that millimolar concentrations of Mg^{2+} inhibit Ca^{2+} release (Meissner, 1984; Nagasaki and Kasai, 1983). Likewise, in single channel recordings, cis 4 mM Mg^{2+} partially inhibits the Ca^{2+}-channel activity by decreasing open state probability of the channel (Lai et al., 1988).

Pessah et al. (1987) indicated that Mg^{2+} at physiological levels inhibits [^{3}H]ryanodine binding by decreasing both the affinity and the number of ryanodine-binding sites by altering the apparent affinity of activator site for Ca^{2+}. Mg^{2+} also significantly retards the association kinetics of ligand binding.

The multiple mechanisms proposed for the Mg^{2+} inhibitory effect are: 1) competitive displacement of Ca^{2+} from its activating high-affinity site (Pessah et al., 1987); 2) binding of Mg^{2+} to the inhibitory low-affinity Ca^{2+} binding site (Kirino et al., 1983; Meissner and El-Hashem, 1992); 3) Mg^{2+} could sterically block the Ca^{2+}-channel as it binds to a site near the conduction pathway in agreement with its known high permeability (Smith et al., 1986)

3. Adenine Nucleotides

ATP enhances Ca^{2+} release from isolated SR (Meissner, 1984; Smith et al., 1985), increases ryanodine binding (Campbell et al., 1987; Liu et al., 1989; Hymel et al., 1988; Michalak et al., 1988; Lai et al., 1988; Zarka and Shoshan-Barmatz, 1993), and activates single Ca^{2+} release channels incorporated into planar bilayers (Smith et al., 1985; Lai et al., 1988; Hymel et al., 1988; Ashley, 1989).

Pessah et al. (1987) reported that 1 mM AMP-PCP (the non-hydrolysable analog of ATP), in the presence of 1 mM Mg^{2+}, nearly triples the ryanodine-binding site affinity while increasing the total ryanodine binding by 2.5-fold. Other adenine nucleotides exhibit the same properties in modulating the [^{3}H]ryanodine-binding site with a potency order of AMP-PCP > cAMP > ADP~adenosine > AMP. They proposed that adenine nucleotides influence the kinetics of channel opening once Ca^{2+} is bound to the activator site, either by increasing the efficiency of the gating mechanism or by increasing the duration of the open state of the channel.

By using the planar lipid bilayer method, Smith et al. (1986b) reported that millimolar ATP increases the frequency of open events and decreases the duration of closed events (P_o = 0.6) in the absence of Ca^{2+}. Millimolar ATP and micromolar 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 ($P_o > 0.99$).

Meissner et al. (1997) indicated that by using AMP, the apparent affinity of the Ca^{2+} inactivation sites for Ca^{2+} is lowered by greater than 3-fold, whereas there is an increase (< 2-fold) in the Ca^{2+} affinity of the channel activation sites. AMP does not substantially increase the affinity of the Ca^{2+} activation sites for the competing cations (Mg^{2+} and monovalent). Thus, the primary effect of AMP is to decrease the Ca^{2+} affinity of the low-affinity channel inactivation sites.

While the specific involvement of an adenine nucleotide binding site in the potentiation of Ca^{2+} release channel activity is well established, the mechanism by which ATP produces its stimulatory effect remains unclear (Shoshan-Barmatz and Ashley, 1998). Other adenine nucleotides (AMP-PCP, ADP, AMP, cAMP, adenosine, and adenine) potentiate Ca^{2+} release (Meissner, 1984) and ryanodine binding (Michalak et al., 1988; Pessah et al., 1987; Zarka and shoshan-Barmatz, 1993), suggesting that activation involves binding to an effector site rather than covalent modification of the channel protein by phosphorylation.

4. Ryanodine

Ryanodine was first characterized after isolation and crystallization from the ground stem wood and root of *Ryania speciosa* Vahl by Rogers et al. (1948). Ryanodine is a neutral plant alkaloid that is soluble in water and alcohol. The structure of ryanodine is shown in Figure 1.3. Bianchi (1968) first suggested that ryanodine interferes with the release and uptake of Ca^{2+} by the sarcoplasmic reticulum (SR). Meissner (1986b) demonstrated that the effect of ryanodine on isolated SR vesicles is either to stimulate or

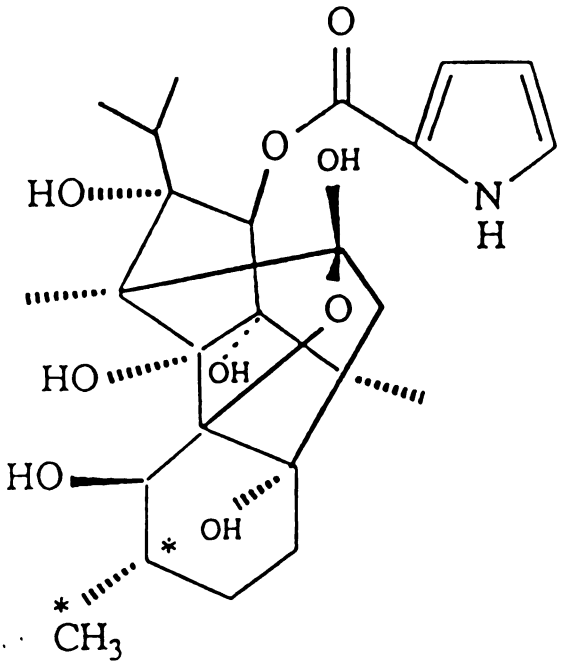


Figure 1.3 Ryanodine structure showing positions of tritium labels (9,21).

to inhibit Ca^{2+} efflux, depending on the experimental conditions. Vesicles are rendered permeable to $^{45}Ca^{2+}$ at a ryanodine concentration of 0.01 μM when diluted into a medium containing the two Ca^{2+} release channel inhibitors, Mg^{2+} and ruthenium red. At ryanodine concentrations greater than 10 μM, $^{45}Ca^{2+}$ efflux is inhibited in channel-activating (5 μM Ca^{2+}) or –inhibiting (10 mM Mg^{2+} plus 10 μM ruthenium red) media.

In both skeletal and cardiac muscle SR, high-affinity sites and a low-affinity [^{3}H]ryanodine-binding sites have been identified. At nM concentrations, ryanodine only binds to the open state of the Ca^{2+}-channel, locking it irreversibly into an open subconductance state. At μM concentrations, ryanodine binding results in closure of the channel. It is generally accepted that there is only a single high-affinity site for [^{3}H]ryanodine per RyR tetramer (Lai et al., 1988). The ratio of high-affinity (K_d = 8 nM) to low-affinity (K_d = 5 μM) binding sites has been reported to be 1:3 (Shoshan-Barmatz and Ashley, 1998). A model based on allosteric negative cooperativity between the four possible ryanodine binding sites in the homotetrameric RyR suggests that binding of ryanodine to one of the four available sites (with high affinity) triggers conversion of the other sites in the tetramer to low-affinity binding sites (Pessah and Zimany, 1991). In addition, the [Ca^{2+}-RyR] complex is very stable, and ryanodine only dissociates very slowly, with a $t_{1/2}$ of about 14 hours (Pessah et al., 1987). This supports the suggestion that the receptor undergoes a conformational change on binding Ca^{2+} and/or ryanodine so that ryanodine becomes physically occluded and cannot diffuse freely off its binding site (Shoshan-Barmatz and Ashley, 1998).

Because ryanodine binds to the open Ca^{2+} channel and keeps it open, and because the binding affinity is high and specific, the analysis of ryanodine binding to the channel

protein can provide important information about the Ca^{2+}-channel's functional state, its modulation by various compounds, and its distribution in various tissue (Shoshan-Barmatz and Ashley, 1998). In particular, ryanodine binding experiments may be used to identify difference in channel protein activity when a mutation has occurred (see section V).

5. Caffeine

The plant alkaloid 1,3,7-trimethylxanthine (caffeine) has been used extensively in studies of Ca^{2+} release (Stephenson, 1981; Sorenson et al., 1986; Endo, 1977; Palade, 1987; Fabiato, 1983; Meissner and Henderson, 1987). Pessah et al. (1987) showed that caffeine (20 mM) in the presence of 1 mM Mg^{2+} decreases the threshold for Ca^{2+} activation of [^{3}H]ryanodine binding by ~20-fold and increases the apparent affinity of the activator site for Ca^{2+} by 17- to 27-fold. Caffeine, therefore, appears to act on a domain which allosterically modulates the sensitivity of the Ca^{2+} activator site for Ca^{2+}, and reversing Mg^{2+} inhibition by selectively increasing the apparent affinity of the Ca^{2+} binding site for Ca^{2+}. Chu et al. (1990) reported that caffeine enhances the ryanodine association rate of the Ca^{2+}-channel protein without a change in the dissociation rate.

Caffeine increases the mean open times of the channels without changing their unit conductances. The mechanism of this activation is shown to involve both a Ca^{2+}-dependent increase in the frequency and duration of openings and a Ca^{2+}-dependent activation at high caffeine concentrations (Rousseau et al., 1988).

C. Dihydropyridine Receptor (DHPR) or L-type Ca^{2+} Channel

The skeletal muscle DHPR is a L-type Ca^{2+} channel, a member of a family of voltage-gated ion channels that includes Na^+, Ca^{2+} and K^+ channels (De Waard et al,

1995; Catterall, 1996), and is localized to the junctional T-tubule membrane. It is called dihydropyridine receptor (DHPR) because it has very high binding affinity for drugs that share the common basic structure of dihydropyridine. Examples of these drugs include nitrendipine, PN200-110 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihypro-2,6-dimethyl-methyoxy-carbonylpyridine-3-carboxylate) and azidopine. Biochemical evidence first indicated that DHPRs were located in T-tubules of adult skeletal muscle (Fosset et al., 1983). In skeletal muscle, DHPRs are a complex of five subunits α1 (185 kDa), α2 (143 kDa), β (54 kDa), γ (30 kDa) and δ (26 kDa) (Figure 1.4). The α1 subunit is the receptor for Ca^{2+} antagonists and can itself function as a voltage-gated ion channel (Lacerda et al. 1991; Mikami et al. 1989), while the other subunits modulate α1 channel activity. The structure of the α1 subunit has four homologous transmembrane 'repeats' (Figure 1.5), loop I, II, III, IV, each with 6 hydrophobic segments (S1-S6), which are thought to span the T-tubular membrane. The positively charged segment 4 (S4) of each repeat contains positively charged (Arg or Lys) residues in every third or fourth position, and is regarded as the voltage-sensing element (Melzer, et al. 1995). In Ca^{2+} channels, the III-IV loop has not been implicated in voltage-dependent inactivation, but S6 in domain I and the cytoplasmic loop linking domains I and II are involved in the inactivation of Ca^{2+} channels (Zhang et al., 1994). The cytoplasmic loop linking domain I and II interacts with the β subunit and the binding of the β subunit may modulate the inactivation process in the α1 subunit (Pragnell et al., 1994). The C-terminal region contains important elements for the regulation of Ca^{2+} channel activity (Wei et al., 1994). The skeletal α1 subunit loop linking domains II and III is involved in skeletal muscle E-C coupling

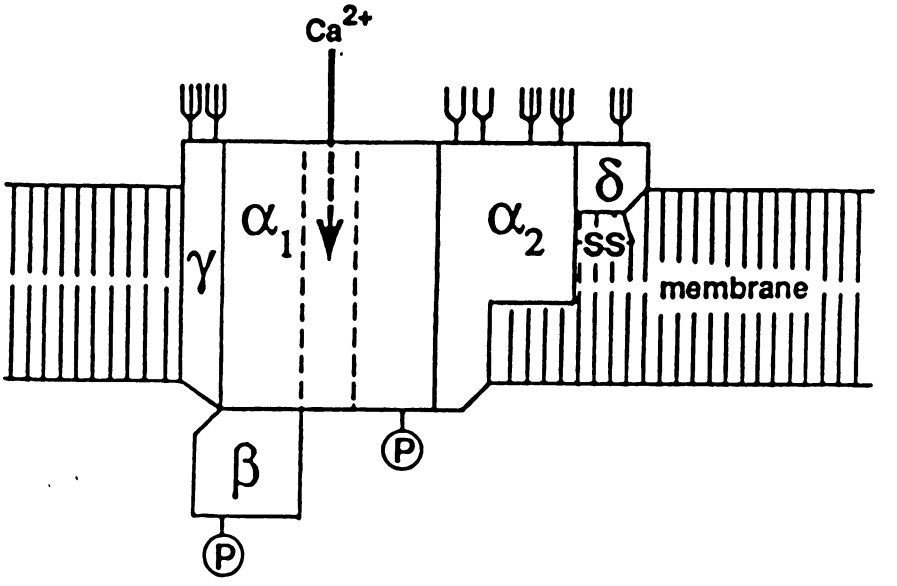


Figure 1.4 Model of the subunit structure of the dihydropyridine receptor (adapted from Catterall, 1988). P, sites of cAMP-dependent protein phosphorylation ; ψ, N-linked glycosylation.

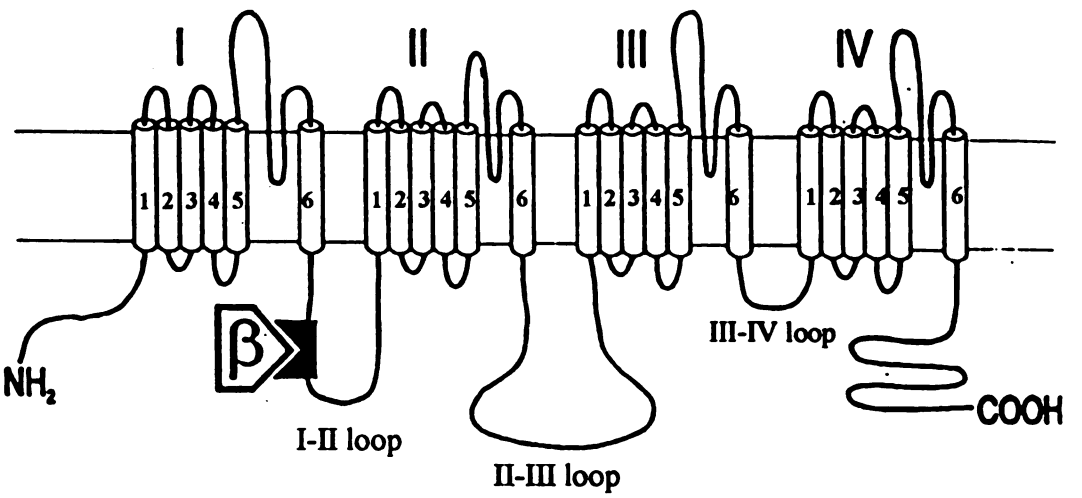


Figure 1.5 The structure of four homologous repeats of the DHPR α_1 subunit (adapted from Pragnell et al., 1994).

(Tanabe et al., 1990), and a mutation in the loop linking domain III and IV has been linked to malignant hyperthermia (Monnier et al., 1997), a condition resulting from abnormal Ca^{2+} release. A mutation in the DHPR III-IV loop is unlikely to alter Ca^{2+} conductance, since this loop does not form part of the channel pore and has not been identified as being important for either activation of inactivation of DHPR (Catterall, 1996), but could modify the coupling between the DHPR and ryanodine receptors by delaying or inhibiting closure of RyR1 upon repolarization (Suda, 1995).

The β hairpins, which form the aqueous pore, control the ion selectivity (Melzer, et al. 1995). The association of the β subunit with α1 increases the efficiency of coupling between voltage sensing and pore opening (Olcese et al. 1994) and increases the current amplitude substantially (Wei et al. 1991). Gregg et al. (1996) demonstrated that β_1-null mice which lack the primary β subunit in skeletal muscle die at birth from asphyxia. Also normal action potential in β_1-null myotubes fails to elicit a Ca^{2+} transient. L-type Ca^{2+} current is decreased 10- to 20-fold in β_1-null cells compared with littermate controls. Immunohistochemistry of cultured myotubes shows that not only is the β_1 subunit absent, but also the α_1 subunit is undetectable. These findings suggest that the β_1 subunit may not only play an important role in the transport or insertion of the α_{1s} subunit into the membrane, but also may be vital for the targeting of muscle DHPR complex to the T-tubule/SR junction (Ogawa et al., 1999).

IV. Protein-protein Interaction Between Ryanodine Receptor (RyR) and Dihydropyridine Receptor (DHPR) in Skeletal Muscle

The first biochemical evidence of direct interaction between proteins in the T-tubule membrane and the sarcoplasmic reticulum membrane was the observation that

^{125}I-labeled T-tubules, when mixed with sarcoplasmic reticulum, transferred ^{125}I to a protein doublet of molecular mass 325 and 300 kDa, which are major proteolytic fragments of RyR1 (Cadwell and Caswell, 1982). RyR1 and DHPR have been coimmunoprecipitated from triad vesicles solubilized in CHAPS, by antibodies directed against DHPR. Immunoprecipitation with antibodies against RyR1 also coprecipitates both RyR1 and DHPR, providing the evidence for binding between RyR1 and DHPR (Marty et al., 1994). Also, the activities of RyR1 and DHPR are reciprocally coupled, such that presence of the RyR1 stimulates the skeletal muscle DHPR α1 subunit and vice versa (Nakai et al., 1996). Yano et al. (1995) showed that by using a fluorescent conformational probe, a conformational change of the Ca^{2+} release channel and Ca^{2+} release are stimulated by depolarization. Nimodipine, an antagonist of the voltage sensor, inhibits both the conformational change and Ca^{2+} release, providing evidence for an interaction between the voltage sensor and the Ca^{2+} release channel that leads to the activation of channel opening (Rios and Brum, 1987).

Block et al. (1988) showed that in the swim-bladder muscle of toadfish, DHPRs are clustered in groups of four and positioned in exact correspondence with RyR1 tetramers so that each DHPR on the T-tubule membrane is located immediately opposite to one of the RyR1 subunits (Figure 1.6). Image analysis has determined that DHPRs are associated with RyR1 tetramers. The same disposition of DHPRs and RyRs is observed in cultured human muscle, mouse, rat and chicken myotubes (Franzini-Armstrong and Jorgensen, 1994; Franzini-Armstrong and Kish, 1995). Because the tetrad is composed of four DHPRs and there are two RyRs for every tetrad, the alternate RyR association pattern would predict a 2:1 ratio of DHPR to RyR (Franzini-Armstrong and Kish, 1995).

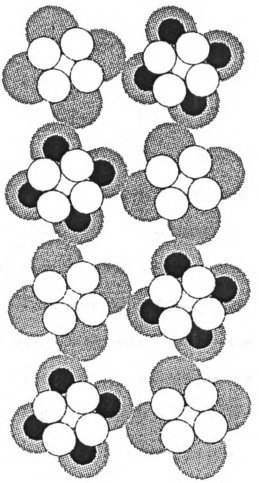


Figure 1.6 Diagram of the spatial relationships between the junctional complex components (adapted from Block et al., 1988). Four large shaded spheres represent each foot; small white spheres are the intramembranous portion of the foot protein; black discs represent the position of the junctional T tubule tetrads. In the diagram, tetrads interact with alternate feet.

However, various ratios from 0.6 to 2.1, determined by biochemical analysis, have been reported for skeletal muscle (Sutko and Airey, 1996).

Two mouse models show that the interaction between RyR1 and DHPR is essential for the E-C coupling. One is the dysgenic mouse model, in which the α_1 subunit of DHPR is knocked out. The muscles of these mice do not contract because of a lack of E-C coupling and they develop poorly (Powell and Fambrough, 1973; Klaus et al. 1983). The other is the RyR1-knockout or dyspedic mouse model in which E-C coupling is blocked (Takeshima et al. 1994). The triad junction does not have foot structures present in the dyspedic mouse (Franzini-Armstrong, 1999).

Protasi et al. (1998) indicated that DHPRs do not associate into tetrads in the absence of RyR1 and that tetrads are restored after "rescue" by RyR1 cDNA. These results provide strong support of two current hypotheses on junctional architecture. One is that DHPRs are linked to RyRs in skeletal muscle and this linkage is responsible for the formation of tetrads (Block et al., 1988; Takekura et al., 1994; Protasi et al., 1997). The other is that the random disposition of cardiac muscle DHPRs within junctional domains is the result of the absence of a link to the cardiac RyRs (Sun et al., 1995).

The interaction of RyR1 and DHPR is also important for the inactivation of Ca^{2+} release. Early studies showed that the Ca^{2+} release that occurs following depolarization is turned off by subsequent repolarization (Melzer et al., 1984, 1987; Schneider and Simon, 1988; Rios and Pizarro, 1991). The Ca^{2+} release flux starts to decay within a second after fiber repolarization, suggesting that there is a direct mechanical interaction between the voltage sensor and the release channel similar to the activation of Ca^{2+} release following depolarization (Rios and Pizarro, 1991).

In cardiac muscle cells, freeze-fracture electron micrograph images show that the pattern of DHPRs disposition differs from that in skeletal muscle cell. Franzini-Armstrong (1999) indicated that the DHPRs are in proximity of the feet, but do not seem to be directly linked to them. Thus, DHPR-RyR2 interaction may be indirect, via a transmitter (e.g., calcium). Sun et al. (1995) and Protasi et al. (1996) also reported that the DHPR is clustered in large intramembrane particles, but they do not form tetrads despite the presence of RyR2. The direct interaction between DHPR and RyR2 is probably missing in the cardiac muscle.

V. Ca^{2+}-channel Related Meat Quality Problem: Malignant Hyperthermia (MH), Porcine Stress Syndrome (PSS), Central Core Disease (CCD)

A. Malignant Hyperthermia and Porcine Stress Syndrome

Malignant hyperthermia (MH) has been recognized in humans since 1960 as an inherited disorder of skeletal muscle characterized by an accelerated muscle metabolism, contracture development, and rapidly rising temperature in response to certain anesthetics (Gronert, 1986; Ellis and Heffron, 1985). The syndrome occurs in about one out of 15,000 anesthetized children and one out of 50,000 anesthetized adult humans (ϕrding, 1996; Britt, 1970; ϕrding, 1985). Malignant hyperthermia can cause neurological, liver, or kidney damage, and is frequently fatal.

Also in the early 1960's, it was noted that certain strains of pigs showed a high frequency of susceptibility to stresses such as heat, crowding, transport, etc. Frequently, these pigs would exhibit an episode of MH similar to that in human MH. The similarity between the two diseases is further suggested by the observation that halothane also triggers MH in the stress-susceptible pigs. Malignant hyperthermia episodes in pigs are

usually fatal. Major economic losses in the pork processing industry result from the development of pale, soft and exudative (PSE) meat that arises from postmortem manifestation of MH in stress susceptible animals (O'Brien, 1987). Today, it is widely accepted that a significant fraction of pork PSE problem is related to the postmorterm hypermetabolic condition induced by stress. At the time of slaughter stress induces in these pigs a tremendous increase in aerobic and anaerobic metabolism, causing the production of heat, CO_2 and lactic acid and the contraction of skeletal muscle. High temperature in combination with rapid pH drop favors the protein denaturation during the conversion of muscle to meat. This results in the loss of protein water-holding-capacity and solubility, two main protein characteristics that determine the quality and acceptability of processed meat products (Cassens, 1975; Strasburg and Byrem, 2000). The gene associated with this syndrome is sometimes called *halothane* or *HAL* gene, stress gene, or PSS gene (Hall et al., 1980; Harrison, 1979). Since two alleles of this gene are presented in mammals, there are three porcine genotypes: homozygous normal (NN), heterozygous (Nn) and homozygous MH-susceptible (MHS) *HAL* gene (nn). The homozygous nn gene 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 than homozygous NN pigs (Zhang et al., 1992).

Studies seeking to define the primary defect in MHS muscle have utilized the porcine model of the syndrome which, both clinically and biochemically, very closely resembles the human syndrome in MHS muscle. Analysis of Ca^{2+} release from isolated SR fractions, skinned muscle fibers, or muscle fiber bundles, suggested a defect

associates with the Ca^{2+}-induced calcium release mechanism of SR or with its control (Mickelson et al., 1988).

A defect in the Ca^{2+}-release mechanism was originally proposed to be the cause of MH because MH muscle is hypersensitive to halothane and caffeine (Endo, 1977). While MH SR seems to have normal Ca^{2+} uptake, several groups reported abnormal Ca^{2+} release from MHS SR vesicles (Endo et al. 1983; Takagi et al. 1983; Nelson 1983; Ohnishi 1987; Kim et al. 1984; Mickelson et al., 1986). Mickelson et al. (1988) used a [^{3}H]ryanodine binding approach to examine the SR Ca^{2+}-channel protein function in PSS-susceptible pigs. Their results suggested that the ryanodine receptor of isolated MHS porcine heavy SR exhibits an altered Ca^{2+} dependence of [^{3}H]ryanodine binding at the inhibitory Ca^{2+} binding site as well as a lower K_d for ryanodine (92 vs 265 nM) when compared to normal porcine SR. The channel protein content of the normal and MHS [^{3}H]ryanodine receptor in heavy SR vesicles (9.3-12.6 pmol/mg) is not significantly different. They concluded that since the SR ryanodine receptor is a Ca^{2+} release channel as well as a component intimately involved in transverse tubule-SR communication, abnormalities in the skeletal muscle ryanodine receptor may be responsible for the abnormal SR Ca^{2+} release and contractile properties demonstrated by MH muscle. The landmark paper published by Fujii et al. (1991) reported the ultimate cause of PSS in pigs is a mutation in the skeletal muscle Ca^{2+}-channel protein at the residue 615, in which arginine is substituted by a cysteine. This discovery has led to the development of a rapid screening test for the presence of the undesirable alleles in breeding stock.

Like the pork industry, the modern turkey industry has successfully bred for rapid growth and heavy muscling in birds to meet consumers' demand for lean and inexpensive

turkey meat products. However, in recent years, difficulties have emerged in further processing of turkey breast muscle. Current problems with the soft texture, poor cohesiveness, and poor juiciness of processed turkey breast muscle result in multi-million-dollar yearly losses to the turkey industry (Foegeding, 1992). Previous studies suggested that several similarities exist between the development of PSE meat from swine and defective muscle quality in domestic turkeys (Sosnicki and Wilson, 1991, 1992; Sosnicki, 1993a,b). Heat, stress and struggling prior to slaughter can trigger acceleration of postmortem glycolysis in both pork (Mitchell and Heffron, 1982) and turkeys (Froning, 1978; McKee and Sams, 1997), and cause rapid pH drop. The combination of high temperature with quick pH decline favors protein denaturation during the conversion of muscle to meat. Pietrzak et al. (1997) showed that the fast postmortem glycolysis group (PSE group) is characterized by lower muscle ATP, higher lactate levels, lower water holding capacity, lower cooking yield, and lighter color compared with the slow glycolysis group (control group). Numerous similarities between development of PSE pork and PSE turkey strongly suggest that the genetic selection for rapid growth rate could result in inadvertent selection for a mutation of the Ca^{2+}-release channel protein in skeletal muscle and subsequent undesirable meat quality problem.

The biochemical approaches used to study the Ca^{2+} release mechanism in stress susceptible pigs were facilitated by the availability of genetically defined normal and MH pigs. Utilization of the same approach in turkeys is hampered by the fact that there are no phenotypic markers to identify normal and abnormal populations. Wang et al. (1999) assumed that the frequency of a mutation would be greater in modern commercial line of turkeys compared to a genetically unimproved line. Sarcoplasmic reticulum was isolated

from these two lines for comparison of ryanodine binding properties. The SR fractions, enriched in the Ca^{2+}-channel protein, from commercial turkeys exhibit a higher mean affinity for ryanodine when compared to that from unimproved turkeys (K_d = 12.2 vs 20.5 nM). A four-fold difference ($p < 0.05$) in mean Ca^{2+}-channel protein content or B_{max} (1.10 vs 4.01 pmol/mg) is observed between commercial and genetically unimproved turkey SR fractions. This suggests that there is an alteration in RyR activity in either or both isoforms of the commercial turkeys compared to controls.

B. Central Core Disease

Central Core Disease (CCD) is a rare, nonprogressive myopathy, presenting in human infancy, which is characterized by hypotonia and proximal muscle weakness. It is inherited as an autosomal dominant trait. An important feature of CCD is its close association with susceptibility to MH (Denborough et al., 1973; Brownell, 1988; Eng et al., 1978; Frank et al., 1980). Diagnosis of CCD is by histologic examination of skeletal muscle biopsy specimens. Typical CCD samples show amorphous central cores in type 1 fibers. Identification of cores is facilitated by staining for oxidative enzyme activity. As the cores are depleted of mitochondria, they appear as negative areas within the normal enzyme activity areas of the surrounding muscle fibers (Ogawa, 1994). There are numerous reports indicating a gene mutation of the RyR1 in the MH/CCD disorder (Table 1.1).

Mills (2000) observed central core fiber damage in commercial turkey leg muscles. She suggested that this could indicate mitochondrial abnormalities or alterations in the distribution of mitochondria due to the large fiber sizes, both of which would affect muscle metabolism and possibly meat quality. The percentage of fibers exhibiting

damage in the breast muscles is significantly greater in the commercial strain and increases linearly with age. Muscle damage only occurs at low levels in the traditional strain with no apparent relationship to age. The commercial birds also show a significantly greater hyperthermia than the traditional birds with a mean rise of 4°C compared to only 2°C in the traditional line ($p < 0.05$). It is possible that these central core fibers have arisen as a result of a mutation in either or both of the ryanodine receptors in commercial turkeys in similar fashion to human with central core disease.

Table 1.1 Mutations in Skeletal Muscle Ca^{2+}-channel Protein (RyR1) in Human MH/CCD (adapted from Jurkat-Rott et al. 2000)

Nucleotide	Substitution	Disorder	Frequency	Ref.
T103C	Cys-35-Arg	MH	1 family	Lynch et al. 1997
C487T	Arg-163-Cys	MH; CCD	2%	Quane et al. 1993
G742A	Gly-248-Arg	MH	2%	Gillard et al. 1992
G1021A	Gly-341-Arg	MH	6%	Quane et al. 1994a
C1209G	Ile-403-Met	CCD	1 family	Quane et al. 1993
A1565C	Tyr-522-Ser	MH; CCD	1 family	Quane et al. 1994b
C1654T	Arg-552-Trp	MH	1 family	Keating et al. 1997
C1840T	Arg-614-Cys	MH	4%	Gillard et al. 1991
G1841T	Arg-614-Leu	MH	2%	Quane et al. 1997
C6487T	Arg-2163-Cys	MH	4%	Manning et al. 1998a
G6488A	Arg-2163-His	MH; CCD	1 family	Manning et al. 1998a
G6502A	Val-2168-Met	MH	7%	Manning et al. 1998a
C6617T	Thr-2206-Met	MH	1 family	Manning et al. 1998a
C6617G	Thr-2206-Arg	MH	1 family	Brandt etal. 1999
G7303A	Gly-2434-Arg	MH	4%	Keating et al. 1994
G7307A	Gly-2435-His	MH; CCD	1 family	Zhang et al. 1993
G7307T	Arg-2435-Leu	MH	1 family	Barone etal. 1999
G7361A	Arg-2454-His	MH	1 family	Barone etal. 1999
C7360T	Arg-2454-Cys	MH	1 family	Brandt etal. 1999
C7372T	Arg-2458-Cys	MH	4%	Manning et al. 1998b
G7373A	Arg-2458-His	MH	4%	Manning et al. 1998b
T14693C	Ile-4898-Thr	CCD	1 family	Lynch et al. 1999

Chapter 2

Modification of experimental conditions for ryanodine binding assay of turkey skeletal muscle

I. Introduction

In skeletal muscle, intracellular Ca^{2+} channels release Ca^{2+} from the sarcoplasmic reticulum (SR) to the sarcoplasm in response to a depolarization potential, which causes muscle contraction. The SR Ca^{2+}-release channel is also known as the ryanodine receptor (RyR) because the channel protein can bind the plant alkaloid ryanodine with very high affinity.

The activity of the mammalian skeletal muscle ryanodine receptor isoform (RyR1) can be modulated by several physiological and pharmacological factors: μM Ca^{2+}, mM adenine nuleotides, caffeine, high salt concentration, and nM ryanodine stimulate the channel activity, whereas mM Mg^{2+}, ruthenium red, mM Ca^{2+} and μM ryanodine inhibit the activity (Nagasaki and Kasai, 1983; Ikemoto et al., 1985; Meissner et al., 1986b; Smith et al., 1985; 1986a; b). RyR1 exists as a tetramer of four identical subunits. There is one high affinity ryanodine binding site per tetramer, and 3-4 low affinity sites. Ryanodine binds to the open state of the channel, and this characteristic has been exploited as a tool to study functional activity of RyR1 under various conditions.

Ryanodine binding has previously been used as a tool to probe the functional activity of the calcium release mechanism in normal and stress-susceptible pigs. A substantial fraction of meat from these stress-susceptible pigs exhibits a quality defect referred to as pale, soft and exuative (PSE) meat. Ryanodine binding studies indicated that SR from stress-susceptible pigs displays higher affinity ryanodine binding activity,

which suggests that there is a mutation in the ryanodine receptor. Fujii et al. (1991) identified a mutation of arginine to cysteine at residue 615 which leads to increased channel activity and is associated with PSE meat. Wang et al. (1999) analyzed the ryanodine binding activity of genetically unimproved and commercial lines of turkeys. They concluded that there is a highly significant difference ($p < 0.05$) in ryanodine receptor activity between these two lines. However, because of variation in the assay results, statistical significance could only be achieved by averaging of several individuals from each of the two populations. This variation makes it impossible to use ryanodine binding activity as a screening tool to identify individual turkeys with altered channel proteins. One reason for this could be a result of differences between the mammalian and avian skeletal muscle E-C coupling mechanisms. Therefore, the objective of this portion of the study was to identify buffer conditions that effectively distinguish individual differences of ryanodine receptor activities in avian muscle. In this set of experiments, the concentrations of sucrose, salt and adenine nucleotide, factors which increase the open state probability and affinity of the channel for ryanodine, were varied to define the optimal ryanodine binding conditions for an eventual correlation of individual ryanodine binding affinity with meat quality.

II. Materials and Methods

A. Materials

1. Skeletal Muscle

Breast muscle samples from two Nicholas turkeys were provided by the Tom Otto turkey farm (Middleville, MI), a commercial producer. Pectoralis major muscles were cut into one-inch cubes and immediately frozen in liquid nitrogen and stored at -80°C.

2. Chemicals

Ryanodine was purchased from Wako Chemical USA, Inc. (Richmond, VA), radioactive ryanodine ([^{3}H]Ryanodine) was purchased from New England Nuclear Life Science (Boston, MA). Phenylmethylsulfonyl fluoride (PMSF), aprotinin, benzamidine, pepstatin, leupeptin were purchased from Sigma (St. Louis, MO).

B. Methods

1. Preparation of Heavy Sarcoplasmic Reticulum Vesicles (HSR)

Skeletal muscle SR was isolated from turkey breast muscle samples by the following procedure adapted from Mickelson et al. (1986) and Wang et al. (1999) with minor modifications. Frozen muscle cubes (200 g) were thawed at 4°C for 20 min, incubated with 5 volumes of Buffer I (0.1 M NaCl, 5 mM Tris-maleate, pH 7.0) for 5 min., and homogenized in a Waring blender for 2 min with intervals of 15 sec. on and 15 sec. off. After 30 min centrifugation at 3300 x g_{max}, the supernatant was collected by filtering through a 16-layer cheesecloth. The pellet was discarded, and the supernatant was centrifuged for 30 min at 16,000 x g_{max}. This pellet was collected, resuspended with 20 ml Buffer II (0.6 M KCl, 5 mM Tris-maleate, pH 7.0), and gently homogenized in a Dounce homogenizer. The homogenized suspension was centrifuged for 40 min. at

150,000 x g_{max} using a Beckman Ti-70 rotor. The pellet was collected and suspended in about 5-7 ml 10% sucrose solution, and gently homogenized in a Dounce homogenizer. The crude SR vesicles obtained by this step were flash-frozen in liquid nitrogen and stored at -80°C.

Heavy SR (HSR) vesicles, enriched in the Ca^{2+} channel protein, were isolated by suspending ~1-2 g crude SR in 2 ml of Buffer III (10% sucrose, 0.4 M KCl, 5 mM Tris-maleate, pH 7.0) and loading the suspension on discontinuous sucrose gradients (22%, 36%, 45%). The gradients were centrifuged for 5 h at 100,000 x g_{max} in a Dupont-Sorvall AH 629 rotor. The HSR was collected at interface between the 45% and 36% sucrose layers. HSR fractions were diluted at least 1:1 with 10% sucrose and centrifuged at 150,000 x g_{max} in the Beckman Ti-70 rotor for 40 min. The pellet was suspended and homogenized in 10% sucrose, flash-frozen and stored at -80°C.

During the HSR protein purification, the following protease inhibitors were added at each step: PMSF at 0.2 mM, and aprotinin, benzamidine, pepstatin and leupeptin at 1 μg/ml, to Buffer I, II, III, 10% sucrose and sucrose gradients. Beta-mecaptoethanol was also added at a final concentration of 30 mM to Buffers I and II.

B. Ryanodine Binding Assays

Fifty μg of HSR protein were incubated in buffer containing various [^{3}H]ryanodine concentrations (2-70 nM), 10 mM MOPS, a $CaCl_2$-EGTA-nitrilotriacetic acid buffer to give a specific $[Ca^{2+}]_{free}$ of 10 μM (pH 7.2), plus combinations of NaCl (0.1 M, 0.5 M, 1 M), sucrose (0.1 M, 0.5 M, 1 M) and AMP-PCP (0, 1 mM) in a 3×3×2 matrix. Triplicate samples of each condition were incubated for 6 h at 37°C, and filtered by Whatman GF/B filters. Filters were washed three times with 5 ml ice-cold buffer (10

mM MOPS, 0.1 M NaCl, 50 μM $CaCl_2$, pH 7.0), and the amount of [^{3}H]ryanodine retained on the filters was determined by scintillation counting. Non-specific binding of ryanodine was determined by adding unlabeled ryanodine at 1000 times the [^{3}H]ryanodine in the incubation buffer. Specific ryanodine binding was determined by subtracting the non-specific binding from the total binding.

The ryanodine binding conditions selected from the 3×3×2 matrix were also compared with the results using the conditions of Wang et al. (1999). In the latter case, the buffer used for ryanodine binding consisted of 0.25 M KCl, 25 mM PIPES (pH 7.0), a $CaCl_2$-EGTA-nitrilotriacetic acid buffer to give a specific $[Ca^{2+}]_{free}$ of 10 μM.

The K_d (ryanodine binding affinity) and B_{max} (maximum binding capacity) values for ryanodine binding by the HSR were calculated by Scatchard analysis using Sigma Plot.

3. Protein Assay

HSR protein concentration was determined by the method of Lowry (1951). Bovine serum albumin was used as a standard.

4. Statistical Analysis

The Durbin-Watson test was used to compare the consistency of both Wang's (Wang et al., 1999) and the current condition.

III. Results and Discussion

The ryanodine binding assay is a useful tool to analyze the function of the Ca^{2+}-channel protein in the SR, because ryanodine only binds to the open state of the Ca^{2+}-channel protein (RyR) with high affinity. The affinity for ryanodine and open state probability of RyR are modulated by various effectors. This property was exploited by Mickelson et al. (1988) to define differences in channel protein activity of normal and stress-susceptible pigs. The buffer condition used for analysis of ryanodine binding of pig SR, however, gives significant variation within individual experiments when adapted for turkey SR, suggesting that optimization of the assay condition was required for turkey SR. The aim of this study was to define conditions that will facilitate the comparison of the individual RyR functional differences between the random-bred and commercial turkey populations.

The ryanodine binding assay was conducted by using two Nicholas commercial turkey breast muscle samples (Turkey 1 and Turkey 2). Three salt concentrations (0.1 M, 0.5 M, 1 M), three sucrose concentrations (0.1 M, 0.5 M, 1 M), and two AMP-PCP concentrations (0, 1 mM), were chosen to optimize the ryanodine binding buffer condition. AMP-PCP is the nonhydrolyzable analog of ATP and was used instead of ATP to avoid possible interference from phosphorylation. The equilibrium constants obtained by Scatchard analysis of [^{3}H]ryanodine binding under different conditions are summarized in Table 2.1. The eighteen combinations of salt, sucrose and AMP-PCP for each sample caused the dissociation constant (K_d) to vary tremendously, while no dramatic difference was found in the total binding capacity (B_{max}) values within each

Table 2.1 Equilibrium Constants of Ryanodine Binding of Heavy Sarcoplasmic Reticulum Fractions from Two Turkey Breast Muscle Samples

		Turkey 1				Turkey 2			
		no AMP-PCP		1 mM AMP-PCP		no AMP-PCP		1 mM AMP-PCP	
[NaCl]	[sucrose]	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d
(M)	(M)	(pmol/mg)	(nM)	(pmol/mg)	(nM)	(pmol/mg)	(nM)	(pmol/mg)	(nM)
0.1	0.1	4.9	23.7	8.1	14.0	17.2	81.9	20.8	22.3
0.5	0.1	6.7	4.9	8.7	5.3	19.3	12.5	18.2	6.5
1.0	0.1	8.2	4.5	10.4	14.0	13.7	3.5	18.9	5.2
0.1	0.5	7.5	19.5	11.4	12.2	16.3	27.9	20.7	13.6
0.5	0.5	8.9	5.7	10.5	6.4	20.1	11.0	23.6	5.0
1.0	0.5	7.7	8.2	9.4	2.3	17.7	4.2	25.4	3.5
0.1	1.0	7.7	9.9	10.6	6.3	18.7	19.0	15.9	8.3
0.5	1.0	7.2	3.9	8.3	3.1	19.6	7.4	17.8	5.3
1.0	1.0	12.6	3.5	11.1	5.0	17.5	2.7	18.2	3.6

sample. For Turkey 1, the lowest K_d value was 2.3 nM, while the highest was 23.7 nM, and the average B_{max} value was 8.9 ± 1.9 pmol/mg. For Turkey 2, the lowest K_d value was 2.7 nM, while the highest was 81.9 nM, and the average B_{max} value was 18.9 ± 2.7 pmol/mg.

The binding affinity of [^{3}H]ryanodine to RyR is markedly dependent on the presence of compounds in the binding buffer, which are known to alter Ca^{2+} release from the SR. As a general rule, conditions that open the channel, such as the presence of micromolar Ca^{2+}, millimolar adenine nucleotide, or high ionic strength, are found to increase the affinity of [^{3}H]ryanodine to the high affinity ryanodine binding site (Meissner et al., 1997). Each of the factors included in this study has been reported to increase the open state probability and affinity of the channel alone.

It was observed that either in the presence or absence of 1 mM AMP-PCP, when the salt concentration was increased, the K_d decreased in both samples. For Turkey 1, in the presence of 0.1 M NaCl and 0.1 M sucrose, the K_d was 23.7 nM in the absence of 1 mM AMP-PCP and 14.0 nM in the presence of AMP-PCP. The K_d's derived from 1 M NaCl plus 1 M sucrose were only 3.5 nM and 5.0 nM in the absence or presence of AMP-PCP, respectively. The same trend was observed from the Turkey 2 skeletal muscle sample. Therefore, high concentrations of NaCl can increase the binding affinity of [^{3}H]ryanodine to the receptors. Chu et al. (1990) reported that the apparent affinity of RyR for ryanodine is increased upon increasing NaCl concentrations from 25 mM to 2 M, via an increase in the rate of association of ryanodine. High salt is known to remove loosely associated proteins and molecules and may, therefore, be enhancing the apparent association of ryanodine by removing an endogenous occupant of the site. However,

because the effects of ionic strength (both high and low) on the properties of the protein appear to be totally reversible, they concluded that it is most likely that the conformation of the binding site is dependent on ionic strength. Meissner et al. (1997) observed that replacement of Cl^- with 2-[N-Morpholino]ethanesulfonic acid (Mes^-) in 0.25 M $choline^+$ media lowered [^{3}H]ryanodine binding (B_{max} = 10.8 vs 12.4 pmol/mg, K_d = 1.8 vs 11.6 nM) . They suggested that an increase in [Cl^-] is responsible for elevating the [^{3}H]ryanodine binding levels by increasing the apparent Ca^{2+} affinity of the Ca^{2+} activation sites and decreasing the apparent Ca^{2+} binding affinity of the channel inactivation site (Meissner et al., 1997).

In contrast to the effect of varied salt concentration on the [^{3}H]ryanodine binding, there is no clear trend of the effect of sucrose on the [^{3}H]ryanodine binding among all the combinations of conditions. However, for turkey 1, under the 0.1 M [NaCl] condition, the K_d was 23.7 nM and 14.0 nM in the absence and presence of 1 mM AMP-PCP, respectively, at a sucrose concentration of 0.1 M. When [sucrose] was raised to 0.5 M, the K_d values were 19.5 nM and 12.2 nM in the absence and presence of 1 mM AMP-PCP, respectively. Then, as the [sucrose] increased to 1 M, the K_d was decreased to 9.91 nM and 6.30 nM in the absence and presence of AMP-PCP, respectively. The data suggested that the [^{3}H]ryanodine binding is increased by sucrose through increase of the affinity under 0.1 M salt concentration. The same trend was also observed in the Turkey 2 sample.

Certain concentrations of salt and sucrose can increase the [^{3}H]ryanodine binding affinity. Salt can change the ionic strength while sucrose changes the osmolarity of the [^{3}H]ryanodine binding buffer. A stimulation of [^{3}H]ryanodine binding (Ogawa and

Harafuji, 1990) and slowing the single channel gating (Tu et al., 1994) by sucrose in the presence of salt suggests that the osmolarity and viscosity of the assay media may play a role in determining channel activity (Meissner, et al., 1997). There are two controversial conclusions about whether it is osmolarity or ionic strength that stimulates the [^{3}H]ryanodine binding. Ogawa and Harafuji (1990) examined the effect of the composition of the reaction medium on ryanodine binding activity of bullfrog skeletal muscle SR. NaCl, KCl and sucrose were tested individually and in combinations at the same osmolarity, and similar ryanodine binding results were obtained. The [^{3}H]ryanodine binding reached a plateau at an osmolarity level of 3.0 osm/liter. This suggests that osmolarity, but not ionic strength, is a crucial factor for ryanodine binding activity. Lai et al. (1989) showed that the tetramer, but not the monomer of the ryanodine binding protein binds ryanodine. The high osmolarity may be favorable for oligomerization of the ryanodine binding protein by reducing the mole fraction of water molecules (Ogawa and Harafuji, 1990). In contrast, Meissner et al. (1997) reported that in experiments on Ca^{2+} dependence of [^{3}H]ryanodine binding to the SR vesicles from rabbit skeletal muscle, the nonionic solution (0.5 M sucrose) with an osmolarity comparable to that of the salts, give very low levels of [^{3}H]ryanodine binding (< 0.05 pmol/mg protein). The results suggested that sucrose can activate the RyR only to a limited extent compared to the effects of salt. Both of these two groups used sucrose and salts at comparable osmolarity, but obtained opposite results. The difference could be the result of the different species they used (mammalian vs amphibian), and/or the different composition of the [^{3}H]ryanodine binding buffer.

In this study, no clear trend was observed to determine whether salt or sucrose has more effect on [^{3}H]ryanodine binding than the other. The combination effect may be playing a greater role rather than either alone.

AMP-PCP was included in some of these experiments to optimize the [^{3}H]ryanodine binding because it is generally recognized that AMP-PCP can enhance ryanodine binding and thus provide a more quantitative measurement of high affinity ryanodine binding (Pessah et al., 1987; Michalak et al., 1988; Zarka and Shoshan-Barmatz, 1993). Among the total thirty-six dissociation constants (K_d) through different test conditions for both samples, 1 mM AMP-PCP decreased the K_d in most cases. Pessah et al. (1987) indicated that 1 mM AMP-PCP with the presence of 1 mM Mg^{2+} nearly triples the [^{3}H]ryanodine binding site affinity, while increasing the B_{max} 2.5-fold, AMP-PCP possibly elicits strong positive cooperativity at the [^{3}H]ryanodine binding site. In this study, 10 μM free Ca^{2+}, 1 mM AMP-PCP were included in the ryanodine binding buffer solution, and this appears to efficiently activate the SR Ca^{2+}release channel and produces channel activation (Smith et al., 1986b; 1988). Under these conditions the open-channel form is predominant, and exhibits a full conductance characteristic for the native channel (Serysheva, et al., 1999).

Salt, sucrose and AMP-PCP are all capable of enhancing the open state probability of the Ca^{2+} channel and thus, enhancing the [^{3}H]ryanodine binding. Different combinations at various concentrations have additive or even synergistic effects. Under the condition of 1 M NaCl, 1 M sucrose and 1 mM AMP-PCP, high [^{3}H]ryanodine binding affinities were obtained (K_d = 5.03 nM and 3.55 nM, Turkey 1 and Turkey 2, respectively), thus reflecting the increased open state of the Ca^{2+}-release channel. The

B_{max} values obtained at these conditions reflect upper limits of maximal binding capacity. However, in order to distinguish differences in RyR activities between random-bred and commercial turkey populations, a lower affinity range (higher K_d) is preferred. Under the condition in which the [^{3}H]ryanodine has high binding affinity for the RyR, little difference in affinity can be distinguished. In comparison of MHS and normal pig SR, for example, Mickelson et al. (1990) reported that the affinity of the MHS RyR for [^{3}H]ryanodine in the presence of 6 μM Ca^{2+} plus 10 mM AMP-PCP (K_d = 6.0 nM) is almost identical to that of the normal SR (K_d = 8.5 nM). However, in the presence of 6 μM Ca^{2+} alone, the K_d values are 92 nM vs 265 nM from MHS and normal pigs, respectively. The K_d's derived from the condition of 0.1 M NaCl, 0.5 M sucrose and no AMP-PCP were 19.54 nM and 27.86 nM, while the B_{max} were 7.52 pmol/mg and 16.34 pmol/mg from Turkey 1 and Turkey 2, respectively. The affinity constants were the second highest among 18 values in each sample, while the total binding capacities were within the average range. Therefore, this condition can provide the best measurement of the receptor content (B_{max}) while giving a high K_d value, and was chosen as the optimal condition for the turkey skeletal muscle ryanodine binding assay.

The results from the current ryanodine binding conditions were compared with those from the conditions of Wang et al. (1999). Twelve assays were conducted using the ryanodine-binding conditions of Wang et al. (1999) and twenty-five assays under current conditions were conducted simultaneously. Two assays were randomly chosen from Wang's condition, and another two assays were chosen from the current condition to match those from Wang's. Durbin-Watson statistical analysis showed that the consistency obtained from current condition is greater than that from Wang's ($p<0.05$). Thus, the

condition selected for future experiments represents an improvement in reproducibility of the ryanodine binding assay for turkey skeletal muscle. This assay condition was chosen to use for the following study to compare the functional differences in ryanodine receptor activity between random-bred and commercial turkey populations.

IV. Conclusion

The optimal buffer conditions for studying the [^{3}H]ryanodine binding varies with muscle type, species, pattern of muscle stimulation, ryanodine concentration, and other non-physiological factors. The ryanodine binding assay is a useful way to investigate the SR Ca^{2+}-release channel function. By testing different combinations of salt, sucrose and AMP-PCP concentrations, a buffer condition consisting of 0.1 M NaCl, 0.5 M sucrose, with 10 μM free Ca^{2+} and 10 mM MOPS (pH 7.2) was determined to be the best conditions for the ryanodine binding assay of the turkey skeletal SR. These assay conditions will provide the necessary stability and reproducibility to: 1) test a single bird's skeletal muscle RyR function, 2) distinguish RyR functional differences would be suggestive of differences in primary structure between the random-bred and commercial turkeys, 3) measure RyR content accurately, 4) eventually correlate the individual ryanodine binding data with meat quality characteristics.

Chapter 3

Ca^{2+}-channel protein activity and regulation associated with turkey PSE meat quality problems

I. Introduction

The modern turkey industry has grown rapidly during the last 30 years to meet consumers' demand for lean and inexpensive turkey meat products. To meet this demand, breeders have intensively selected for heavy muscling and rapid growth of turkeys. Over the past three decades, the market age of turkeys has shortened from 26-28 weeks to 14-16 weeks while the market weight has increased from about 20 lb to about 35 lb. However, the improvement in size and growth efficiency has come with a cost: increased incidence of meat quality defects including PSE (pale, soft, exudative) meat. This product is characterized by soft texture, lighter color, decreased water holding capacity and poor cooking yield.

The development of PSE turkey meat during the postmortem conversion of muscle to meat is remarkably similar to that of PSE pork. Decades of research have shown that incidence of PSE pork is influenced by both environmental factors and by genetic factors. Intensive genetic selection in the pork industry led to inadvertent appearance of an inherited trait called Porcine Stress Syndrome (PSS). Pigs with PSS are more likely than non-stress-susceptible pigs to yield PSE pork. This meat quality defect is observed in about 10-20% of the total US pork production (Vansickle, 1989). Porcine stress syndrome causes substantial economic losses to farmers, owing to death of animals from stresses of transportation, heat, crowding, etc., before reaching market (Louis, 1993). Stress-susceptible pigs which go through the slaughter process exhibit increased

glycolytic rates during the conversion of muscle to meat, leading to higher carcass temperatures in combination with acidic pH that favors protein denaturation. This results in decreased protein solubility and poor water holding capacity.

It has been known since 1980's that abnormal calcium homeostasis is a key factor in the development of PSE pork (Gronert 1980; Nelson, 1983; Cheah et al., 1984). The major breakthrough in the field was the colocalization of the malignant hyperthermia gene with the SR Ca^{2+} channel (RyR) to the same region of chromosome 19 in human (MacLennan et al., 1990; McCarthy et al., 1990). Fujii et al., (1991) reported that a single point mutation in the porcine gene for the ryanodine receptor (RyR) was correlated with PSS in five major breeds of lean, heavily muscled swine.

The activity of RyR is modulated by several ligands including Ca^{2+}, Mg^{2+} and adenine nucleotides. It is also regulated by direct coupling with a protein called the dihydropyridine receptor (DHPR). DHPR in skeletal muscle appears to have a dual function as both a calcium channel and voltage sensor mediating excitation-contraction coupling. As the voltage sensor, which is the main function of skeletal DHPR, it transduces the sarcolemma/T-tubule depolarization into a signal for opening of the SR Ca^{2+} release channel (RyR) (Rios and Brum, 1987). DHPR's role as a Ca^{2+} channel appears to be minor. Mickelson et al. (1994) measured RyR and DHPR content in heavy SR, crude SR, and total muscle homogenates. They observed that the crude membrane fraction and total muscle homogenates from MH pigs exhibited only 61-81% of the DHPR and RyR content of that from normal pigs. However, the ratio of DHPR to RyR in the muscle homogenates was not different between MH and normal pigs. They concluded that although the stoichiometry of the DHPR to RyR is not altered, the presence of the

MH RyR allele during muscle development results in a decreased relative content of these two proteins. This is probably due to a lower junctional membrane content and may be an important ultrastructural consequence of the altered sarcoplasmic Ca^{2+} regulation in MHS muscle (Mickelson et al., 1994).

Based on the numerous similarities between development of PSE pork and PSE turkey, we hypothesize that a defect in one or both of the turkey Ca^{2+}-channel isoforms and/or the regulation of Ca^{2+}-release channel isoforms contributes to the increased incidence of PSE turkey meat in modern commercial turkeys. To test this hypothesis, we have adapted the ryanodine binding strategy used by Mickelson et al. (1988), coupled with improvements in the assay developed in the previous chapter, to determine whether biochemical differences exist between RyRs from a control population of random-bred, genetically unimproved turkeys and modern commercial population. We have also measured DHPR content and determined the stoichiometry of DHPR: RyR binding in the two lines to determine whether difference in stoichiometry of DHPR:RyR might be related to the incidence of PSE turkey meat.

II. Materials and Methods

A. Materials

1. Skeletal Muscle

Two populations of turkeys were used in this study. A group of random-bred, genetically unimproved turkeys (McCartney, 1964) was used as a control population. These turkeys were provided by Dr. Karl Nestor of the Ohio Agricultural Research and Development Center (Wooster, Ohio). Commercial turkey breast muscle samples from Cargill/Plantation Hatchery were provided by Dr. Alan Sams (Texas A&M University). Two lots of these commercial samples were obtained approximately one and half years apart, group I (1998) and group II (2000). Breast muscles were cut into one-inch cubes and immediately frozen in liquid nitrogen, stored at -80°C.

2. Chemicals

Ryanodine was purchased from Wako Chemical USA, Inc. (Richmond, VA), nifedipine was purchased from Sigma (St. Louis, MO). Radioactive ryanodine ([^{3}H]ryanodine) and radioactive PN200-110 ([^{3}H]P200-110) were purchased from New England Nuclear Life Science (Boston, MA). Protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), aprotinin, benzamidine, pepstatin, leupeptin, were purchased from Sigma.

B. Methods

1. Preparation of Crude Sarcoplasmic Reticulum Vesicles (CSR)

Skeletal muscle SR was isolated from turkey breast muscle samples by procedures of Mickelson et al. (1986) and Wang et al. (1999) with minor modification. The 200 g frozen muscle cubes were thawed at 4°C for 20 min, incubated with 5 volumes

of Buffer I (0.1 M NaCl, 5 mM Tris-maleate, pH 7.0), for 5 min., and homogenized in a Waring blender for 2 min with intervals of 15 sec. on and 15 sec. off. After 30 min centrifugation at 3300 x g_{max}, the supernatant was collected by filtering through a 16-layer cheesecloth. The pellet was discarded, and the supernatant was centrifuged for 30 min at 16,000 x g_{max}. This pellet was collected, resuspended with 20 ml Buffer II (0.6 M KCl, 5 mM Tris-maleate, pH 7.0), and gently homogenized in a Dounce homogenizer. The homogenized suspension was centrifuged for 40 min. at 150,000 x g_{max} using a Beckman Ti-70 rotor. The pellet was collected and suspended in 10% sucrose solution, and gently homogenized in a Dounce homogenizer. The crude SR vesicles obtained by this step were flash-frozen in liquid nitrogen and stored at -80°C.

During the crude SR vesicles purification, the following protease inhibitors were added to each buffer, PMSF at 0.2 mM, and aprotinin, benzamidine, pepstatin and leupeptin at 1 μg/ml. Beta-mecaptoethanol was also added to a final concentration of 30 mM to Buffers I and II.

2. Preparation of Heavy Sarcoplasmic Reticulum Vesicles (HSR)

Heavy SR (HSR) vesicles, enriched in the Ca^{2+} channel protein, were isolated by sucrose gradient centrifugation. Crude SR(~1-2 g) was suspended in 2 ml of Buffer III (10% sucrose, 0.4 M KCl, 5 mM Tris-maleate, pH 7.0) and loaded on discontinuous sucrose gradients (22%, 36%, 45%). The gradients were centrifuged for 5 h at 100,000 x g_{max} in a Dupont-Sorvall AH 629 rotor. The HSR was collected at interface between the 45% and 36% sucrose layers. HSR fractions were diluted at least 1:1 with 10% sucrose and centrifuged at 150,000 x g_{max} in the Beckman Ti-70 rotor for 40 min. The pellet was suspended and homogenized in 10% sucrose, flash-frozen and stored at -80°C.

During the HSR purification, the following protease inhibitors were added at each step, PMSF at 0.2 mM, and aprotinin, benzamidine, pepstatin and leupeptin at 1 μg/ml, to Buffer III, 10% sucrose and sucrose gradients.

3. Ryanodine Binding Assays

Fifty μg of HSR protein were incubated in buffer containing 10 mM MOPS (pH 7.2), a $CaCl_2$-EGTA-nitrilotriacetic acid buffer to give a specific $[Ca^{2+}]_{free}$ of 10 μM, 0.1 M NaCl, 0.5 M sucrose, [^{3}H]ryanodine (2-70 nM as final concentration) was added to each tube. Triplicate samples of each [^{3}H]ryanodine concentration were incubated for 6 h at 37°C, and filtered by Whatman GF/B filters. Filters were washed three times with 5 ml ice-cold buffer (10 mM MOPS, 0.1 M NaCl, 50 μM $CaCl_2$, pH 7.0), and the amount of [^{3}H]ryanodine retained on the filter was determined by scintillation counting. Non-specific binding of ryanodine was determined by adding unlabeled ryanodine at 1000 times the [^{3}H]ryanodine in the incubation buffer. Specific ryanodine binding was determined by subtracting the non-specific binding from the total binding.

The K_d (ryanodine binding affinity) and B_{max} (maximum binding capacity) values for ryanodine binding by the HSR or CSR were calculated by Scatchard analysis using Sigma Plot.

4. PN200-110 Binding Assays

The PN200-110 binding assays are based on those of Mickelson et al. (1994) with minor modifications. Crude SR membranes (30 μg) were incubated in media containing 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 0.5 M sucrose, and various concentrations (0.1 - 4 nM) of [^{3}H]PN200-110. After 45 min incubation of duplicate samples at room temperature (25°C), samples were filtered by Whatman GF/B filters. Filters were washed

five times with 5 ml ice-cold buffer (200 mM Choline-Cl, 20 mM Tris-HCl), and the amount of [^{3}H]PN200-110 retained on the filter was determined by scintillation counting. Nonspecific binding was determined by adding 1 μM unlabeled nifedipine in the incubation buffer. Specific binding was determined by subtracting the non-specific binding from the total binding. All the procedures were conducted under low lighting condition to minimize photodegradation of PN200-110.

B_{max} (maximum binding capacity) value for [^{3}H]PN200-110 binding by the crude SR was calculated by Scatchard analysis using Sigma Plot.

5. Protein Assay

CSR and HSR protein concentration was determined by the method of Lowry (1951). Bovine serum albumin was used as a standard.

6. Statistical Analysis

Student's t-test was used to compare the K_d, B_{max} values for the crude and heavy SR, and ratio of DHPR/RyR, between random-bred and commercial turkeys.

III. Results and Discussion

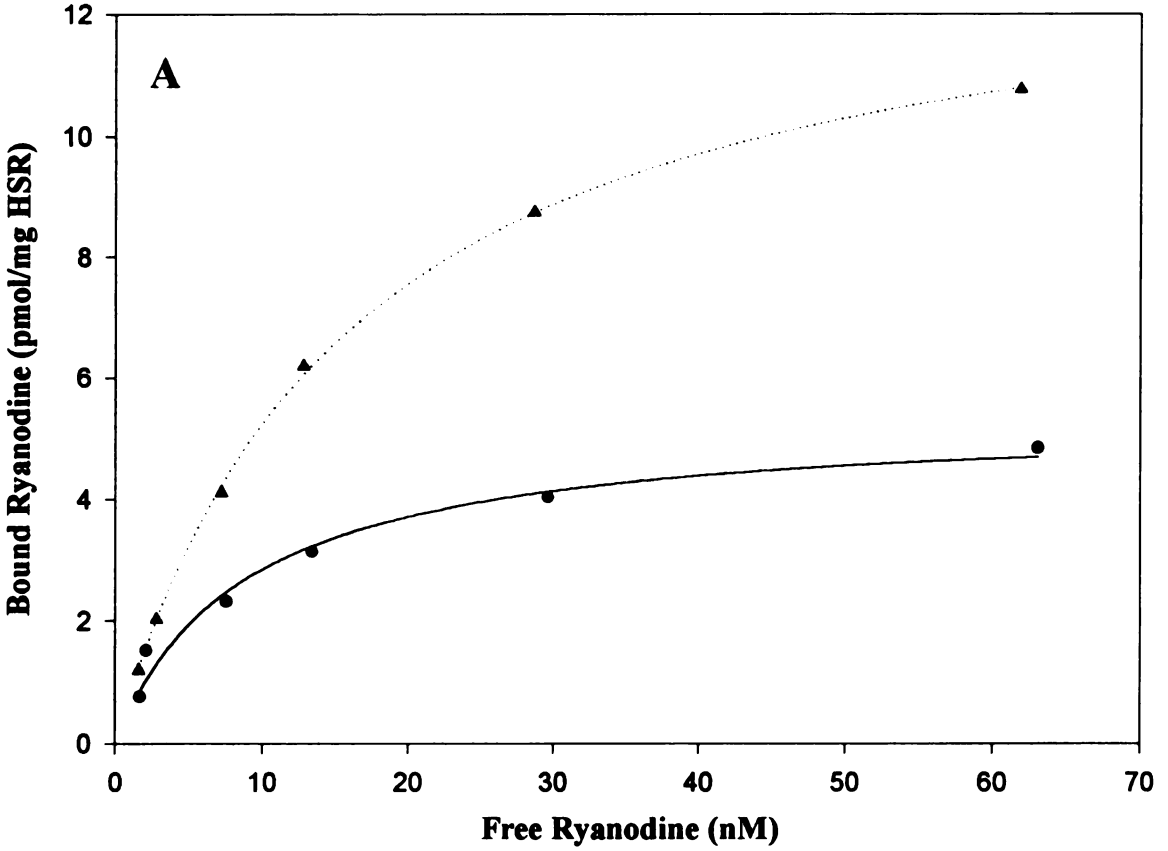
A. Ryanodine-dependence of [^{3}H]ryanodine binding to Ca^{2+}-release channel protein in heavy SR from random-bred and commercial turkey populations

The [^{3}H]ryanodine binding experimental conditions optimized in the previous study for the turkey skeletal muscle SR vesicles was chosen to analyze SR from random-bred and commercial turkeys for functional differences in RyR's. Since ryanodine only binds to the open state of the channel proteins with high affinity, the affinity of RyR for ryanodine is a useful probe of the functional difference in channel protein activity. If a mutation exists in the RyR isoform(s), this may be manifested as a difference in [^{3}H]ryanodine binding affinity as compared with a control sample.

[^{3}H]Ryanodine binding activities of commercial and random-bred turkey skeletal muscle heavy SR vesicles were measured over a range of ryanodine concentrations (2-70 nM). A total of eight random-bred turkey samples were compared with seventeen commercial samples obtained in 1998 (group I) and four commercial samples obtained in 2000 (group II). Representative binding data from one individual turkey from group I and random-bred turkeys are shown in Figure 3.1. The equilibrium binding constants (K_d and B_{max}) of [^{3}H]ryanodine binding of heavy SR and crude SR for each individual random-bred and commercial turkey sample are shown in Table 3.1 and Table 3.2, respectively. The average values for each group are summarized in Table 3.3.

Commercial turkeys from group I exhibited a higher ($p < 0.01$) affinity for ryanodine ($K_d = 8.4 \pm 1.7$ nM, $n = 17$) compared to that from genetically unimproved turkeys ($K_d = 16.0 \pm 2.2$ nM, $n = 8$). The mean Ca^{2+} channel protein content (B_{max}) from

Figure 3.1 Ryanodine dependence of ryanodine binding to random-bred(▲) and commercial (●) representative turkey skeletal muscle heavy sarcoplasmic reticulum vesicles (HSR) at 10 μM Ca^{2+}. A) Specific ryanodine binding by random-bred and commercial turkey HSR. B) Scatchard plot of ryanodine binding by random-bred and commercial turkey HSR.


A
Bound Ryanodine (pmol/mg HSR)
Free Ryanodine (nM)
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70

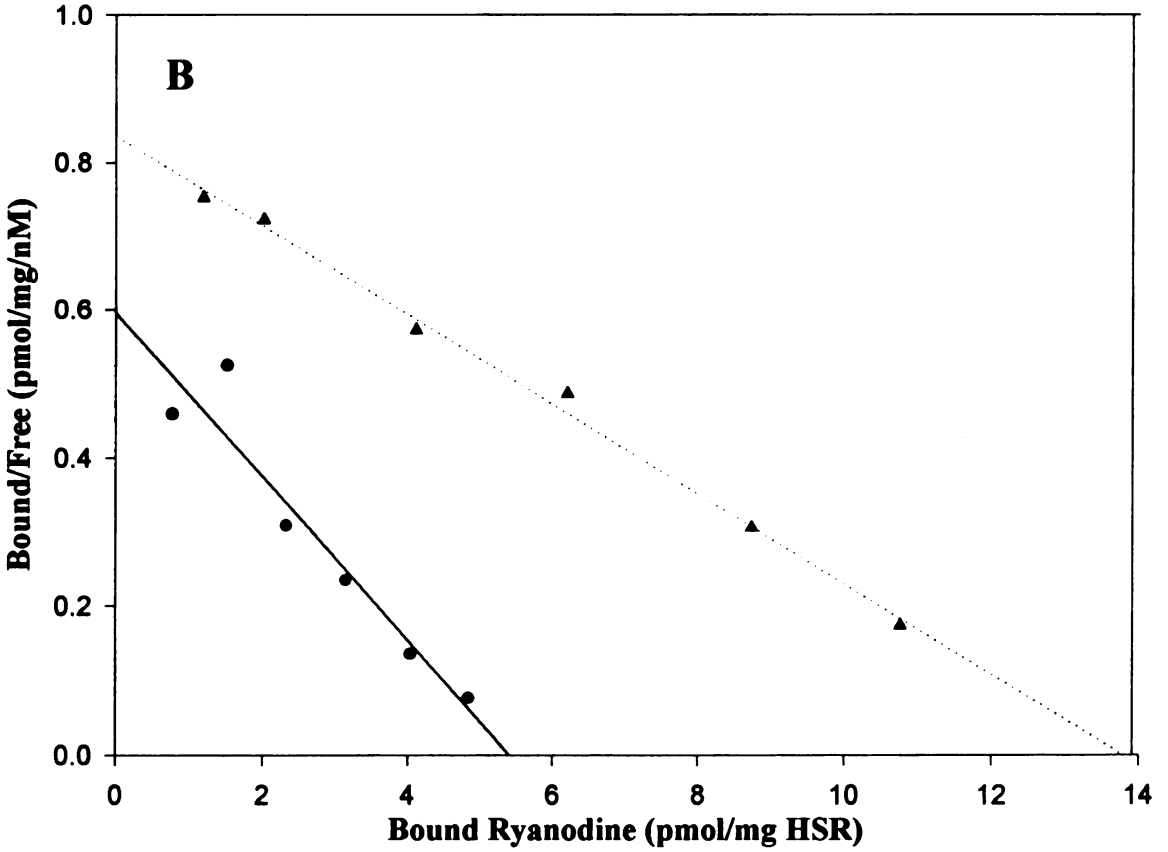

B
Bound/Free (pmol/mg/nM)
Bound Ryanodine (pmol/mg HSR)
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10
12
14

Table 3.1 Equilibrium Constants of Individual [^{3}H]Ryanodine Binding of Heavy SR from Random-bred and Commercial Turkeys (group I and II)

Random-bred Group			Commercial Group (I)			Commercial Group (II)		
Turkey ID	B_{max}	K_d	Turkey ID	B_{max}	K_d	Turkey ID	B_{max}	K_d
A	15.7	16.1	209	5.7	5.5	4a	9.2	20.0
B	17.1	14.5	303	5.1	8.0	5a	3.0	16.9
C	8.0	13.5	527	3.6	8.3	6a	1.1	20.2
D	13.7	16.4	663	5.0	7.9	8a	4.4	19.3
E	12.1	15.6	11	2.1	10.0			
F	10.7	15.0	36	7.1	7.8			
G	8.4	15.6	117	5.4	9.1			
H	14.7	20.9	435	7.6	7.1			
			451	5.1	7.4			
			459	9.1	6.7			
			468	12.5	9.2			
			567	4.2	6.0			
			283	5.3	8.9			
			75	8.3	9.2			
			323	5.4	12.8			
			290	2.8	9.3			
			664	6.4	9.7			
n	**8**	**8**		**17**	**17**		**4**	**4**
AVG	**12.5**	**16.0**		**5.9**	**8.4**		**4.4**	**19.1**
SD	**3.4**	**2.2**		**2.5**	**1.7**		**3.4**	**1.5**

Table 3.2 Equilibrium Constants of Individual [^{3}H]Ryanodine Binding of Crude SR from Random-bred and Commercial Turkeys (group II)

	Random-bred Group			Commercial Group (II)		
	Turkey ID	B_{max}	K_d	Turkey ID	B_{max}	K_d
	A	3.1	24.4	1a	1.4	22.7
	D	2.1	20.1	1b	2.4	26.6
	E	1.2	17.3	2b	1.2	22.5
	F	1.7	24.2	3b	2.8	27.5
	H	1.1	22.3	4b	2.0	23.0
n		**5**	**5**		**5**	**5**
AVG		**1.9**	**21.7**		**1.9**	**24.5**
SD		**0.8**	**3.0**		**0.7**	**2.4**

Table 3.3 Equilibrium Constant Means of [^{3}H]Ryanodine Binding of Crude and Heavy SR from Random-bred and Commercial Turkeys (group I, II)

	K_d (nM)	B_{max} (pmol/mg)
Random-bred		
Heavy SR (n=8)	16.0 ± 2.2^a	12.5 ± 3.4^e
Crude SR (n=5)	21.7 ± 3.0^b	1.9 ± 0.8^f
Commercial (group I)		
Heavy SR (n=17)	8.4 ± 1.7^c	5.9 ± 2.5^g
Commercial (group II)		
Heavy SR (n=4)	19.1 ± 1.5^d	4.4 ± 3.4^g
Crude SR (n=5)	24.4 ± 2.4^b	1.9 ± 0.7^f

[a,b,c,d] Different letters within a column indicate significant differences ($p < 0.05$)

[e,f,g] Different letters within a column indicate significant differences ($p < 0.05$)

genetically unimproved turkeys (B_{max} = 12.5 ± 3.4 pmol/mg) is higher ($p < 0.01$) than that from group I commercial turkeys (B_{max} = 5.9 ± 2.9 pmol/mg).

The K_d value obtained from the group II commercial turkey heavy SR vesicles was 19.1 ± 1.5 nM, which was significantly higher than that from both random-bred turkeys (16.0 ± 2.2 nM) ($0.01 < p < 0.05$) and group I commercial turkeys (8.4 ± 1.7 nM) ($p < 0.01$). The total binding capacity (B_{max}) from group II commercial turkey heavy SR was not significantly different compared to group I (4.4 ± 3.4 vs 5.9 ± 2.5 pmol/mg). The B_{max} from both commercial groups were significantly lower than that from random-bred turkeys (12.5 ± 3.4 pmol/mg) ($p < 0.01$).

The data of the random-bred turkeys and commercial group I samples compare favorably with the population averages obtained by Wang et al. (1999). They showed that the heavy SR fractions from commercial turkeys exhibited a higher ($P < 0.05$) mean affinity for ryanodine when compared to that from unimproved turkeys (K_d = 12.2 vs 20.5 nM, respectively). A fourfold higher Ca^{2+}-channel protein content (B_{max} = 1.10 vs 4.01 pmol/mg) in heavy SR fractions from random-bred turkeys compared to that from commercial turkeys was also observed. Differences in the B_{max} are probably the result of changes in buffer conditions which improve quantitation of B_{max} as well as improvement in the preparation of HSR.

These results also extend those of Wang et al. (1999) by showing that individual binding characteristics can be determined for each individual turkey. Moreover, we have shown for the first time that there is variation in ryanodine binding within a commercial line which may be the result of genetic differences in ryanodine receptors.

High ryanodine affinity is correlated with increased open state probability of channels. This suggests that there could be more Ca^{2+} released from the sarcoplasmic reticulum to the sarcoplasm during postmortem conversion of muscle to meat in commercial turkeys with high ryanodine binding activity. This could cause accelerated glycolysis and rapid pH drop, resulting in protein denaturation, decreased water holding capacity and protein solubility, as observed in meat from stress-susceptible pigs.

Studies on the pork PSE problem showed that isolated SR vesicles from skeletal muscle of MHS pigs release more Ca^{2+} than vesicles from normal pigs under various physiological and pharmacological conditions (Nelson, 1983; Kim et al., 1984; Mickelson et al., 1986). Vesicles from stress-susceptible pigs bind ryanodine with a 3-fold greater affinity (K_d = 92 vs 265 nM) than vesicles from normal pigs indicating a greater open-state probability of MHS RyR1's (Mickelson et al., 1988). Moreover, in single channel experiments using planar lipid bilayers, the mean percent open times P_o for MHS channels (30.0 ± 3.5%) was significantly greater ($p < 0.0001$) than the mean P_o from normal channels (4.4 ± 0.6%), so the functional alteration of RyR1 is consistent with increased Ca^{2+} release in stress-susceptible pigs that yield PSE pork (Shomer et al., 1995). Therefore, our results showing differences in ryanodine binding in turkeys selected for rapid growth are consistent with those obtained from pigs.

There is only one ryanodine receptor isoform expressed in mammalian skeletal muscle (RyR1), whereas there are two in the avian skeletal muscle, α and β. Both isoforms are expressed in equal abundance in avian skeletal muscle (Lai, et al., 1992; Olivares, et al. 1991). The [^{3}H]ryanodine binding affinity differences observed in this study suggests that there are functional differences in one or both isoforms of Ca^{2+}-

release channel protein in these turkeys. The differences in channel content in heavy SR from the two turkey lines further suggest functional differences in Ca^{2+} regulation in the muscle cell.

The difference in B_{max} value between the commercial and random-bred control group HSR could have resulted from the artifactual differences in distributions of the RyR in the HSR membrane during isolation of SR. To address this question, [^{3}H]ryanodine binding experiments on the total SR vesicle fraction from both groups were conducted and compared.

The [^{3}H]ryanodine binding to the crude SR vesicles yielded a K_d of 21.7 ± 3.0 nM and a B_{max} of 1.9 ± 0.8 pmol/mg from random-bred turkeys (Table 3.3). The results were not different from that of group II commercial turkeys (K_d = 24.5 ± 2.4 nM and a B_{max} = 1.9 ± 0.7 pmol/mg). These results contrast with those observed by Wang et al. (1999), in which the K_d and B_{max} obtained from commercial turkey CSRs were only half of that obtained from random-bred turkeys ($p < 0.05$).

Thus, both the HSR and CSR [^{3}H]ryanodine binding data suggest that in the commercial lines of turkeys, there may be different subpopulations. The group I and the commercial group that Wang et al. (1999) used could be from one subpopulation that carries altered-functional RyR, while group II turkeys, which exhibits similar RyR function as that from random-bred control population, could be from another subpopulation.

SR vesicles isolated from heterozygous pigs exhibit Ca^{2+} release channel properties that are intermediate between those of SR isolated from homozygous MH and homozygous normal pigs (Hawkes et al., 1992; Mickelson et al., 1989). Mickelson et al.

(1994) showed that the total [^{3}H]ryanodine binding capacity of muscle homogenates from heterozygous pigs was not significantly different from binding to homogenates of either type of homozygote, although it was numerically intermediate. Shomer et al. (1995) also indicated that the activities of the heterozygous Ca^{2+} release channels are intermediate even at the single channel level. They concluded that the skeletal muscle SR Ca^{2+} release channels of heterozygous pigs are composed of heterotetramers with unique properties. Likewise, if the group II commercial birds were from a subpopulation with unique RyR properties different from either group I commercial birds or random-bred birds, then no difference for B_{max} or K_d between random-bred and commercial group II turkeys might be detected.

B. DHPR and RyR content in random-bred and commercial turkeys

In mammalian skeletal muscle, a mechanical coupling between RyR1 and the DHPR is proposed to trigger Ca^{2+}-release from the SR (Meissner and Lu, 1995). Avian muscle, however, which consists of two RyR isoforms, is believed to have the DHPR coupled only to the α-RyR isoforms. No studies have been reported on the stoichiometry of DHPR:RyR in avian muscle. Moreover, it is possible that difference in the coupling ratio of DRHP to RyR could account for differences in regulation of Ca^{2+} homeostasis in the commercial turkeys. Therefore, the stoichiometric relationship of these two receptors were determined by using [^{3}H]ryanodine binding and [^{3}H]PN200-110 binding assays.

Crude SR fractions from both commercial and random-bred turkey groups were prepared and analyzed for [^{3}H]ryanodine binding and [^{3}H]PN200-110 binding activities. Results are shown in Table 3.4. Group II commercial turkeys exhibited almost identical

Table 3.4 Ratio of Dihydropyridine and Ryanodine Receptors Between Random-bred and Commercial Turkey Skeletal Muscle Crude SR Vesicles

	PN200-110 B_{max} (pmol/mg)	Ryanodine B_{max} (pmol/mg)	Ratio DHPR/RyR
Random-bred	1.98 ± 0.69	1.85 ± 0.81	1.18 ± 0.49
Commercial	1.66 ± 0.39	1.94 ± 0.67	0.91 ± 0.30

There was no significant difference
n=5

total binding capacity (B_{max} = 1.7 ± 0.4 vs 1.9 ± 0.7 pmol/mg) for [^{3}H]PN200-110 and for [^{3}H]ryanodine, as the random-bred turkeys (B_{max} = 2.0 ± 0.7 vs. 1.9 ± 0.8 pmol/mg). The ratio of [^{3}H]PN200-110 binding to [^{3}H]ryanodine binding was ~1 and there was no significant difference between the two groups.

Franzini-Armstrong and Kish (1995) indicated that since a tetrad is composed of four DHPRs and there are two RyRs for every tetrad, the alternate RyR association pattern would predict a 2:1 ratio of DHPR to RyR. However, the measured ratios from different skeletal muscles and different species range from 0.5-2.0 (Anderson et al., 1994; Bers and Stiffel, 1993; Block et al., 1988; Margreth et al., 1993; Mickelson et al., 1994). Mickelson et al. (1994) observed that the ratio of DHPR:RyR was ~1 for muscle homogenates of all three pig genotypes (MHS, heterozygote and normal). The DHPR content and RyR content of MHS pig muscle homogenates were reduced to 80% and 81%, respectively, of that of normal muscle. Therefore, in MHS muscle, even though there was a slightly reduced content of both the triadic junctional proteins (DHPR and RyR), the stoichiometric relationship between these proteins is not altered in MH vs normal muscle (Mickelson et al., 1994). They also suggested that the reduced DHPR and RyR content in MH muscle could reflect lower junctional membrane protein content which may have functional consequence for Ca^{2+} homeostasis. It should be noted that our results, which show no difference in DHPR or RyR levels were based on samples only from group II, these samples showed no difference in ryanodine binding activity from the random-bred turkeys and thus, may be expected to show no difference in expression levels of these proteins.

Anderson et al. (1994) compared the DHPR/RyR ratio in frog and rabbit skeletal muscle. The value of 0.61 for the frog as compared with rabbit ratio of 0.98 indicates, according to the authors, that one-half as many RyRs in the frog are coupled to DHPRs. They explain this difference by the presence of two RyR isoforms in amphibian muscle, which are expressed in approximately equal amount (Lai, et al., 1992; Olivares, et al.,1991), and of which possibly only one is linked to the DHPR. In our study, which is the first to examine avian muscle, a ratio of 1.0 was obtained which is comparable to that of Margreth et al. (1993) using frog sartorius muscle. This would seem to predict an excess of ryanodine receptors. However, it is possible that there are non-junctional RyRs which contribute to the overall binding (Sutko and Airey, 1996).

IV. Conclusion

Using the ryanodine binding conditions optimized for turkey skeletal muscle, two distinct subpopulations of a commercial turkey line were observed. Both subpopulations differed in [^{3}H]ryanodine binding activity from that of the random-bred control turkey population. The HSR from one commercial subpopulation exhibited a higher ryanodine binding affinity (K_d = 8.4 vs 16.0 nM) and lower Ca^{2+}-release channel content (B_{max} = 5.9 vs 12.5 pmol/mg) than random-bred control population. This suggests that the RyR function in this subpopulation of commercial birds is altered, and these differences in RyR activity could predispose these turkeys to a higher incidence of PSE meat.

The other subpopulation of commercial turkeys yielded similar ryanodine binding affinity of crude SR vesicles and of heavy SR as that of the random-bred control (24.5 vs 21.7 nM for CSR, $p > 0.05$, 19.1 vs 16.0 nM for HSR, $0.01 < p < 0.05$, respectively). The contents of the RyR in crude SR preparations from these two populations were also similar ($p > 0.05$). The results suggest that this subpopulation might be less likely to exhibit PSE meat than turkeys from group I.

Direct interaction between DHPR and RyR is essential for skeletal muscle E-C coupling. RyR regulation by the DHPR is also important for the Ca^{2+} release and subsequent muscle contraction and glycolysis. The coupling ratio of DHPR:RyR in this study was 1.0 and no DHPR:RyR ratio between commercial turkeys and random-bred control turkeys (0.91 vs 1.18) was observed. The reasons could be: 1) function of RyR from the commercial turkey subpopulation used for this study is similar to that from random-bred control turkeys and therefore, less likely to be predisposed to yielding PSE

meat. 2) DHPR/RyR ratio might remain constant even if a mutation is present in one of the RyR isoforms as was observed in pig muscles.

CONCLUSIONS

In skeletal muscle, excitation-contraction coupling is essential for the Ca^{2+} release and muscle contraction. Protein-protein interaction between two receptors, dihydropyridine receptor (DHPR) and ryanodine receptor (RyR), plays an important role in this process. But the specific mechanisms involved in the regulation of Ca^{2+} release and the relationship between abnormal Ca^{2+} regulation and meat quality problems are still not fully understood.

The PSE (pale, soft, exudative) meat problem, which was first observed in pork industry, has now become prevalent in the turkey industry. A Ca^{2+}-release channel (RyR) gene mutation detected by Fujii et al. (1991) is correlated directly with porcine stress syndrome (PSS), which is one of the causes of the PSE pork. Based on the similarities between turkey and pork PSE problems, we hypothesized that a mutation in one or both of the turkey Ca^{2+}-channel isoforms and/or differences in the regulation of Ca^{2+}-release channel isoforms contribute to the incidence of PSE turkey meat in modern commercial turkeys.

In the first part of this work, the [^{3}H]ryanodine binding assay was optimized for turkey skeletal muscle. By testing different combinations of salt, sucrose and AMP-PCP concentrations, a buffer condition of 0.1 M NaCl, 0.5 M sucrose, with 10 μM free Ca^{2+} and 10 mM MOPS was determined to be the best condition for the ryanodine binding assay for the turkey skeletal muscle SR.

In the second part of this work, two groups of commercial turkeys were compared with a random-bred control group. The Ca^{2+}-release channel biochemical functions from one subgroup of commercial turkeys was different from the random-bred control turkeys.

The SR vesicles from seventeen commercial turkeys showed a two-fold increase in ryanodine affinity while the total Ca^{2+} channel content were decreased by half compared to those from eight random-bred control turkeys. The altered affinity of the Ca^{2+} channel protein for ryanodine suggests that there is a channel functional defect in this subgroup of commercial turkeys. The channel content difference may suggest that there are some factors that alter the gene expression or protein stability. Another group of commercial turkeys was found that yields similar Ca^{2+} channel properties to those of random-bred controls. Four HSR and five CSR vesicles from this subpopulation showed similar ryanodine binding affinity compared to control eight turkeys. These results suggest that there are at least two subpopulations of commercial turkeys, one subpopulation that may possess altered Ca^{2+} channel function, which is predisposed to yielding PSE meat, while another subpopulation possesses the relatively normal Ca^{2+} channel function, and thus is less likely to yield PSE meat. The stoichiometry of DHPR:RyR was not different between five commercial turkeys and eight control turkeys. This could mean that either the subpopulation of commercial turkeys studied doesn't have obvious functional defect of Ca^{2+} channel, or there might not be a stoichiometric DHPR/RyR ratio difference between random-bred and commercial turkey lines.

FUTURE RESEARCH

This study showed that the SR Ca^{2+}-release channel in a subpopulation of commercial turkeys exhibited a higher ryanodine binding affinity and lower Ca^{2+}-release channel content. Another subpopulation appeared to be similar to the random-bred controls. This suggested that there is a Ca^{2+}-release channel functional defect in the former commercial turkey subpopulation. However, since there are two isoforms of Ca^{2+}-release channel in turkey skeletal muscle, the method used in this study cannot identify directly which isoform(s) is specifically responsible for this defect. In order to answer this question, the two isoforms need to be purified from both random-bred and commercial turkeys, and biochemical characteristics from both isoforms need to be compared (eg. ryanodine binding data) between these two populations. If ryanodine binding activity is different from purified Ca^{2+}-release channel isoform(s) between random-bred and commercial turkey population, then this would offer more direct evidence that: 1) there is a biochemical defect in the commercial turkey subpopulation, 2) which isoform has the mutation, 3) which isoform(s) has the direct interaction with dihydropyridine receptor. If a biochemical defect is identified in specific isoform(s), then cDNA sequencing of the isoforms could be done to identify the specific mutation.

This study focused on the biochemical changes in the commercial turkey SR based on ryanodine binding properties. However, there are no meat quality results comparing early postmortem pH, water holding capacity, color etc., for both random-bred turkey and commercial turkey populations slaughtered under identical circumstances. The hypothesis of this study was that the mutation of a Ca^{2+}-release channel is more frequently found in commercial turkey population. A higher incidence of PSE is believed

to exist in the commercial turkeys than in random-bred, genetically unimproved turkey population. To prove this hypothesis and also to investigate the PSE meat incidence in the commercial subpopulations, it is necessary to collect both ryanodine binding data and turkey meat quality data from individual turkeys from both random-bred and commercial turkey populations. The correlation of meat quality data with corresponding skeletal muscle ryanodine binding activity data could provide more evidence for the relationship between altered Ca^{2+}-release channel function and PSE meat incidence in commercial turkey populations.

REFERENCES

REFERENCES

Anderson, K., Cohn, A.H. and Meissner, G. 1994. High-affinity [^{3}H]PN200-110 and [^{3}H]ryanodine binding to rabbit and frog skeletal muscle. Am. J. Physiol. 266: C462-C466.

Airey, J.A., Beck, C.F., Murakami, K., Tanksley, S.J., Deerinck, T.J., Ellisman, M.H., and Sutko, J.L. 1990. Identification and localization of two triad junctional foot protein isoforms in mature avian fast twitch skeletal muscle. J. Biol. Chem. 65: 14187-14194.

Airey, J.A., Grinsell, M.M., Jones, L.R., Sutko, J.L., Witcher, D. 1993a. Three ryanodine receptor isoforms exist in avian striated muscle. Biochemistry 32: 5739-5745.

Airey, J.A., Baring, M.D., Beck, C.F., Chelliah, Y., Deerinck, R.J., Ellisman, M.H., Houenou, L.J., McKemy, D.D., Sutko, J.L., and Talvenheimo, J. 1993b. Failure to make normal α ryanodine receptor is an early event associated with the crooked neck dwarf (cn) mutation in chicken. Dev. Dyn. 197: 169-188.

Airey, J.A., Deerinck, T.J., Ellisman, M.H., Houenou, L.J., Ivanenko, A., Kenyon, J.L., McKemy, D.D., and Sutko, J.L. 1993c. Crooked neck dwarf (cn) mutant chicken skeletal muscle cells in low-density primary cultures fail to express normal α ryanodine receptor and exhibit a partial mutant phenotype. Dev. Dyn. 197: 189-202.

Ashley, R.H. 1989. Activation and conductance properties of ryanodine-sensitivity calcium channels from brain microsomal membranes incorporated into lipid lipid bilayers. J. Membr. Biol. 111: 179-189.

Bers, D.M., and Stiffel, V.M. 1988. Ratio of ryanodine to dihydropyridine receptors in cardiac and skeletal muscle and implications for E-C coupling. Am. J. Physiol. 264: C1587-C1593.

Bianchi, C.P. 1968. Pharmacological actions on excitation-contraction coupling in striated muscle. Fed. Proc. 27: 126-131.

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

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

Brownell, A.K. 1988. Malignant hyperthermia: relationship to other diseases. Br. J. Anaesthes. 60: 303-308.

Bull, R., Marengo J.J., Suarez-Isla, B.A., Donoso, P., Sutko, J.L., and Hidalgo, C. 1989. Activation of calcium channels in sarcoplasmic reticulum from frog muscle by nanomolar concentrations of ryanodine. Biophys. J. 56: 749-756.

Bull, R., and Marengo J.J. 1993. Sarcoplasmic reticulum release channels from frog skeletal muscle display two types of calcium dependence. FEBS Lett. 331: 223-227.

Cadwell, J.J., and Caswell, A.H. 1982. Identification of a constituent of the junctional feet linking terminal cisternae to transverse tubules in skeletal muscle. J. Cell Biol. 93: 543-550.

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

Cannell, M.B., Cheng, H., and Lederer. W.J. 1995. The control of calcium release in heart muscle. Science 268: 1045-1049.

Cassens, R.G., Marple, D.N. and Eikelenboom, G. 1975. Animal physiology and meat quality. Adv. Food Res. 12: 71-155.

Caswell, A.H., and Brandt, N. 1989. Does muscle activation occur by direct mechanical coupling of transverse tubules to sarcoplasmic reticulum? TIBS. 14: 161-165.

Catterall, W.A. 1996. Molecular properties of sodium and calcium channels. J. Bioenerg. Biomembr. 28: 219-230.

Cheah, K.S., Cheah, A.M., Crosland, A.E., Casey, J.C., Webb, A.J. 1984. Relationship between Ca^{2+} release, sarcoplasmic Ca^{2+}, glycolysis and meat quality in halothane-sensitive and halothane insensitive pigs. Meat Sci. 10:117-130.

Cheng, H. Lederer, M.R., Xiao, R.P., Gomez, A.M., Zhou, Y.Y., Ziman, B., Spurgeon, H., Lakatta, E.G., Lederer, W.J. 1996. Excitation-contraction coupling in heart: new insights from Ca^{2+} sparks. Cell Calcium 20: 129-140.

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

Chu, A., Fill, M., Stefanis, E., and Entman, M.L. 1993. Cytoplasmic Ca^{2+} does not inhibit the cardiac muscle sarcoplasmic reticulum ryanodine receptor Ca^{2+} channel,

although Ca^{2+}-induced Ca^{2+} inactivation of Ca^{2+} release is observed in native vesicles. J. Membr. Biol. 135: 49-59.

Coronado, R., Morrissette, J., Sukhareva, M., and Vaugham, D.M. 1994. Structure and function of ryanodine receptors. Am. J. Physiol. 266: C1485-C1504.

Denborough, M.A., Deett, X., Anderson, R.M. 1973. Central-core disease and malignant hyperthermia. BMJ. 1: 272-273.

De Waard, M. and Campbell, K.P. 1995 Subunit regulation of the neuronal alpha 1A Ca^{2+} channel expressed in *Xenopus* oocytes. J. Physiol. (London) 485: 619-634.

De Waard, M., Witcher, D.R., Pragnell, M., Liu, H., and Campbell, K.P. 1995. Properties of the alpha 1-beta anchoring site in voltage-dependent Ca^{2+} channels. J. Biol. Chem. 270: 12056-12064.

Ebashi, S. 1991. Excitation-contraction coupling and the mechanism of muscle contraction. Annu. Rev. Physiol. 53: 1-16.

Ellis, F.R. and Heffron, J.J.A. 1985. In Recent Advances in Anaesthesia and Analgesia (Atkison, R.S., and Adams, A.P., eds) No. 15, pp. 173-207, Churchill-Livingstone, London

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. Physiol. Rev. 37: 71-108.

Endo, M., Yagi, S., Ishizuka, T., Horiuti, K., Koga, Y., Amaha, K. 1983. Changes in the Ca^{2+} release mechanism in sarcoplasmic reticulum from a patient with malignant hyperthermia. Biomed. Res. 4: 83-92.

Eng. G.D., Epstein, B.S., Engel, W.K., McKay, D.W., and McKay, R. 1978. Malignant hyperthermia and central core disease in a child with congenital dislocating hips. Arch Neurol. (Chicago) 35: (4) 189-197

Fill, M., Coronado, R., Mickelson, J.R., Vilven, J., Ma, J.J., Jacobson, B.A., and Louis, C.F. 1990. Abnormal ryanodine receptor channels in malignant hyperthermia. Biophys. J. 57: 471-475.

Fabiato, A. 1983. Calcium-induced release of calcium from cardiac sarcoplasmic reticulum. Am. J. Physiol. 245: C1-C14.

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

Foegeding, E.A. 1992. Factors affecting texture and yield of cooked turkey. Zootechnica Intl. 2: 24-27.

Fosset, M., Jaimovich, E., Delpont, E., and Lazdunski M., 1983. [^{3}H]Nitrendipine receptors in skeletal muscle: properties and preferential location in transverse tubules. J. Biol. Chem. 258: 6086-6092.

Frank, J.P., Harati, Y., Butler, I.J., Nelson, T.E., and Scott, C.I. 1980. Central core disease and malignant hyperthermia syndrome. Ann. Neurol. 7: 11-17.

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

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

Franzini-Armstrong, C., and Kish, J.W. 1995. Alternate disposition of tetrads in peripheral couplings of skeletal muscle. J. Muscle Res. Cell Motil. 16: 319-324.

Franzini-Armstrong, C., and Protasi, F. 1997. The ryanodine receptor of striated muscles, a complex capable of multiple interactions. Physiol. Rev. 77: 699-729.

Franzini-Armstrong, C. 1999. The sarcoplasmic reticulum and the control of muscle contraction. FASEB J. 13 (Suppl.): S266-S270.

Froning, G.W., Babji, A.S., Mather F.B. 1978. The effect of pre-slaughter temperature, stress, struggle, and anaesthetization on color and textural characteristics of turkey muscle. Poultry Sci. 57: 630-633.

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

Greaser, M.L. 1986. Conversion of muscle to meat. In: P. Bechtel (Ed.) Muscle as Food. Pp 37-102. Academic Press, New York.

Gregg, R.G., Messing, A., and Strube, C. 1996. Absence of the beta subunit (cchb1) of the skeletal muscle dihydropyridine receptor alters expression of the alpha 1 subunit and eliminates excitation-contraction coupling. Proc. Natl. Acad. Sci. U.S.A. 93: 13961-13966.

Gronert, G.A. 1980. Malignant hyperthermia. Anesthesiology. 53: 395-423.

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

Hall, G.M., Lucke, J.N., and Liser, D. 1980. Malignant hyperthermia: Pearls out of swine. Brit. J. Anaesth. 52: 165-171.

Harrison, G.G. 1979. Porcine malignant hyperthermia. Intl. Anaesth. Clinics. 17: 25-61.

Hawkes, M.J., Nelson, T.E., Hamilton, S.L. 1992. [^{3}H]ryanodine as a probe of changes in the functional state of the Ca^{2+}-release channel in malignant hyperthermia. J. Biol. Chem. 267: 6702-6709.

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

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

Ivanenko, A., McKemy, D.D., Kenyon, J.L., Airey, J.A., and Sutko, J.L. 1995. Embryonic chicken skeletal muscle cells fail to develop normal excitation-contraction coupling in the absence of the alpha ryanodine receptor. Implications for a two-ryanodine receptor system. J. Biol. Chem. 270: 4220-4223.

Jaggar, J.H., Porter, V.A., Lederer, W.J., and Nelson, M.T., 2000. Calcium sparks in smooth muscle. Am. J. Physiol. Cell. Physiol. 278: C235-C256.

Jeyakumar, L.H., Copello, J.A., O'Malley, A.M., Wu, G.M., Grassucci, R., Wagenknecht, T., and Fleischer, S. 1998. Purification and Characterization of Ryanodine Receptor 3 from Mammalian Tissue J. Biol. Chem. 273: 16011-16020.

Kim, D.H., Sreter, F.A., Ohnishi, S.T., Ryan, J.F., Roberts, J., Allen, P.D., Meszaros, L.G., Antoiu, B., and Ikemoto, N. 1984. Kinetic studies of Ca^{2+} release from sarcoplasmic reticulum of normal and malignant hyperthermia susceptible pig muscles. Biochim. Biophys. Acta 775: 320-327.

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

Klaus, M.M., Scordilis, S.P., Rapalus, J.M., Briggs, R.T., and Powell, J.A. 1983. Evidence for dysfunction in the regulation of cytosolic Ca^{2+} in excitation-contraction uncoupled dysgenic muscle. Dev. Biol. 99: 152-165.

Klein, M.G., Cheng, H., Santana, L.F., Jiang, Y.H., Lederer, W.J., and Schneider, M.F. 1996. Two mechanisms of quantized calcium release in skeletal muscle. Nature (London). 379: 455-458.

Lacerda, A.E., Kim, H.S., Ruth, P., Perez-Reyes, E., Flockerzi, V., Hofmann, F. Birnbaumer, L., and Brown, A.M. 1991. Normalization of current kinetics by

interaction between the alpha1 and beta subunits of the skeletal dihydropyridine-sensitive Ca^{2+} channel. Nature (London) 352: 527-530.

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

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

Lai, F.A., Misra. M., Xu, L., Smith, H.A., and Meissner, G. 1989. The ryanodine receptor-Ca^{2+} release channel complex of skeletal muscle sarcoplasmic reticulum. Evidence for a cooperatively coupled, negatively charged homotetramer. J. Biol. Chem. 264: 16776-16785.

Lai, F.A., Liu, Q.Y., Xu, L., El-Hashem, A., Kramarcy, N.R., Sealock, R., and Meissner, G. 1992. Amphibian ryanodine receptor isoforms are related to those of mammalian skeletal or cardiac muscle. Am. J. Physiol. 263: C365-C372.

Leeson, T.S., and Leeson, C.R. 1970. Histology, 2nd Ed. Saunders, Philadelphia, Pennsylvania.

Leong, P., and MacLennan, D.H. 1998. Complex interactions between skeletal muscle ryanodine receptor and dihydropyridine receptor proteins. Biochem. Cell Biol. 76: 681-694.

Liu, Q.Y., Lai, A., Rousseau, E., Jones, R.V., and Meissner, G., 1989. Multiple conductance states of the purified calcium release channel complex from skeletal sarcoplasmic reticulum. Biophys. J. 55: 415-425.

Lopez-Lopez, J.R., Shacklock, P.S., Balke, C.W., and Wier, W.G. 1995. Local calcium transients triggered by single L-type calcium channel currents in cardiac cells. Science 268: 1042-1045.

Louis, C.F. 1993. Porcine stress syndrome: Biochemical and genetic basis of this inherited syndrome of skeletal muscle. Reciprocal Meat Conference Proceeding. 46:89-96.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 65-275.

MacLennan D.H., Duff, C., Zorzato, F., Fujii, J., Phillips, M., Korneluk, R.G., Frodis, W., Britt, B.A., and Worton, R.G. 1990. Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia. Nature. 343: 559-561.

Margreth, A., Damiani, E. and Tobaldin, G. 1993. Ratio of dihydropyridine to ryanodine receptors in mammalian and frog twitch muscles in relation to the mechanical hypothesis of excitation-contraction coupling. Biochem. Biophys. Res. Commun. 197: 1303-1311.

Martonosi, A.N. 1984. Mechanisms of Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle. Physiol. Rev. 64: 1204-1319.

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

Meissner, G. 1975. Isolation and characterization of two types of sarcoplasmic reticulum vesicles. Biochim. Biophys. 389: 51-68.

Meissner, G. 1984. Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum. J. Biol. Chem. 259: 2365-2371.

Meissner, G., Darling, E., and Eveleth, J. 1986a. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. Biochemistry 25: 236-244.

Meissner, G. 1986b. Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. J. Biol. Chem. 261: 6300-6306.

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

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

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

Meissner, G., and Lu, X. 1995. Dihydropyridine receptor-ryanodine receptor interactions in skeletal muscle excitation-contraction coupling. Biosci. Rep. 15: 39-408.

Meissner, G., Rios, E., Tripathy, A., and Pasek, D.A. 1997. Regulation of skeletal muscle Ca^{2+} release channel (ryanodine receptor) by Ca^{2+} and monovalent cations and anions. J. Biol. Chem. 272(3): 1628-1638.

Melzer, W., Rios, E., and Schneider, M.F. 1984. Time course of calcium release and removal in skeletal muscle fibers. Biophys. J. 45: 637-641.

Melzer, W., Rios, E., and Schneider, M.F. 1987. A general procedure for determining the rate of calcium release from the sarcoplasmic reticulum in skeletal muscle fibers. Biophys. J. 51: 849-863.

Melzer, W., Herrmann-Frank, A., and Luttgau, H.C. 1995. The role of Ca^{2+} ions in excitation-contraction coupling of skeletal muscle fibers. Biochim. Biophys. Acta. 1241: 59-116.

McCarthy, T.V., Healy, J.M.S., Heffron, J.J.A. Lehane, M., Deufel, T. Lehmannhorn, F., Farrall, M., and Johnson, K. 1990. Localization of the malignant hyperthermia susceptibility locus to human-chromosome 19Q12-13.2. Nature 343: 562-564.

McCartney, M.G. 1964. A randombred control population of turkeys. Poultry Sci. 43: 739-744.

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

McKee, S.R. and Sams, A.R. 1997. The effect of seasonal heat stress on rigor development and the incidence of pale, exudative turkey meat. Poultry Sci. 76: 1616-1620.

Michalak, M., Dupraz, P., and Shoshan-Barmatz, V. 1988. Ryanodine binding to sarcoplasmic reticulum membranes: comparison between cardiac and skeletal muscle. Biochim. Biophys. Acta 939: 587-594.

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

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

Mickelson, J.R., Gallant, E.M., Rempel, W.E., Johnson, K.M., Litterer, L.A, Jacobson, B.A., and Louis, C.F. 1989. Effects of halothane-sensitivity gene on sarcoplasmic reticulum function. Am. J. Physiol. 257: C787-C794.

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

Mickelson, J.R., Ervasti, J.M., Campbell K.P., and Louis, C.F. 1994. Skeletal muscle junctional membrane protein content in pigs with different ryanodine receptor genotypes. Am. J. Physiol. 267 (Cell Physiol. 36): C282-C292.

Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S., and Numa, S. 1989. Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. Nature (London) 340: 230-233.

Mills, J. 2000. The effect of selection for fast growth rate and high meat yield on skeletal muscle in commercial turkeys. 23rd Technical Turkey Conference, UK.

Mitchell, G. and Heffron, J.J.A. 1982. Porcine stress syndromes. Adv. Food Res. 28: 167-230.

Monnier, N., Procaccio, V., Stieglitz, P., and Lunardi, J. 1997. Malignant-hyperthermia susceptibility is associated with a mutation of the alpha 1-subunit of the human dihydropyridine-sensitive L-type voltage-dependent calcium-channel receptor in skeletal muscle. Am. J. Hum. Genet. 60: 1316-1325.

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

Murayama, T., and Ogawa, Y. 1992. Purification and characterization of two ryanodine-binding protein isoforms from sarcoplasmic reticulum of bullfrog skeletal muscle. J. Biochem. 112: 514-522.

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

Nakai, J., Dirksen, R.T., Nguyen, H.T., Pessah, I.N., Beam, K.G., and Allen, P.D. 1996. Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. Nature (London) 380: 72-75.

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

O'Brien, P.J. 1987. Etiopathogenetic defect of malignant hyperthermia: hypersensitive calcium-release channel of skeletal muscle sarcoplasmic reticulum. Vet. Res. Commun. 11: 527-559.

O'Brien, P.J., Meissner, G., and Block, B.A. 1993. The fastest contracting skeletal muscle of non-mammalian vertebrates express only one isoform of the ryanodine receptor. Biophys. J. 65: 2418-2427.

O'Brien, P.J., Valdivia, H.H., and Block, B.A. 1995. Physiological difference between the α and β ryanodine receptors of fish skeletal muscle. Biophysical J. 68: 471-482.

Ogawa, Y., and Harafuji, H. Effect of temperature on [^{3}H]ryanodine binding to sarcoplasmic reticulum from bullfrog skeletal muscle. 1990. J. Biochem. 107: 887-893.

Ogawa, Y. 1994. Role of ryanodine receptor. Criti. Rev. Biochem. Molec. Biol. 29: 229-274.

Ogawa, Y., Murayama, T., and Kurebayashi, N. 1999. Comparison of properties of Ca^{2+} release channels between rabbit and frog skeletal muscles. Mol. Cell. Biochem. 190: 191-201.

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

Olcese, R., Qin N., Schneider, T., Neely, A., Wei, X., Stefani, E., and Birnbaumer, L. 1994. The amino terminus of a calcium channel beta subunit sets rates of channel interaction independently of the subunit effect on activation. Neuron, 13: 1433-1438.

Olivares, E.B., Tanksley, S.J., Airey, J.A., Beck, C.F., Ouyang, Y., Deerinck, T.J., Ellisman, M.H., and Sutko, J.L. 1991. Nonmammalian vertebrate skeletal muscles express two triad junctional foot protein isoforms. Biophys. J., 59: 1153-1163.

ϕrding, H. 1985. Incidence of malignant hyperthermia in Denmark. Anesthes Analges. 64: 700-704.

ϕrding, H. 1996. Investigation of malignant hyperthermia susceptibility in Denmark. Dan Med Bull. 43: 111-125.

Orlova, E.V., Serysheva, I.I., Heel, M.V., Hamilton, S.L., and Chiu, W. 1996. Two structural configurations of the skeletal muscle calcium release channel. Nature Structural Biol. 3: 547-551.

Oyamada, H., Murayama, T., Takagi, T., Iino, M., Iwabe, N., Miyata, T., Ogawa, Y., and Endo, M. 1994. Primary structure and distribution of ryanodine-binding protein isoforms of the bullfrog skeletal muscle. J. Biol. Chem. 269: 17206-17214.

Palade, P. 1987. Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum; II. Release involving a Ca^{2+}-induced Ca^{2+} release channel. J. Biol. Chem. 262: 6135-6141.

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

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

Pessah, I.N., and Zimany, I. 1991. Characterization of multiple [^{3}H]ryanodine binding sites on the calcium release of sarcoplasmic reticulum from skeletal and cardiac muscle: Evidence for a sequential mechanism in ryanodine action. Mol. Pharmacol. 39: 679-689.

Pietrzak, M., Greaser, M.L. and Sosnicki, A.A. 1997. Effect of rapid rigor mortis processes on protein functionality in pectoralis major muscle of domestic turkeys. J. Anim. Sci. 75: 2106-2116.

Powell, J.A. and Fambrough. D.M. 1973. Electrical properties of normal and dysgenic mouse skeletal muscle in culture. J. Cell Physiol. 82: 21-38.

Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T.P., and Campbell, K.P. 1994. Calcium channel beta-subunit binds to a conserved motif in the I-II cytoplasmic linker of the alpha I-subunit. Nature (London). 368: 67-70.

Protasi, F., Sun, X.H., and Franzini-Armstrong, C. 1996. Formation and maturation of calcium release apparatus in developing and adult avian myocardium. Dev. Biol. 173: 265-278.

Protasi, F., Franzini-Armstrong, C., and Flucher, B.E. 1997. Coordinated incorporation of skeletal muscle dihydropyridine receptors and ryanodine receptors in peripheral couplings of BC_3H1 cells. J. Cell Biol. 137: 859-870.

Protasi, F., Franzini-Armstrong, C., and Allen, P.D. 1998. Restoration of tetrads by transfection of dyspedic cells with RyR-1. Biophys. J. 74: A141.

Rios, E., and Brum, G. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. Nature (London). 325: 717-720.

Rios, E., and Pizarro, G. 1991. Voltage sensor of excitation-contraction coupling in skeletal muscle. Physiol. Rev. 71: 849-908.

Rogers, E.F., Koniuszy, F.R., Shavel, J., Fr., and Folker, K. 1948. Plant insecticides. I. Ryanodine, a new alkaloid from *Ryania speciosa* Vahl. J. Am. Chem. Soc. 70: 3086-3088.

Rousseau E, Ladine, J., Liu, Q.Y., Meissner, G. 1988. Activation of the Ca^{2+}-release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. Arch Biochem. Biophys. 267: 75-86.

Saito, A., Inui, M., Radermacher, M., Frank, J., and Fleischer, S. 1988. Ultrastructure of the calcium release channel of sarcoplasmic reticulum. J. Cell Biol. 107: 211-219.

Serysheva, I.I., Schatz, M., van Heel, M., Chiu, W., and Hamilton, S.L. 1999. Structure of the skeletal muscle calcium release channel activated with Ca^{2+} and AMP-PCP. Biophys. J. 77: 1936-1944.

Schneider, M.F., and Simon, B.J. 1988. Inactivation of calcium release from the sarcoplasmic reticulum in frog skeletal muscle. J. Physiol. (London) 405: 727-745.

Schwartz, L.M., McCleskey, E.W. and Almers, W. 1985. Dihydropyridine receptors in muscle are voltage-dependent but most are not functional calcium channels, Nature (London) 314: 747-751.

Shirokova, N., and Rios, E. 1997. Small event Ca^{2+} release: a probable precursor of Ca^{2+} sparks in frog skeletal muscle. J. Physiol. (London) 502: 3-11.

Shirokova, N., and Rios, E. 1998. Local calcium release in mammalian skeletal muscle. Biophys. J. 74: A269.

Shomer, N.H., Mickelson, J.R. and Louis, C.F. 1995. Ca^{2+} release channels of pigs heterozygous for malignant hyperthermia. Muscle and Nerve 18: 1167-1176.

Shoshan-Barmatz, V., and Ashley, R.H. 1998. The structure, function, and cellular regulation of ryanodine-sensitive Ca^{2+} release channels. Intl. Rev. Cytology 183: 185-270.

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

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

Smith, J.S., Coronado, R., Meissner, G. 1986b. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Mg^{2+}. J. Gen. Physiol. 88: 573-588.

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

Soeller, C., and Cannell, M.B. 1997. Numerical simulation of local calcium movements during L-type calcium channel gating in the cardiac diad. Biophys. J. 73: 97-111.

Sorenson, M.M., Coelho, H.S.L., and Reuben, J.P. 1986. Caffeine inhibition of calcium accumulation by the sarcoplasmic reticulum in mammalian skinned fibers. J. Membr. Biol. 90: 219-230.

Sosnicki, A.A. and Wilson, B.W. 1991. Pathology of turkey skeletal muscle: Implications for the poultry industry. Food Struct. 10: 317-326.

Sosnicki, A.A. and Wilson, B.W. 1992. Relationship of focal myopathy of turkey skeletal muscle to meat quality. Proc. 19th World's Poult. Congr. 3: 43-47.

Sosnicki, A.A. 1993a. Focal myonecrosis effects in turkey muscle tissue. Proc. 46th Annu. Recip. Meat Conf. 46: 97-102.

Sosnicki, A.A. 1993b. Is the modern turkey prone to pale, soft, exudative (PSE) breast muscle conditions? Meat Focus Int. 2: 75-78.

Sosnicki, A.A. 1995. The domestic turkey: A model of the impact selection production practices on meat quality. In: A. Ouali, D.I. Demeyer, and F.J.M. Smulders (Ed.) Expression of tissue to proteinases and regulation of protein degradation as related to meat quality. pp. 363-380. Audet Tijdschriften bv, Nijmegen, The Netherlands.

Stephenson, E.W. 1981. Activation of fast skeletal muscle: contributions of studies on skinned fibers. Am. J. Physiol. 240: C1-C19.

Strasburg, G.M., and Byrem, T.M. 2000. Red meats. In: Food Chemistry: Principles and applications, edited by G. Christen and Smith, J.S.. ADNC Press, California.

Suda, N. 1995. Involvement of dihydropyridine receptor in terminating Ca^{2+} release in rat skeletal myotubes. J. Physiol. (London), 486: 105-112.

Sun, X-H., Protasi, F., Takahashi, M., Takeshima, H., Gerfuson, D.G., and Franzini-Armstrong, C. 1995. Molecular architecture of membranes involved in excitation-contraction coupling of cardiac muscle. J. Cell. Biol. 129: 659-671.

Sutko, J.L., and Airey, J.A. 1996. Ryanodine receptor Ca^{2+} release channels: does diversity in form equal diversity in function? Physiol. Rev. 76: 1027-1071.

Takagi, A., Suohara, N., Ishizuka, T., Nonaka, I., and Sugita, H. 1983. Malignant hyperthermia and related neuromuscular diseases: caffeine contracture of the skinned muscle fibers. Muscle Nerve 6: 510-514.

Takekura, H., Bennet. L., Tanabe, T., Beam, K.G., and Franzini-Armstrong, C. 1994. Restoration of junctional tetrads in dysgenic myotubes by dihydropyridine receptor cDNA. Biophys. J. 67: 793-804.

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and Numa, S. 1989. Nature 339: 439-445.

Takeshima, H. 1993. Primary structure and expression from cDNAs of the ryanodine receptor. Ann. N.Y. Acad. Sci. 707: 165-177.

Takeshima, H., Iino, M., Takekura, H., Nishi, M., Kuno, J., Minowa, O., Takano, H. Noda, T. 1994. Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. Nature 369: 556-559.

Tanabe, T., Beam, K.G., Adams, B.A., Niidome, T., and Numa, S. 1990. Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. Nature (London). 346: 567-569.

Tu, Q., Velez, P., Brodwick, M., and Fill, M. 1994. Slowing of single cardiac muscle ryanodine receptor channel gating by viscous solutions. Biophys. J. 66: A418.

Valdivia, H.H., Fuentes, O., and Block, B.B. 1994. Imperatoxin A, a selective activator of skeletal ryanodine receptors (RYR), distinguishes between α and β RYR isoforms in non-mammalian muscle. Biophys. J. 66: A418.

Vansickle, J. 1989. PSE problems in U.S. pork. National Hog Farmer, October 15.

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

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

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

Wagenknecht, T., and Radermacher, M. 1997. Ryanodine receptors: structure and macromolecular interactions. Curr. Opin. Struct. Biol. 7: 258-265.

Wang, L., Byrem, T.M., Zarosley, J., Booren, A.M. and Strasburg, G.M. 1999. Skeletal muscle calcium channel ryanodine binding activity in genetically unimproved and commercial turkey populations. Poultry Sci. 78: 792-797.

Wei, X., Perez-Reyes, E., Lacerda, A.E., Schuster, G., Brown, A.M., and Birnbaumer, L. 1991. Heterologous regulation of the cardiac Ca^{2+} channel alpha 1 subunit by

skeletal muscle beta and gamma subunits. Implications for the structure of cardiac L-type Ca^{2+} channels. J. Biol. Chem. 266: 21943-21947.

Wei, X., Neely, A., Lacerda, A.E., Olcese, R., Stefani, E., Perez-Reyes, E., and Birnbaumer, L. 1994. Modification of Ca^{2+} channel activity by deletions as the carboxyl terminus of the cardiac alpha 1 subunit. J. Biol. Chem. 269: 1635-1640.

Yano, M., El-Hayek, R., and Ikemoto, N. 1995. Conformational changes in the junctional foot protein/Ca^{2+} release channel mediate depolarization-induced Ca^{2+} release from sarcoplasmic reticulum. J. Biol. Chem. 270: 3017-3021.

Yamazawa, T., Takeshima, H., Sakurai, T., Endo, M., and Iino, M. 1996. Subtype specificity of the ryanodine receptor for Ca^{2+} signal amplification in Excitation-Contraction coupling. EMBO J. 15: 6172-6177.

Zarka, A., and Shoshan-Barmatz, V. 1993. Characterization ad photoaffinity labeling of the ATP binding site of the ryanodine receptor from skeletal muscle. Eur. J. Biochem. 214: 147-155.

Zhang, J.F., Ellinor, P.T., Aldrich, R.W., and Tsien, R.W. 1994. Molecular determinants of voltage-dependent inactivation in calcium channels. Nature (London). 372: 97-100.

Zhang, W., Kulers, D.L., and Rempel, W.E. 1992. Halothane gene and swine performance. J. Amin. Sci. 70: 1307-1313.

Zarka, A., and Shoshan-Barmatz, V. 1993. Characterization and photoaffinity-labeling of the ATP binding-site of the ryanodine receptor from skeletal muscle. Eur. J Biochem. 213: 147-154.

Zot, A.S., and Potter, J.D. 1987. Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. Annu. Rev. Biophys. Biochem. 16: 535-559.