Dissertation:

Glycogen Extraction From Skeletal Muscle Sarcoplasmic Reticulum: Structural and Functional Modifications

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Abstract

Glycogen extraction From Skeletal Muscle Sarcoplasmic Reticulum: Structural And Functional Modification

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In this investigation, skeletal muscle sarcoplasmic reticulum (SR) was purified from female Sprague Dawley rats (200-250 g). SR samples were subjected to two different biochemical glycogen-extraction protocols. Both amylase and removal of EDTA (No-EDTA) from the homogenization and storage buffers reduced the amount of glycogen associated with the SR. Neither treatment caused impaired SR calcium (Ca^{2+}) handling when exogenous ATP was utilized for SR Ca^{2+} transport. In fact, these treatments caused small increases in both SR Ca^{2+}-uptake and release rates. As expected, glycogen phosphorylase content was reduced as a result of glycogen extraction in the presence of amylase, however this was not the case for No-EDTA samples. Interestingly, many other proteins differed in content after glycogen extraction. These treatments resulted in a greater recovery of the sarco(endo)plasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA) and a substantial loss of glycogen phosphorylase and glycogen debranching enzyme (AGL) in amylase-treated samples. Creatine kinase (CK) and pyruvate kinase (PK) contents were increased as a result of both glycogen-extraction conditions. It was imperative to consider these altered protein contents while analyzing the data and assessing the effects of glycogen extraction on SR Ca^{2+} handling.

For endogenously synthesized ATP-supported SR Ca^{2+}-uptake experiments, normalizing data to protein content (either CK and SERCA or PK and SERCA) revealed that amylase-treated samples had lower SR Ca^{2+}-uptake rates, compared to control samples. Although not significant, SR Ca^{2+}-uptake rates for No-EDTA samples were also lower than control samples. These data suggest that changes in endogenously supported SR Ca^{2+}-uptake due to glycogen extraction affected the source of ATP synthesis (either PK or CK), the effectiveness of energy utilization for Ca^{2+} transport (SERCA), or altered the metabolic channeling properties.

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Chapter One: Introduction

Statement Of Problem

Skeletal muscle fatigue is a phenomenon that manifests with repeated or sustained muscle contraction. It is characterized by a reduced shortening velocity (Edman and Mattiazzi, 1981; Crow and Kushmerick, 1983; de Haan et al. 1989; Westerblad and Lännergren, 1994), prolongation of relaxation (Edman and Mattiazzi, 1981; Westerblad and Lännergren, 1991), and decreased maximum force generated by a muscle, due to decreases in both the calcium (Ca^{2+}) sensitivity of the contractile apparatus (Westerblad and Allen, 1991; Westerblad and Allen, 1993) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Allen et al. 1989; Gyorke et al. 1993; Lee et al. 1991; Kabbara et al. 1999). Skeletal muscle fatigue is commonly thought of in terms of athletic performance. Furthermore, athletic training and physical activity are focused on the resistance to muscle fatigue for a given exercise intensity in the athletic arena. However, muscle fatigue is not only experienced by the physically fit, in fact, quite the opposite is true. Everybody experiences muscle fatigue in their lives, many times in their daily routine. For example, carrying groceries up a flight of stairs, or lifting boxes to and from a moving truck. Although moving does not seem like a daily routine for most of us, according to the United States Postal Service (http://www.usps.com/news/2002/press/), 17% of the nations population moved in 2002 (corresponding to about 44,000,000 address changes). The more physically inactive a person's life style is, the more susceptible they will be to muscle fatigue in daily routine. This is problematic because muscle fatigue can lead to injury as a result of improper lifting (breakdown of technique due to muscle fatigue) or loss of balance. More seriously, skeletal muscle fatigue can be a debilitating symptom of certain clinical conditions like congestive heart failure (Buller et al. 1991; Minotti et al. 1993). Despite a plethora of research on the topic, the exact mechanism of skeletal muscle fatigue is unknown.

Decreased muscle function associated with fatigue is accompanied by depressed sarcoplasmic reticulum (SR) function (Allen et al. 1989; Gyorke et al. 1993; Lee et al. 1991; Westerblad et al. 1991; Luckin et al. 1992; Baker et al. 1993; Belcastro et al. 1993; Favero et al. 1993; Westerblad et al. 1993; Ward et al. 1998; Williams et al. 1998; Kabbara et al. 1999; Ørtenblad et al. 2000). The SR in skeletal muscle serves as the

storage site for Ca^{2+} as well as allowing for Ca^{2+}-release and uptake, which mediates skeletal muscle contraction. Although the exact mechanisms of muscle fatigue are unknown, it is likely that there are many different contributing factors, some of which involve SR Ca^{2+} handling.

Repeated and sustained muscle contractions often result in reductions in muscle glycogen content. In fact, there is a strong relationship between skeletal muscle glycogen content and exercise exhaustion (Bergstrom et al. 1967; Hargreaves et al. 1984; Coyle et al. 1986; Coggan and Coyle 1989; Romijn 1993). Despite this vast body of research on influences of glycogen on skeletal muscle function, very little work has been aimed at determining the effects of glycogen extraction (glycogen removal without muscle contraction) on SR Ca^{2+} handling.

Glycogen is an extremely important source of energy for the contracting muscle (Ahlborg et al. 1967; Bergström et al. 1967; Galbo et al. 1979; Hargreaves et al. 1984; Coyle et al. 1986; Coggan and Coyle, 1989; Romijn 1993; Chin and Allen, 1997; Stephenson et al. 1999). Interestingly, glycogen has been found to be specifically associated with the SR in skeletal muscle (Entman et al. 1976; Fridén et al. 1989; Cuenda et al. 1994; Lees et al. 2000; Lees et al. 2001). It seems reasonable that energy storage sites are conveniently located at sites of energy utilization within the cell. Perhaps an even more interesting aspect of glycogen associated with the SR is that its mere presence serves as a signal for SR Ca^{2+} handling properties. Indeed, there are some data to support this hypothesis. It has been shown that glycogen extraction from skeletal muscle results in impaired SR Ca^{2+}-uptake (Chin and Allen, 1997) and release (Stephenson et al. 1999).

Compartmentation is a model that describes the components of the muscle fiber as being immobilized at the site of utilization. In terms of local adenosine triphosphate (ATP) production for SR Ca^{2+} handling, compartmentation seems to be important for decreasing diffusion distance of metabolites, improving Ca^{2+} transport efficiency, and creating a microenvironment, whereby local changes in metabolite concentrations can be detected. Glycolytic, glycogenolytic, and Ca^{2+}-transporting enzymes, including sarco(endo)plasmic

reticulum ATPase (SERCA, SR Ca^{2+}-ATPase) are found to be closely associated with the SR (Entman et al. 1976; Entman et al. 1980; Han et al 1992; Cuenda et al 1994; Xu et al. 1995; Xu and Becker, 1998). Not only are purified SR samples able to support Ca^{2+}-uptake via endogenous ATP production, but also it seems that they may do so more efficiently than when exogenous ATP is added (Xu et al 1995).

Creatine kinase (CK) and pyruvate kinase (PK) are found to be located at the SR (Baskin and Deamer, 1970; Rossi et al. 1990; Korge and Campbell, 1992; Korge et al. 1993; Korge and Campbell, 1994; Xu et al. 1995; Duke and Steele, 1999). Not only does the presence of CK and PK located at the SR aid in the maintenance of local adenosine diphosphate (ADP) concentration, but they also seem to play an integral role in optimal Ca^{2+}-uptake (Wolosker et al. 1996; de Groof et al. 2002). Both CK and PK will support SR Ca^{2+}-uptake in the presence of ADP and creatine phosphate (CP) or phosphoenol-pyruvate (PEP), respectively. In terms of skeletal muscle fatigue, a condition which is known to result in reduced glycogen content, a lowering in CK-supported SR Ca^{2+}-uptake was found to be more severe compared to ATP-supported uptake (Korge and Campbell, 1992).

Significance Of Study

Although there have been a few studies that investigated the effects of glycogen extraction on the properties of the SR (protein content and function), none have quantified the magnitude of glycogen extraction. Also, despite a wealth of information indicating the importance of glycogen for optimal muscle function and extensive research in the area of fatigue and impaired SR Ca^{2+} handling, the importance of localized SR glycogen for Ca^{2+} handling is unknown.

Many studies utilize intact skeletal muscle fibers to probe force production and relaxation rate and/or rate of SR Ca^{2+}-release and uptake. Because most of the normal physiological mechanisms remain intact in this preperation, the contributions of metabolic channeling between these enzymes and SERCA are included, but not isolated. Certainly the cellular localization of CK and glycogenolytic and glycolytic enzymes to the SR, combined with

the fact that these systems are able to support SR Ca^{2+}-uptake, indicates their physiological importance. However, there has been no published research on the effects of glycogen extraction on glycolytic and CK-supported SR Ca^{2+}-uptake.

Specific Aims

1. To determine if glycogen and glycogen phosphorylase associated with the SR are decreased either after treatment with α-amylase or the exclusion of ethylenediaminetetraacetic acid (EDTA) from the homogenizing and storage buffers.

2. To determine if glycogen extraction alters the Ca^{2+} handling characteristics of the isolated SR fractions.

3. To determine whether either PEP + ADP or CP + ADP-supported Ca^{2+}-uptake into the SR will be diminished to a similar extent as ATP-supported Ca^{2+}-uptake after glycogen extraction.

4. To determine whether glycogen extraction is associated with structural changes at the ATP-binding site of SERCA.

Specific Aim 1

The first specific aim was to determine if glycogen and glycogen phosphorylase associated with the SR are decreased after either treatment with α-amylase or the exclusion of EDTA from the homogenizing and storage buffers.

Rationale

It has been shown that glycogen particles are associated with SR preparations (Wanson et al. 1972, and Entman et al. 1980). Because it has been established that glycogen phosphorylase is associated to the SR via glycogen, SR glycogen extraction should result in diminished SR glycogen phosphorylase (Meyer et al. 1970, Wanson et al. 1972, Entman 1980, Cuenda 1994, and Cuenda 1995). In fact, SR purified either from animals

fasted for 48 hours or from treatment of purified SR vesicles with α-amylase has been shown to be dramatically reduced in glycogen and glycogen phosphorylase content (Entman et al. 1976; Cuenda et al. 1994). In addition, prolonged muscle contraction reduces both glycogen particles located in regions of skeletal muscle where the SR is situated (Fridén et al. 1989) and also reduces glycogen and glycogen phosphorylase associated with SR vesicles purified from fatigued muscles (Lees et al. 2000 and 2001).

Exclusion of EDTA from the homogenizing and storage buffers is hypothesized to decrease glycogen and glycogen phosphorylase associated with SR purified from skeletal muscle. EDTA is often found in extraction buffers. EDTA binds divalent metal ions that can react with thiol groups on proteins. EDTA, therefore, chelates the divalent metal ion Ca^{2+}, which is involved in glycogen phosphorylase activation. In terms of glycogen extraction, the presence of EDTA prevents glycogen breakdown via activation of endogenous glycogen phosphorylase (see Tate et al. 1991 for review).

Hypothesis

It was hypothesized that incubation either with α-amylase or without EDTA would decrease the amount of glycogen and glycogen phosphorylase associated with the SR.

Specific Aim 2

The second specific aim was to determine if glycogen extraction alters the Ca^{2+} handling characteristics of the SR samples.

Rationale

Muscle glycogen is an important substrate for energy production in the cell. Glycogen breakdown provides glucose-1-phosphate, which is subsequently converted to glucose-6-phosphate and enters glycolysis. It is well established that prolonged muscle contraction results in decreased muscle glycogen concentration (Kelso et al. 1987). Furthermore, glycogen depletion has been associated with decreased muscle performance (Stephenson et al., 1999; Chin et al., 1997; Galbo et al., 1979; Ahlborg et al., 1967; and Bergström et al., 1967).

Glycogen located at the SR is of specific importance for the proposed study. There is ample evidence that fatiguing muscle contraction results in impaired SR Ca^{2+}-uptake and ATPase activity (Byrd et al. 1989a, b; Luckin et al. 1991; Biederman et al. 1992; Ward et al. 1998; Williams et al. 1998; Wilson et al. 1998) and release rates (Allen et al. 1989; Gyorke et al. 1993; Lee et al. 1991; Westerblad and Allen, 1991; Luckin et al. 1992; Baker et al. 1993; Belcastro et al. 1993; Favero et al. 1993; Westerblad et al. 1993; Ward et al. 1998; Williams et al. 1998; Kabbara et al. 1999; Ørtenblad et al. 2000). Although there are a few studies that examine the effects of glycogen extraction on SR Ca^{2+} handling, none of these have measured glycogen associated with the SR (Brautigan et al. 1979; Chin and Allen 1997; Stephenson et al. 1999). Therefore, the proposed study seeks to determine if glycogen extraction, without muscle contraction, will result in diminished SR Ca^{2+} handling.

Hypothesis

It was hypothesized that the glycogen extraction would result in diminished Ca^{2+} handling characteristics (peak uptake and release rates) of the SR samples.

Specific Aim 3

The third specific aim was to determine whether either PEP + ADP or CP + ADP-supported Ca^{2+}-uptake into the SR would be diminished to a similar extent as ATP-supported Ca^{2+}-uptake after glycogen extraction.

Rationale

Glycolytic enzymes, as well as CK, associated with the SR are not only able to support Ca^{2+}-uptake (Xu et al. 1995), but there seems to be a link between localized glycolytic ATP synthesis and regulation of Ca^{2+}-release as well (Han et al. 1992). Although there are some data indicating that glycogen extraction causes impaired SR Ca^{2+}-uptake in skinned muscle fibers (Brautigan et al. 1979), there are no data on glycogen extraction and SR Ca^{2+}-uptake supported by endogenous glycolytic ATP synthesis.

Ca^{2+}-uptake via ATP hydrolysis seems to be coupled to SR-bound CK (Korge et al. 1993; Xu and Becker, 1998). CK acts as an ADP sensor and serves to maintain a minimal local [ADP]. In fact, CK associated with SR Ca^{2+}-uptake seems to be an integral aspect of cellular metabolism to keep up with the energetic demands of repeated muscle contraction (Korge et al. 1993). Korge and Campbell (1992) found that SR purified from fatigued muscles exhibited a greater change in SR Ca^{2+}-uptake supported by the CK system than when exogenous ATP was used as the source of energy.

The present study sought to determine whether SR glycogen status is a regulator of the CK and glycolytic systems associated with SR Ca^{2+} handling.

Hypothesis

It was hypothesized that Ca^{2+}-uptake into the SR, energetically supported by endogenous CK and PK, would be decreased by a larger extent than that of ATP-supported Ca^{2+}-uptake.

Specific Aim 4

The fourth specific aim was to determine whether glycogen extraction is associated with structural changes at the ATP-binding site of SERCA.

Rationale

Ca^{2+}-uptake and ATPase activity measured in SR isolated from fatigued skeletal muscle, is diminished without any change in the intracellular milieu (Byrd et al. 1989a and b; Luckin et al. 1991; Biederman et al. 1992; Ward et al. 1998; Williams et al. 1998; Wilson et al. 1998). These data may not simply reflect changes due to the purification process because they compare well to intracellular Ca^{2+} transient data (Allen et al. 1989; Lee et al. 1991; Westerblad and Allen, 1991). Therefore, it seems that changes in Ca^{2+}-uptake are associated with an intrinsic change in SERCA itself.

Fluorescein isothiocyanate (FITC) is a fluorescent compound often used to assess SERCA conformation. FITC binds competitively with ATP in the ATP-binding pocket

of SERCA (Mitchinson et al. 1982). In 1991, Luckin et al. investigated FITC binding in SR vesicles purified from rat gastrocnemius muscles after one bout of prolonged exercise. These investigators reported a 40% decline in maximal FITC binding in fatigued SR compared to control, with no apparent change in FITC binding sensitivity. However, Schertzer et al. (2003) reported no differences in fluorescence emission of SR-bound FITC in vesicles purified from rat gastrocnemius muscles after a fatiguing bout of treadmill running, compared to control.

Hypothesis

It was hypothesized that SR glycogen extraction would decrease the FITC binding, indicating that a reduced proportion of SERCA will be in the ATP-binding conformation.

Chapter Two: Review of the Literature

Introduction

Skeletal muscle fatigue is a phenomenon that manifests with repeated or sustained muscle contraction. It is characterized by a reduced shortening velocity (Edman and Mattiazzi, 1981; Crow and Kushmerick, 1983; de Haan et al. 1989; Westerblad and Lännergren, 1994), prolongation of relaxation (Edman and Mattiazzi, 1981; Westerblad and Lännergren, 1991), and decreased maximum force generated by a muscle, due to decreases in both the calcium (Ca^{2+}) sensitivity of the contractile apparatus (Westerblad and Allen, 1991; Westerblad and Allen, 1993) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Allen et al. 1989; Gyorke et al. 1993; Lee et al. 1991; Kabbara et al. 1999). Decreased muscle function associated with fatigue is accompanied by depressed sarcoplasmic reticulum (SR) function (Allen et al. 1989; Gyorke et al. 1993; Lee et al. 1991; Westerblad et al. 1991; Luckin et al. 1992; Baker et al. 1993; Belcastro et al. 1993; Favero et al. 1993; Westerblad et al. 1993; Ward et al. 1998; Williams et al. 1998; Kabbara et al. 1999; Ørtenblad et al. 2000). The SR in skeletal muscle serves as the storage site for Ca^{2+} as well as allowing for Ca^{2+}-release and uptake, which mediates skeletal muscle contraction. SR Ca^{2+} handling utilizes approximately 1/3 of the total energy cost of muscle contraction in mammalian skeletal muscle (Stienen et al. 1995). Although the exact mechanisms of muscle fatigue are unknown, it is likely that there are many different contributing factors (see Williams and Klug, 1995 for review). A metabolic signal in skeletal muscle seems logical, in that muscle metabolism must match the energy demands of contraction in order to prevent rigor and irreversible cellular damage (Maier and Pette, 1987). Compartmentation of glycogen, glycolytic enzymes, creatine kinase (CK) associated with the SR in skeletal muscle are likely integral components of normal physiological Ca^{2+} handling (Han et al 1992; Xu et al. 1995; Wolosker et al. 1996; Chin and Allen, 1997; Stephenson et al. 1999; de Groof et al. 2002). The role of SR compartmentation and changes in glycogen status as a metabolic signal for regulation of SR Ca^{2+} handling will be discussed.

Skeletal Muscle Excitation-Contraction Coupling

Excitation-contraction coupling (ECC) is the process by which electrical activation of the skeletal muscle cell induces the muscle to contract. The muscle contraction process begins with the activation of the soma of an α-motor neuron. Neural depolarization is carried down the axon and delivers the electrical signal to the nerve terminal. Depolarization of the axon results in the release of acetylcholine (ACh) from the nerve terminus into the synaptic space of the neuro-muscular junction. ACh binds to receptors on the muscle membrane (sarcolemma). Binding of ACh to these receptors causes a local increase in sodium (Na^+) permeability of the sarcolemma, thereby, making the membrane potential of the sarcolemma less negative. Depolarization of the sarcolemma will occur once a threshold potential is reached. Sarcolemmal depolarization is propagated the length of the cell and down through the transverse tubules (T-tubules) (reviewed in Williams, 1994). Subsequently, this propagation activates the voltage sensitive dihydropyridine receptors (DHPR). The DHPR, in turn, causes Ca^{2+}-release from the SR via the ryanodine receptor (RyR) through an unknown mechanism (Ebashi, 1976; Endo, 1977; Ebashi, 1991). Calcium released into the cytoplasm of the cell binds to troponin (the C subunit), which relieves the inhibitory affect troponin (the I subunit) normally has on energy utilization of the contractile apparatus. The development of force is then accomplished through cross-bridge cycling of the contractile apparatus via the hydrolysis of adenosine triphosphate (ATP) by myosin ATPase (Brenner, 1987; Brenner, 1991; Chalovich, 1991; Chalovich, 1992). Cross-bridge cycling is the alternation of force generating and non-force generating interactions between the thick and thin filaments of the contractile apparatus while Ca^{2+} is continuously being released and re-sequestered by the RyR and sarco(endo)plasmic Ca^{2+} adenosine triphosphatase (SERCA), respectively (for review see Gordon et al. 2000). At the cessation of sarcolemmal depolarization, Ca^{2+} is no longer released from the SR and the concentration Ca^{2+} in the cytoplasm decreases, thereby, allowing for muscle relaxation (Martonosi, 1995).

Skeletal Muscle Fatigue

Skeletal muscle fatigue is a phenomenon that can occur with repeated or sustained muscle contraction. It is characterized by a reduced shortening velocity (Edman and Mattiazzi, 1981; Crow and Kushmerick, 1983; de Haan et al., 1989; Westerblad and Lännergren, 1994), prolongation of relaxation (Edman and Mattiazzi, 1981; Westerblad and Lännergren, 1991), and decreased maximum force generated by a muscle, due to decreases Ca^{2+} sensitivity of the contractile apparatus (Westerblad and Allen, 1991; Westerblad and Allen, 1993) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Allen et al. 1989; Gyorke et al. 1993; Lee et al. 1991; Kabbara et al. 1999). Although the exact mechanisms of muscle fatigue are unknown, it is likely that there are many different contributing factors. Further, the mechanisms of muscle fatigue may be dependent upon the stimulation intensity and frequency. For example, the mechanism of fatigue in a muscle that was stimulated at a low intensity for a prolonged period of time may be different than the mechanism for a muscle that was stimulated at a high intensity for a short period of time.

The Role Of The Sarcoplasmic Reticulum In Skeletal Muscle Fatigue

The SR regulates $[Ca^{2+}]_i$ concentration within the skeletal muscle cell. Ca^{2+} is released from the SR through the RyR and is sequestered via the SERCA. Ca^{2+}-uptake via the SERCA is achieved through the hydrolysis of ATP (discussed in more detail later). The energy derived from the hydrolysis of one molecule of ATP is sufficient to transport two molecules of Ca^{2+} into the SR.

Ca^{2+} Release

Changes in SR regulation of $[Ca^{2+}]_i$ may be responsible for decreased force production normally found after prolonged muscle contraction. Many studies have shown that Ca^{2+}-release rates are decreased in isolated SR preparations from exercised muscles, compared to those isolated from rested muscles (Luckin et al. 1992; Belcastro et al. 1993; Favero et al. 1993; Ward et al. 1998; Williams et al. 1998; Ørtenblad et al. 2000). This notion is

also supported by studies that have shown decreased tetanic $[Ca^{2+}]_i$ in repetitively stimulated single muscle fibers isolated from amphibian (Allen et al. 1989; Gyorke et al. 1993; Lee et al. 1991; Kabbara et al. 1999) and mammalian muscle (Westerblad et al. 1991; Westerblad et al. 1993; Baker et al. 1993).

Ca^{2+} Uptake

The SERCA may also play a role in altered Ca^{2+} handling after prolonged muscle contraction. It has been postulated that dysfunction of this enzyme may result in either increased relaxation time (Allen et al. 1989; Lee et al. 1991; Westerblad and Allen, 1991), decreased Ca^{2+} availability for release (for review see Allen et al. 2002), or both. The function of this enzyme can be measured using an enzyme activity assay, Ca^{2+}-uptake rates, relaxation rates, or $[Ca^{2+}]_i$. Each of which will be addressed separately.

Isolated SR vesicles can be purified using differential centrifugation or sucrose gradient techniques. Because SR proteins and membranes isolated by these techniques are re-suspended in storage buffers, changes in function cannot be attributed to changes in intracellular milieu. Researchers have shown that decreased SR Ca^{2+}-uptake and Ca^{2+}-ATPase activity generally accompany fatigue induced by either prolonged (Byrd et al. 1989a; Luckin et al. 1991; Biederman et al. 1992; Wilson et al. 1998) or short term muscle contraction (Byrd et al. 1989b; Ward et al. 1998; Williams et al. 1998). These reductions in SR Ca^{2+}-ATPase activity range from about 20-60%. However, decreases in both SR Ca^{2+}-uptake and Ca^{2+}-ATPase activity do not always occur together. Belcastro et al. (1993) ran groups of rats on a treadmill for 2, 15, 30, 45, and 130 minutes. They found depressed SR Ca^{2+}-ATPase activity in all exercised groups, compared to control, with 70% of the total reduction being observed after the first two minutes. However, SR Ca^{2+}-uptake rates were reported to have increased in the exercise group, compared to the control. Bonner et al. (1976) also reported no changes in SR Ca^{2+}-ATPase activity isolated from untrained rats after exercise to exhaustion, compared to control samples. In fact, Bonner et al. (1976) found that SR vesicles isolated from rats trained to run on a treadmill for 30 minutes daily, five days per week had increased Ca^{2+}-ATPase activities after exercised to exhaustion. Similarly, Green et al. (1998) found that high resistance

training, in human subjects, resulted in decreases in SR Ca^{2+}-ATPase activities after a prolonged exercise bout that were less pronounced than those found before training after the same exercise protocol.

Ferrington et al. (1996) investigated changes in Ca^{2+}-ATPase activity isolated from rats during post exercise recovery after two hours of treadmill running. They found that increases in Ca^{2+}-ATPase activity correlated with time. Gollnick et al. (1991) found similar results from a study done on humans. Maximum voluntary contractile strength tests (MVC) were performed and muscle biopsies were taken before, immediately after, and 30 minutes after a fatiguing bout of a one leg kicking exercise. They found depressed SR Ca^{2+}-ATPase activity and MVC immediately after the exercise, compared to before the exercise. After 30 minutes of recovery, both SR Ca^{2+}-ATPase activity and MVC increased from those measured immediately after exercise, but were still lower than the before exercise measures. In 1998, Hargreaves et al. also found increased SR Ca^{2+}-uptake and ATPase activity in humans after 90 minutes of rest following three 30 second "all-out" sprint bouts on a cycle ergometer. However, Booth et al. (1997) found little recovery of SR Ca^{2+}-uptake and ATPase activities in human muscle homogenates after 60 minutes rest following prolonged exercise (incremental exercise on a cycle ergometer until volitional fatigue). These investigators did find significant decreases in half relaxation time ($RT_{1/2}$) following involuntary twitch contractions, but no changes in $RT_{1/2}$ following involuntary tetanic contractions after 60 minutes rest. It is important to note, however, that $RT_{1/2}$ may not be an ideal measure of SR function.

Whole muscle homogenates can also be used to investigate SR Ca^{2+}-uptake and Ca^{2+}-ATPase activity. Whole muscle homogenates are prepared simply by homogenizing the muscle sample and directly adding an aliquot to the desired assay. In 1999, Yasuda et al. found decreased SR Ca^{2+}-ATPase activity in whole muscle homogenates of rat soleus muscle after short (average time 2.8 minutes) and long term (average time 87.7 minutes) exercise to exhaustion. Similarly, researchers have shown decreased SR Ca^{2+}-uptake and ATPase activity in humans following three 30 second "all-out" sprint bouts on a cycle ergometer (Hargreaves et al. 1998) and following prolonged exercise (Green et al. 1992;

Parsons et al. 1992; Booth et al. 1997; Green et al. 1998). However, Chin et al. (1995 and 1996) found depressed Ca^{2+}-uptake rates in both isolated SR vesicles and homogenate fractions, while there were no differences in SR Ca^{2+}-ATPase activities in either of these fractions, compared to control samples.

In 2000, Ørtenblad et al. investigated changes in Ca^{2+} handling due to short-term high frequency fatigue using whole muscle homogenates. The investigators stimulated the extensor digitorum (EDL) muscles from rats *in vitro* for 4 minutes at 60 Hz, with a duty cycle of 150 milliseconds every second. Although this stimulation protocol reduced force to 14% of pre-fatigue values, these researchers found no changes in either SR Ca^{2+}-ATPase or uptake rates.

Skinned skeletal muscle fibers (the term "skinned" refers to muscle fibers that do not have an intact sarcolemma) can be used to assess SR function as long as the SR is kept intact. Williams et al. (1997 and 1993) stimulated semitendinosus muscles of male grass frogs for 5 minutes (tetanic contractions elicited every two seconds for 100 msec at 80 Hz). Skinned fibers were isolated from both stimulated muscles and contra-lateral control muscles. They found decreased Ca^{2+}-uptake rates of the SR estimated by the force-time integral after caffeine contractures.

$[Ca^{2+}]_i$ measurements are used to detect transient changes in myoplasmic $[Ca^{2+}]$ during muscle contraction. Measurement of $[Ca^{2+}]_i$ is done using fluorescent Ca^{2+} indicators either microinjected into muscle fibers (Westerblad et al. 1993) or used with skinned fibers (Lamb et al. 1999). SR Ca^{2+}-uptake can be estimated by the rate of decrease of measured $[Ca^{2+}]_i$. Westerblad et al. (1993) found reduced rates of decrease in $[Ca^{2+}]_i$ after the last tetanus as well as elevated resting $[Ca^{2+}]_i$ after 30 minutes of rest. However, these Ca^{2+} handling properties were accompanied by dramatically reduced tetanic $[Ca^{2+}]_i$, which in turn resulted in decreased force production.

Summary

Despite the use of several laboratory techniques and exercise protocols, there is the common finding. Although the mechanism(s) that mediate the altered SR Ca^{2+} handling are not yet known, it is clear that the SR plays a role in depressed skeletal muscle function under fatigued conditions.

Glycogen Influences Skeletal Muscle Function

It is well established that prolonged muscle contraction results in decreased muscle glycogen concentration (Kelso et al. 1987). Furthermore, glycogen depletion has been associated with decreased muscle performance (Ahlborg et al. 1967; Bergström et al. 1967; Galbo et al. 1979; Hargreaves et al. 1984; Coyle et al. 1986; Coggan and Coyle, 1989; Romijn 1993; Chin and Allen, 1997; Stephenson et al. 1999). For example, the rate of glycogen depletion in exercising muscle is dependent on the intensity of the exercise (Romijn, 1993). Furthermore, the time to exhaustion during endurance exercise is linked to the level of pre-exercise muscle glycogen (Bergström et al. 1967) and carbohydrate ingestion during exercise increases time to fatigue (Coyle et al. 1986; Coggan and Coyle, 1989) as well as increases sprint performance following prolonged exercise (Hargreaves et al. 1984). Because glycogen is an important substrate for energy production in the muscle cell, glycogen depletion may be a causal link to decreased muscle function. In view of the fact that the SR regulates $[Ca^{2+}]_i$ needed for force production, it seems reasonable that the SR may be sensitive to changes in muscle glycogen content. Although a mechanism for such a relationship has not yet been established, there is evidence that some glycogen is tightly bound to the SR (Entman et al. 1980; Fridén et al. 1989; Cuenda et al. 1994; Lees et al. 2000; Lees et al. 2001).

Glycogen Associated With The Sarcoplasmic Reticulum

Glycogen has been shown to be specifically associated with the SR using different methods (Entman et al. 1976; Fridén et al. 1989; Cuenda et al. 1994; Lees et al. 2000;

Lees et al. 2001). Both Cuenda et al. (1994) and Entman et al. (1976) used a phenol-sulfuric acid assay on isolated SR vesicles for their investigations, whereas Fridén et al. (1989) used periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP) staining of ultra-thin muscle sections. Using a sucrose density gradient isolation procedure, Entman et al. (1976) reported a range of 300 to 700 μg of glycogen per mg of SR membrane protein isolated from dog cardiac muscle. Cuenda et al. (1994), using a differential centrifugation SR isolation procedure, reported 32 μg glycogen per mg of SR membrane protein isolated from rat skeletal muscle. The binding of glycogen particles to the SR membrane may be achieved by the hydrophobic tail of the glycogen-associated form of protein phosphatase 1 (Hubbard et al. 1989 and 1993).

In 1989, Fridén et al. used PA-TSC-SP staining of ultra-thin muscle sections from human vastus lateralis. The staining revealed compartmentalized distribution of glycogen within the muscle cell. These researchers suggested that glycogen is stored at 5 topographically different sites: subsarcolemmal, intermyofibrillar, para-Z-disc (in between the thin filaments at either side of the Z-disc), N_2-line (the lateral end of the I-band), and the H-zone (flanking the bare region of the myofibrillar M-band). After exercise (sixty, 8 second sprint cycling bouts), glycogen depletion was particularly noticeable at the N_2-line. It is important to note that the SR has been shown to be present at the I-band (Sigel et al. 1969; Pette, 1975).

In 2000, Lees et al. examined the glycogen content of heavy and light SR fractions purified from rat skeletal muscle using two different types of assays. The first method was ethanol extraction, sulfuric acid hydrolysis and phenol determination and the second method was glucoamylase digestion and fluorometric measurement of β-NADP in an enzymatic system. The effects of fatiguing muscle contraction on SR glycogen content, via stimulation of the sciatic nerve, were investigated. They found that both SR fractions purified from the fatigued muscle contained significantly less glycogen as a result of fatiguing muscle contraction and that the glucoamylase digestion and fluorometric measurement of β-NADP in an enzymatic system was the more sensitive assay.

Glycogen Phosphorylase Associated With The Sarcoplasmic Reticulum

Glycogen phosphorylase, an enzyme involved in glycogenolysis, can be found in skeletal muscle in either its inactive b form (dephosphorylated) or its active a form (phosphorylated). Glycogen phosphorylase has also been shown to be associated with the SR (Wanson et al. 1972; Entman et al. 1980). Specifically, glycogen phosphorylase associated with the SR is more than 95% in its b (inactive, dephosphorylated) form (Cuenda et al. 1995). Interestingly, glycogen phosphorylase may be associated with the SR via its binding to the glycogen particles (Meyer et al. 1970; Wanson et al. 1972; Entman et al. 1980; Cuenda et al. 1994). A schematic representation of SR-bound glycogen phosphorylase is shown in Figure 2. Moreover, glycogenolysis of SR glycogen may release glycogen phosphorylase. Cuenda et al. (1994) showed that preparing SR from animals that were starved for 48 hours (a treatment that causes glycogen depletion) resulted in a 2 to 4 fold decrease in glycogen phosphorylase activity and content (measured using SDS-PAGE and PLP concentration). Similarly, it was shown that amylase digestion of endogenous glycogen resulted in 95% depletion of glycogen phosphorylase (measured via glycogen phosphorylase activity) (Entman et al. 1980). More recently, Lees et al. (2001) have shown that the level of SR glycogen as well as glycogen phosphorylase are reduced following repetitive stimulation of rat gastrocnemius muscle.

Sarcoplasmic Reticulum Bound Fluorescein Isothiocyanate

Fluorescein isothiocyanate (FITC) is a lysine reactive-fluorophore that specifically binds to lysine-515 of the SERCA in SR preparations (Mitchinson et al. 1982). FITC binds to SERCA competitively with ATP, however, ATP reacts with a different residue in the ATP-binding pocket. Lysine-515 is located in the ATP-binding pocket of SERCA. There is a positive correlation between FITC binding and the E2 conformation and, consequently, FITC has been used extensively to study changes in SERCA conformation (see Bigelow and Inesi 1992 for review). During the catalytic cycle of SR Ca^{2+}

translocation, SERCA binds two molecules of Ca^{2+}, which shifts the enzyme from the E2 to the E1 conformation. SERCA can bind ATP in the E2 conformation, but transfer of the high-energy phosphate will only occur after the binding of Ca^{2+}. Subsequent ATP hydrolysis results in phosphorylation of the aspartate-351 residue in the ATP-binding pocket, which transfers the chemical energy required for Ca^{2+} transport. In the E1 conformation, the chemical energy of the phosphorylated aspartate is used to reorient the Ca^{2+} sites towards the SR lumen (see Figure 2) (see Jorgensen and Anderson, 1988 and MacLennan et al. 1997 for review). Because the fluorescent emission intensity of FITC is different in the E1 and E2 conformation states, this method can be used to assess SERCA conformation state (Pick and Karlish 1980; Pick 1981).

Cuenda et al. (1991) found that glycogen phosphorylase b status of the SR affected the conformation of the SR Ca^{2+}-ATPase. These investigators found that, as glycogen phosphorylase b content increased, the fluorescent emission intensity of FITC bound to the SERCA also increased. As stated earlier, about 95% of glycogen phosphorylase bound to the SR is in the b form. These data suggest that increased glycogen phosphorylase concentration, which is accompanied by glycogen, may induce a shift in the SERCA conformation. Increasing the proportion of SERCA in the E2 conformation may allow for increased ATP binding, subsequently allowing for increased Ca^{2+} transport into the SR.

In 1991, Luckin et al. investigated FITC binding in SR vesicles purified from rat gastrocnemius muscles after one bout of prolonged exercise. These investigators reported a 40% decline in maximal FITC fluorescent emission intensity in fatigued SR compared to control. However, Schertzer et al. (2003) reported no differences in FITC fluorescent emission intensity with SR vesicles purified from rat gastrocnemius muscles after a fatiguing bout of treadmill running. One potential difference that might explain the discrepancy between the results from these two studies is tissue processing. Luckin et al. (1991) only harvested the deep red (oxidative) fibers from the gastrocnemius, whereas, Schertzer et al. (2003) used the whole gastrocnemius. Muscle fibers not recruited for treadmill running, may mask differences in Schertzer et al. (2003), whereas

Luckin et al. (1991) attempted to minimize this problem by only purifying SR from the more oxidative region (more likely recruited for prolonged running) of the gastrocnemius.

Sarcoplasmic Reticulum Glycogen Extraction

As stated earlier, glycogen extraction from purified SR has been associated with changes in SR protein profile (Entman et al. 1980; Cuenda et al. 1994). Although there are a few studies that examine the effects of glycogen extraction on SR Ca^{2+} handling, none of these have measured glycogen associated with the SR (Brautigan et al. 1979; Chin and Allen 1997; Stephenson et al. 1999). In 1997, Chin and Allen used a stimulation model to decrease cellular glycogen. These investigators measured Ca^{2+} handling in fibers that were allowed to recover (60 minute recovery) in the presence of glucose compared to those that recovered without glucose. These investigators reported complete glycogen recovery with glucose, which corresponded to a recovery of tetanic $[Ca^{2+}]_i$ to 82% of initial values. Under conditions where glucose was removed from the extracellular environment, they reported sustained glycogen depression, which corresponded to a recovery of tetanic $[Ca^{2+}]_i$ to 64% of initial values. These results emphasize the point that glycogen content is likely only one aspect of skeletal muscle status that influences contractile function. Also, it is important to note that using a stimulation model makes it impossible to isolate glycogen status as the one independent variable.

Stephenson et al. (1999) reported that 84% of the glycogen in skinned fibers from cane toad was considered to be part of a non-washable fraction. Reducing the non-washable glycogen pool by either T-system depolarization-induced Ca^{2+}-release or bathing the fibers in either a relaxing solution or a Ca^{2+}-activating solution correlated highly with T-system activation (a measure of ECC). Perhaps most importantly, these researchers reported that depressed responses to T-system depolarization as result of decreased glycogen content were still evident when exogenous ATP and CP were added to bathing solutions (i.e., glycogen was not required as an energy source). Brautigan et al. (1979)

also used a skinned fiber model, but they reduced glycogen stores via α-amylase. These researchers reported decreased SR Ca^{2+}-uptake rates when compared to control group.

Compartmentation And Sarcoplasmic Reticulum ATP Production

The "metabolic soup" model which describes all the components of metabolic pathways freely diffusing within a muscle fiber and interactions between molecules defined by random probability, is no longer accepted. Compartmentation is a model that describes the components of the muscle fiber as being targeted at the site of utilization. In terms of local ATP production for SR Ca^{2+} handling, compartmentation seems to be important for decreasing diffusion of metabolites, improving Ca^{2+} transport efficiency, and creating a microenvironment, whereby local changes in metabolite concentrations can be detected. Glycolytic, glycogenolytic, and Ca^{2+}-accumulating enzymes are found to be closely associated with the SR (Figure 3) (Entman et al. 1976; Entman et al. 1980; Xu et al. 1995; Xu and Becker, 1998). The energy demands of SERCA in proportion to the total energy demands of an active muscle fiber can vary depending on the type of muscle fibers that predominate in a particular muscle. For example, the plantaris muscle is predominantly comprised of "fast" muscle fibers; whereas, the soleus muscle is comprised predominantly of "slow" muscle fibers. These muscles vary in their protein composition based on their physiological function (see Rome and Lindstedt, 1998 for review). The best estimation of the proportion of ATP utilized in Ca^{2+}-uptake in skeletal muscle is about 1/3 (Stienen et al. 1995). This is quite a substantial amount of the total energy cost, consequently it is not surprising to find many of the constituents of ATP synthesis targeted to the SR.

Glycolytic Enzymes Associated With The Sarcoplasmic Reticulum

There is ample published data that support the notion that the SR membrane is targeted by many glycolytic enzymes as well as the actual Ca^{2+} transporting proteins themselves (Pierce and Philipson, 1985; Han et al. 1992; Xu et al. 1995; Xu and Becker, 1998).

Pierce and Philipson (1985) reported that, although glyceraldehyde-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase were lost during the purification (high concentrations of either NaCl or KCl) of cardiac SR membranes, exogenously added enzyme bound to the SR in relatively large quantities. Given these findings, these investigators concluded that the binding of these enzymes to the SR membrane likely occurs *in vivo* via a charge-related mechanism. Xu et al. (1995) reported similar findings from SR purified from both skeletal and cardiac muscle. These investigators reported the presence of several glycolytic enzymes associated with their SR preparation, including pyruvate kinase, enolase, phosphoglyceromutase, GAPDH, phosphoglycerate kinase, and aldolase. These investigators also found that endogenous glycolytic ATP production supported SR Ca^{2+}-uptake with the addition of the required substrates and cofactors. In fact, endogenous ATP production was more efficient than exogenous ATP in supporting SERCA Ca^{2+} transport when glyceraldehyde 3-phosphate, nicotinamide adenine dinucleotide, P_i, and ADP were added. In 1998, Xu and Becker investigated the ultrastructural localization of PK, aldolase, and GAPDH using electron microscopy and double immunogold labeling. These investigators found that SERCA seems to be located at adjacent positions on SR vesicles with all three of these glycolytic enzymes. This study provides anatomical evidence of channeling of glycolytic substrates for the production of ATP to support SR Ca^{2+}-uptake.

Compartmentation of endogenous glycolytic ATP production also seems to be integral for the triadic region of the SR involved in Ca^{2+}-release. Han et al. (1992) reported the presence of aldolase, GAPDH, triosephosphate isomerase (TIM), phosphoglyceromutase, enolase, PK, lactate dehydrogenase, and adenylate kinase in a terminal cisternae (triadic) SR purification preparation. This preparation was able to produce endogenous ATP most effectively when fructose 1,6-biphosphate was used as a substrate. The endogenous ATP synthesized did not seem to be in equilibrium with bulk ATP. Also, the addition of a known protein phosphatase inhibitor, fluoride, altered the ATP-synthesis kinetics dramatically. These investigators concluded that this compartmentation of ATP synthesis might be important in the phosphorylation/dephosphorylation of regulatory proteins involved in Ca^{2+}-release including the RyR.

CK coupling to SERCA

Several groups have reported creatine kinase (CK) to be associated with both purified SR isolated from skeletal muscle (Baskin and Deamer, 1970; Rossi et al. 1990; Korge and Campbell, 1992; Korge et al. 1993; Korge and Campbell, 1994) and in single skinned fiber preparations (Duke and Steele, 1999). Korge and Campbell (1992) reported that creatine phosphate (CP) plus ADP-supported Ca^{2+}-uptake into the SR at a rate that was 65% of ATP-supported uptake. When SR vesicles were isolated from fatigued plantaris muscles (stimulated *in situ* until force declined to 30% of rested value), ATP-supported Ca^{2+}-uptake rates declined. Interestingly, the CP plus ADP-supported Ca^{2+}-uptake rate declined to a greater extent than that of ATP-supported uptake. After fatiguing stimulation, CP plus ADP-supported Ca^{2+}-uptake rates declined to 68% of those found under rested conditions, whereas ATP-supported uptake declined to 84% compared to rested conditions.

The CP shuttle (or phospho-creatine circuit) is a metabolic model that describes the role of CK and CP in terms of energy supply to specific sites in the cell (see Wallimann et al. 1989 for review). Briefly, specifically targeted CK within the cell aids in the local regeneration of ATP using ADP and CP as substrates. There are many examples of CK localized to a specific region of the cell that are coupled to a specific energy demand. For example, myofibril-bound CK is coupled to the myosin ATPase and sarcolemmal-bound CK is coupled to Na^+/K^+-ATPase activity (see Wallimann et al. 1992 for review). SR-targeted CK can be in close structural proximity to SERCA, such that CK-derived ATP may be preferentially utilized by adjacent SERCA. Conversely, ADP production by SERCA may be preferentially utilized by adjacent CK. In fact, metabolic coupling between CK and SERCA was demonstrated by Korge et al. (1993) when CK-supported uptake in isolated SR continued in the presence of two different ATP traps (one being the addition of a competing ATPase and the other being the addition of hexokinase and glucose).

Under conditions of contractile activity, rates of ATP hydrolysis are high, thus increasing the cellular [ADP]. There are, of course, cellular pathways that facilitate minimal

accumulation of ADP because of its deleterious effects on free energy of hydrolysis (ΔG, see equation 1). In terms of Ca^{2+}-transporting efficiency (coupling ratio of 2 Ca^{2+} ions per ATP hydrolyzed), SERCA is affected by changes in local [ADP] accumulation (Verjovski-Almeida et al. 1978; Orlowski et al. 1988). A reduction of the actual free energy of hydrolysis of ATP, such that it is less than that required to translocate 2 Ca^{2+} ions per ATP (SERCA requires 52 kJ/mol in order to translocate 2 Ca^{2+} ions into the SR), will subsequently affect Ca^{2+}-transporting efficiency and result in an uncoupling of catalytic and transport activities of SERCA (Kammermeier, 1987). In fact, it has been shown that the CK system is essential for optimal Ca^{2+} transport (Wolosker et al. 1996; de Groof et al. 2002). Wolosker et al. (1996) reported that S-nitrosoglutathione, a CK inhibitor, resulted in decreased Ca^{2+}-uptake rates in purified SR. de Groof et al. (2002), reported that electrically stimulated CK-deficient myotubes exhibited impaired Ca^{2+} transport, compared to wild-type.

Duke and Steele (1999) investigated the role of CK on Ca^{2+} handling in single skinned skeletal muscle fibers. This group investigated rates of caffeine-induced Ca^{2+}-release and subsequent uptake under two conditions that effectively block CK activity. First, they removed CP from the perfusing solution and second, they added 2,4-dinitro-1-fluorobenzene (DNFB), an inhibitor of CK. Under both treatment conditions, they reported a decrease in the amplitude and a prolonged decline of the Ca^{2+} signal. Also, they reported an elevated basal Ca^{2+} signal in response to removal of CP. These data are consistent with impaired Ca^{2+} handling normally found in fatigue (see Fitts, 1994; Allen et al. 2002 for reviews).

Summary

Despite extensive research, researchers have failed to delineate the mechanism(s) of skeletal muscle fatigue. Although there are likely several factors contributing to diminished SR Ca^{2+} handling in skeletal muscle fatigue, it is probable that SR glycogen status may be involved. Furthermore, a metabolic signal for impaired skeletal muscle function seems logical when one approaches the phenomenon as matching energy utilization with energy production. There is not only the question of energy supply, but

the consequences of energy depletion as well (Maier and Pette, 1987). Unfortunately the answer is not as simple as energy supply itself. There are changes at the sites of energy utilization that are not easily reversed (Allen et al. 1989; Gyorke et al. 1993; Lee et al. 1991; Westerblad et al. 1991; Luckin et al. 1992; Baker et al. 1993; Belcastro et al. 1993; Favero et al. 1993; Westerblad et al. 1993; Ward et al. 1998; Williams et al. 1998; Kabbara et al. 1999; Ørtenblad et al. 2000). The structure of the SR includes several important components of ATP synthesis from stored energy (in the form of glycogen) and systems for optimizing function (Han et al. 1992; Korge et al. 1993; Xu et al. 1995; Xu and Becker, 1998). In order to better understand mechanism(s) for impaired Ca^{2+} handling in skeletal muscle fatigue, we must investigate potential metabolic signals. Glycogen is a major storage site for energy vital for optimal exercise performance, therefore, glycogen status must explored as a metabolic signal of muscle fatigue.

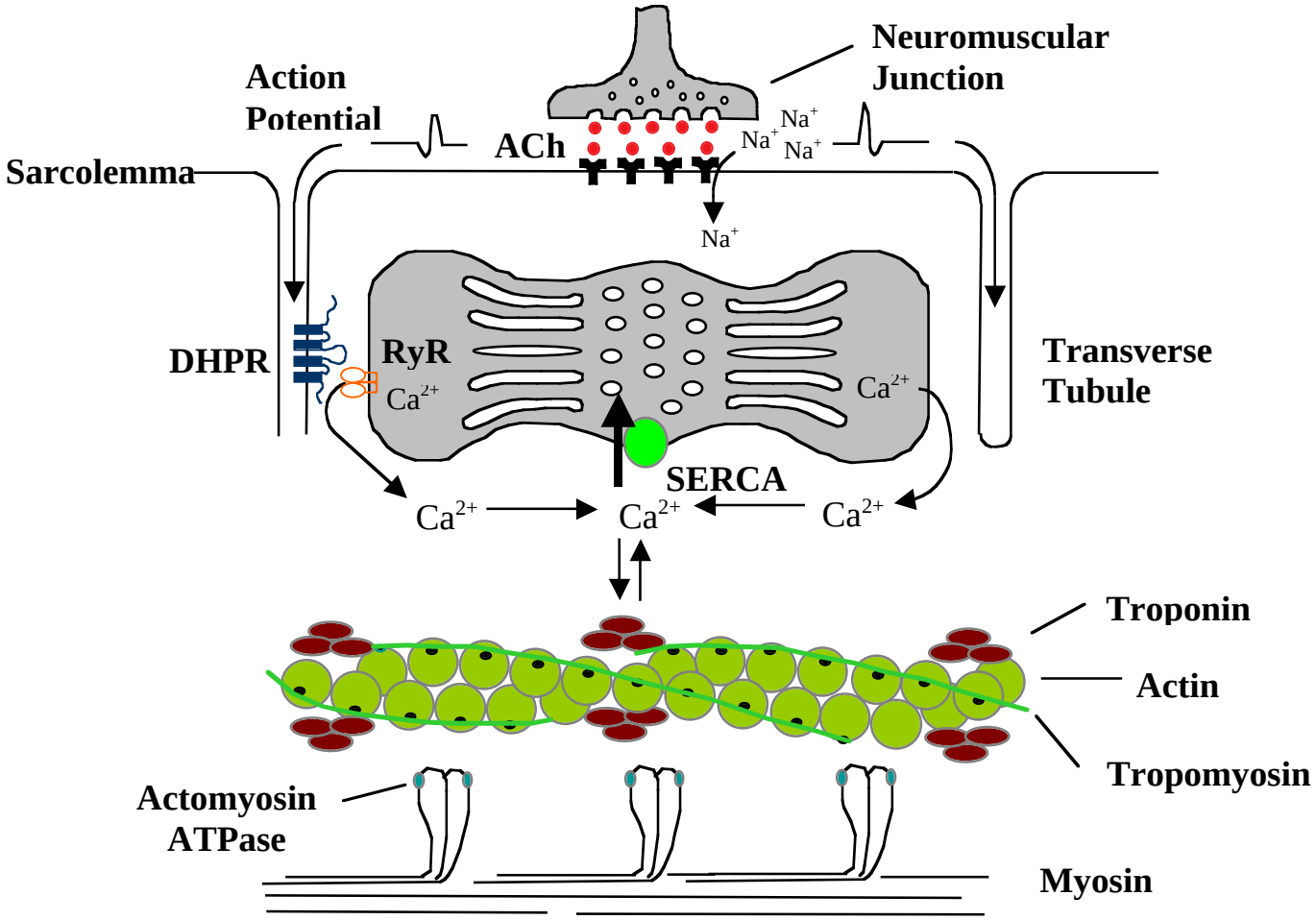

Figure 1. Schematic representation of ECC
The process of membrane excitation linked to muscle contraction is known as excitation-contraction coupling (ECC). In skeletal muscle, acetylcholine (ACh) released from the neuromuscular junction causes an action potential to travel the length of the muscle fiber. The action potential travels down the transverse tubules where it signals Ca^{2+}-release through its interaction with the dihydropyridine receptor (DHPR). Ca^{2+} is released through the ryanodine receptor (RyR) of the SR, subsequently binds to troponin, and initiates muscle contraction. Ca^{2+} is re-sequestered via SERCA and when sarcolemma depolarization stops, muscle relaxation ensues.

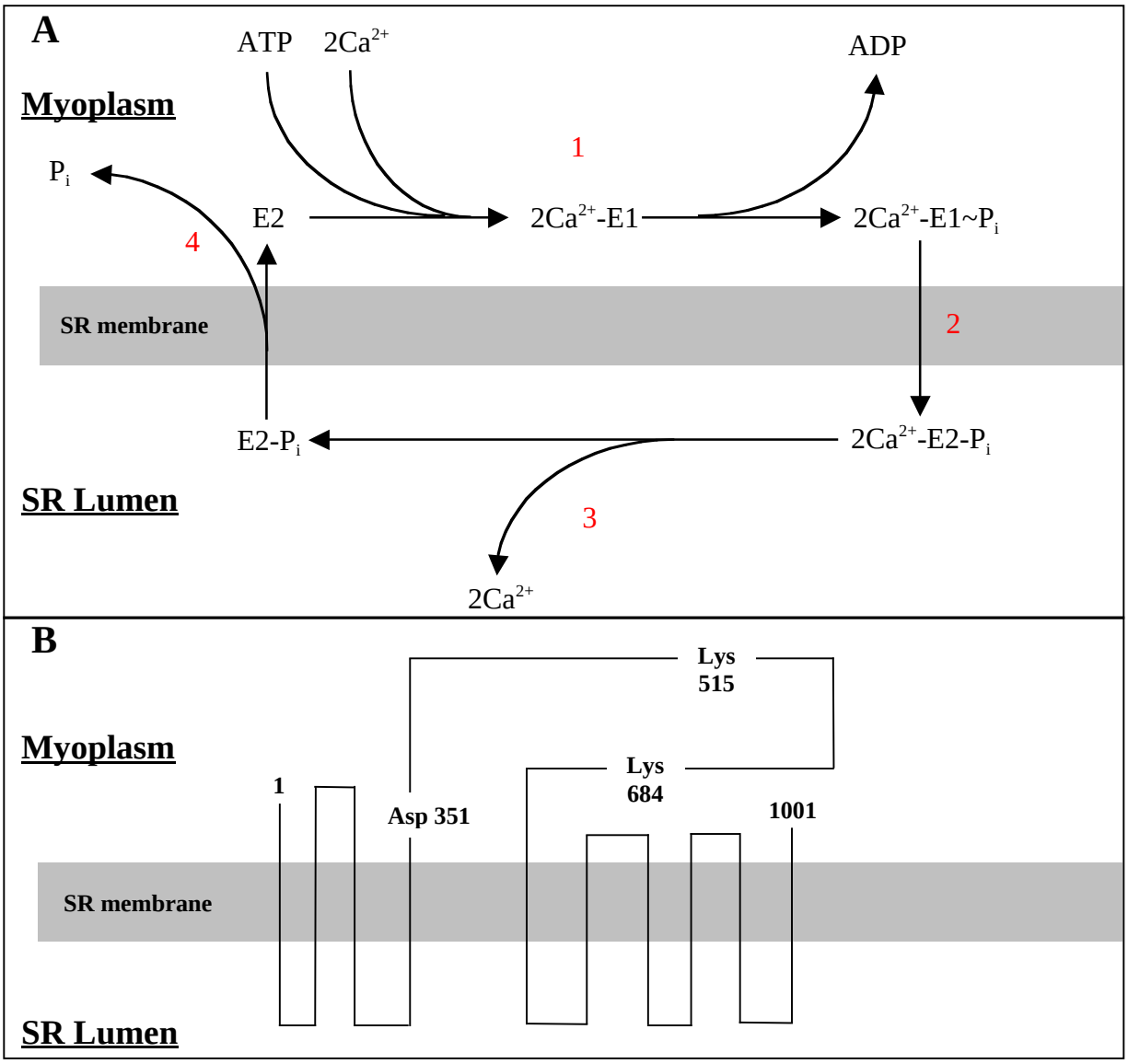

Figure 2. SR Ca^{2+} transport
Schematic representation of sarcoplasmic reticulum (SR) Ca^{2+} transport in skeletal muscle. A) 1: The binding of 2 Ca^{2+} ions at the myoplasmic high affinity binding sites causes the inter-conversion between the E2 and E1 conformation. The binding of ATP is possible in the E2 conformation, however, the transfer of the high-energy phosphoryl group may only occur after the Ca^{2+} ions have bound to SERCA. 2: The chemical energy of the phosphorylated aspartate (~P_i) is used to reorient the Ca^{2+} sites towards the SR lumen. 3: After reorientation, the now low affinity Ca^{2+} binding sites of the E2 conformation are able to release the 2 Ca^{2+} ions into the lumen of the SR. 4: Inorganic phosphate (-P_i) is release into the myoplasm. B) Topological orientation of the FITC binding site (Lys 515), ATP-binding site (Lys 684) and the phosphorylation site of SERCA (Asp 351) (Bigelow and Inesi 1992). The relative distances between the indicated binding sites are not physiologically acurate, nor do they indicate any specific conformation.

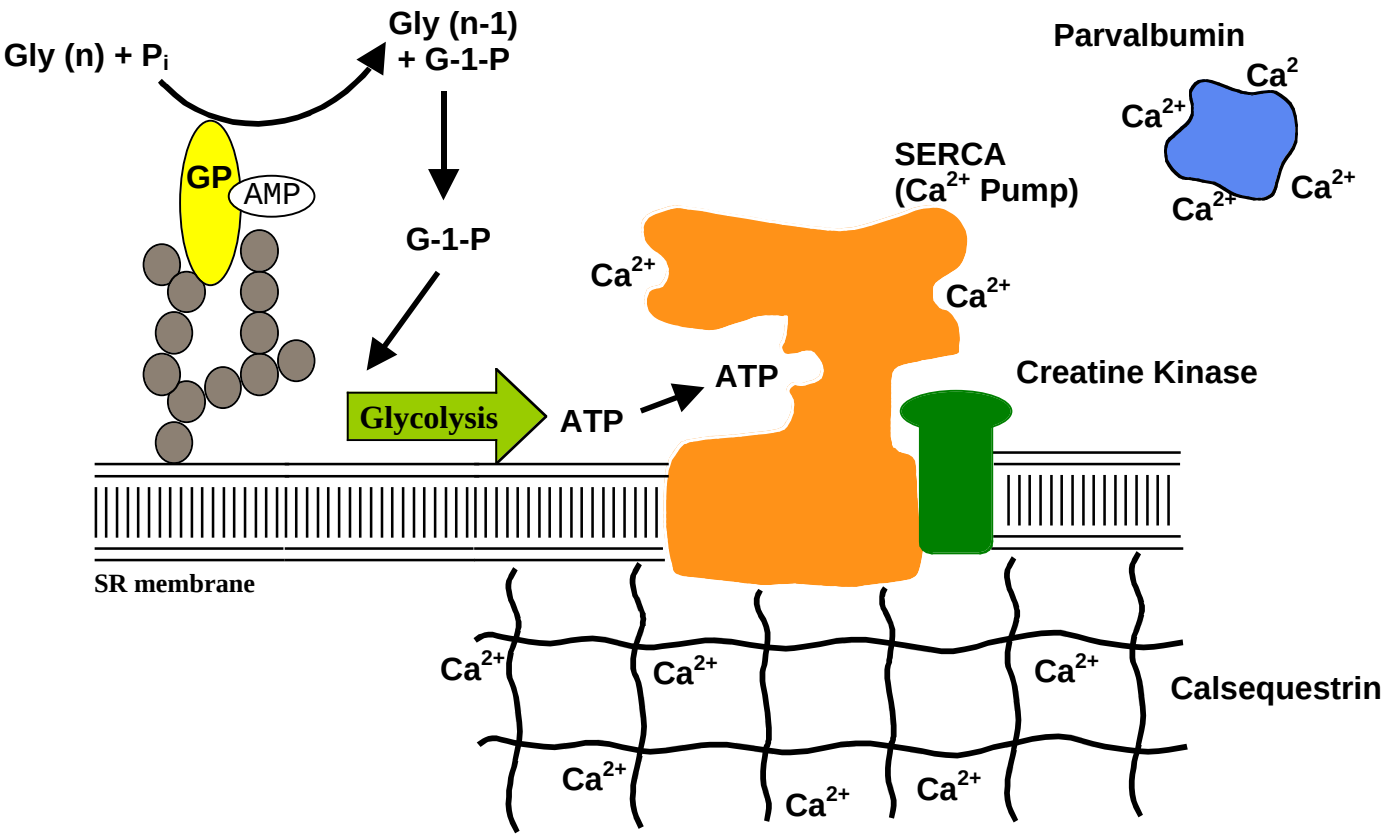

Figure 3. Schematic representation of the SR
This diagram includes the proposed compartmentation of glycogen, glycogen phosphorylase (GP), glycolytic enzymes and creatine kinase along with the Ca^{2+} transporting proteins at the sarcoplasmic reticulum (SR). Calsequestrin binds Ca^{2+} in the lumen of the SR in order to lower the free [Ca^{2+}]. Parvalbumin is a Ca^{2+} binding protein predominantly found in fast skeletal muscle that helps stabilize myoplasmic [Ca^{2+}]. The specific location of glycogen phosphorylase bound to the glycogen is not intended to represent actual *in vivo* binding. The actual binding of glycogen phosphorylase in glycogen granules is thought to be near the surface and possibly mediated via interactions with protein phosphatase 1 (Shearer and Graham, 2002; Newgard et al. 2000).

Chapter Three: Experimental Design And Methods

Animals

The Virginia Tech Institutional Animal Use and Care Committee approved all procedures for present investigation. Female Sprague Dawley rats from Harlan were used (200-250 g, approximately 8-10 weeks of age) in this investigation. Animals were housed two per cage and allowed free access to Harlan Teklad 2018 rodent chow and water. Prior to removal of the tissue, the animals were anesthetized with an intra-peritoneal (i.p.) injection of ketamine (80 mg/kg, from a 10 ml bottle of Ketaset 100 mg/ml) and xylazine (10 mg/kg, from a 20 ml bottle of Rompum 20 mg/ml). Following the tissue removal the animals were euthanised by surgically removing the diaphragm.

Experimental Design

The present investigation was carried out as 2 separate studies. Flow diagrams (Figures 4 and 5) depict the purification steps, treatments and incubation times.

Study 1

The gastrocnemius/plantaris muscles from 24 rats were removed, minced with scissors, homogenized, centrifuged at 8,000xg for 15 minutes, and then incubated for one hour on ice. The muscles collected from one leg served as the treatment samples and the muscles harvested from the contra-lateral leg served as the control samples (control/amylase and control/No-EDTA). For 12 of these animals, the standard homogenizing buffer was used for tissue collected from both legs. Just prior to incubation, α-amylase was added to the samples collected from one leg of each animal. For another group of 12 animals, the standard homogenizing buffer was used for the muscles collected from one of the legs, whereas a homogenizing buffer lacking EDTA was used for the contra-lateral leg. After the incubation, two SR fractions were purified, heavy SR (HSR) and light SR (LSR) (Figure 23). Some of these samples were stored without sucrose in the storage buffer because it interferes with the SR glycogen assay. However, sucrose is normally needed as a cryoprotectant (Anchordoguy et a. 1987; MacDonald et al. 1994). Therefore, one set of 10 animals was used only for the measurement of SR glycogen and another set of 14

animals was used for all other measurements. Glycogen concentration for all the SR samples was measured and compared.

In order to determine glycogen phosphorylase content of these samples, SDS-PAGE (sodium-dodecyl-sulfate polyacrylamide gel electrophoresis) was performed. The optical density of the band that represents glycogen phosphorylase at about 97 kDa was compared between control and glycogen-extracted samples. Peak SR Ca^{2+}-uptake rates supported by ATP, ATPase activities and peak SR Ca^{2+}-release rates were measured using the SR vesicles purified from the groups described above.

Study 2

The gastrocnemius/plantaris muscles from 12 rats were removed, minced with scissors, homogenized, centrifuged at 8,000xg for 15 minutes, and then incubated for one hour on ice. The muscles collected from one leg served as the treatment samples and the muscles harvested from the contra-lateral leg served as the control samples (control/amylase and control/No-EDTA). For 6 of these animals, the standard homogenizing buffer was used for tissue collected from both legs. Just prior to incubation, α-amylase (EC 3.2.1.1) was added to the samples collected from one leg of each animal. For another group of 6 animals, the standard homogenizing buffer was used for the muscles collected from one of the legs, whereas a homogenizing buffer lacking ethylenediaminetetraacetic acid (EDTA) was used for the contra-lateral leg. After the incubation, a single SR fraction was purified (total SR) (Figure 23).

In order to determine glycogen phosphorylase, 4-α-glucanotransferase amylo-1,6-glucosidase (EC 2.4.1.25+3.2.1.33, glycogen debranching enzyme or AGL), creatine kinase (CK) and pyruvate kinase (PK) content of the samples, SDS-PAGE was performed. The optical density of the bands that represent these proteins were compared between control and glycogen-extracted samples. Peak SR Ca^{2+}-uptake rates supported by ATP were measured using the SR vesicles purified from the groups described above. Peak SR Ca^{2+}-uptake rates supported by ADP + CP (substrates for ATP synthesis via CK) and ADP + PEP (substrates for ATP synthesis via PK) were measured using the SR

vesicles purified from the groups described above. Maximal fluorescence intensity of SR-bound FITC was measured using the SR vesicles purified from the groups described above.

Study 1		Study 2
LSR	**HSR**	**Total SR**
Glycogen Concentration	Glycogen Concentration	
	Gly Phos Content	Gly Phos Content
	SERCA Content	SERCA Content
		AGL Content
		CK Content
		PK Content
	ATPase Activity	
	ATP-supported Ca^{2+}-Uptake	ATP-supported Ca^{2+}-Uptake
	SR Ca^{2+}-Release	
		CP + ADP-Supported SR Ca^{2+}-Uptake
		PEP + ADP-Supported SR Ca^{2+}-Uptake
		FITC Binding

Table 1. Overview of experimental measurements made in study 1 and 2
In study 1, purified sarcoplasmic reticulum (SR) was isolated in 2 different fractions, heavy SR (HSR: 8,000-12,000 x g) and light SR (LSR: 12,000-49,000 x g). In study 2, only 1 SR fraction was purified representing both the HSR and the LSR, total SR (total SR: 8,000-49,000 x g). Gly Phos, glycogen phosphorylase; SERCA, sarco(endo)plasmic reticulum ATPase; AGL, glycogen debranching enzyme; CK, creatine kinase; PK, pyruvate kinase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PEP, phosphoenol-pyruvate; FITC, fluorescein isothiocyanate.

Limitations And Basic Assumptions

1. In some cases, SR glycogen extraction was induced by the addition α-amylase, which is not a physiological mechanism. In other cases, SR glycogen extraction was induced by eliminating EDTA from the homogenization and storage buffers, which may have other effects on the SR in addition to glycogen extraction.
2. Assays were performed *in vitro,* which can only mimic the intracellular mileu.
3. The subjects were limited to only one species and gender (female Sprague-Dawley rats (200-250 g)).
4. Rats were disease and pathogen free.
5. Rats were well fed and hydrated.
6. The SR vesicles were undamaged by the isolation protocol.
7. There were no underlying factors within the muscle that would affect normal function.

Tissue Processing And Sarcoplasmic Reticulum Purification

Both gastrocnemius/plantaris muscle groups were utilized for all experiments. Surgical removal of the gastrocnemius/plantaris muscles was done as follows: the hind limbs were shaved using clippers and then the skin of the limbs was removed using scissors and forceps. Once the muscles of the hind limbs were exposed, the tissue covering the gastrocnemius was removed and then the achilles tendon was cut at the distal end. Using forceps, the distal end of the achilles tendon was held taught as the gastrocnemius/plantaris muscles were freed from the hind limb working from the distal end towards the proximal end. Once the origin of the soleus muscle exposed, the soleus was removed beginning at the proximal end and working back towards the distal end. After the removal of the soleus, the gastrocnemius/plantaris muscle were removed from the hind limb by cutting the tendons at the proximal end. Once the gastrocnemius/plantaris muscles were removed from the animal, the muscles were cleaned of any extraneous tissue and weighed.

The muscles from one leg of each animal were homogenized in standard buffer which contained 20mM N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid] (HEPES), 0.2% sodium azide (NaN_3), 1mM EDTA, AEBSF (0.1 mM), Aprotinin (80 nM), Leupeptin (2.2 nM), Bestatin (4 nM), Pepstatin A (1.5 nM), and E-64 (1.4 nM). Two procedures were used on the contra-lateral muscles to extract glycogen from the sarcoplasmic reticulum (SR). These muscles were homogenized in either no-EDTA buffer or in a standard buffer to which 0.1% α-amylase (EC 3.2.1.1) was added for the incubation step (see below). Muscles were removed, placed in ice-cold homogenizing buffer, minced and homogenized (VirtiShear, 3 x 15s). Homogenates were centrifuged at 8,000 x *g* for 15 min. at 4°C. The supernatant was filtered through four layers of gauze and 600mM potassium chloride (KCl) was added. After the addition of KCl, samples were incubated on ice, with gentle shaking, for 1 hour. SR purification for study 1 entailed separation of the SR into 2 different fractions, heavy SR (HSR) and light SR (LSR). HSR was the resulting pellet after the supernatants were centrifuged for 45 minutes at 12,000 x g at 4ºC. The supernatant was removed and centrifuged at 49,000 x g for 60 minutes at 4ºC. The pellet from this centrifugation was the LSR. SR purification for study 2 utilized only 1 fraction (total SR) that included both the HSR and the LSR. After the 1 hour incubation, supernatants were centrifuged at 43,025 x g for 120 min. In all cases, resulting SR pellets were re-suspended in 500 µl of storage buffer (homogenization buffer containing 300mM sucrose and 150mM KCl, however, storage buffer for SR vesicles that were used for SR glycogen measurement will not have sucrose) and stored at –80°C. These samples were used for SR function measurements, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescein isothiocyanate (FITC) binding. The SR vesicles isolated from some animals in study 1were stored in storage buffer containing no sucrose. These samples were only used to determine the amount of glycogen associated with the SR.

Protein Estimation Assay

The protein concentration of the re-suspended SR vesicles was determined using the Bradford dye-binding assay adapted by Bio-Rad using bovine serum albumin as a standard (Bradford, 1976). The binding of basic and aromatic amino acid residues to

Coomassie® Brilliant Blue G-250 dye shifts the maximum absorbance from 465 nm to 595 nm (Sedmack and Grossberg, 1977; Reisner et al., 1975).

Sarcoplasmic Reticulum Glycogen

Glycogen associated with the SR was measured by digesting the glycogen and measuring the glucose released enzymatically following a method modified from Keppler and Decker (1984) (Lees et al. 2001). Re-suspended SR (a volume containing 50 μg of protein) was incubated in 0.5 ml of an acetic acid (174 mM) solution containing glucoamylase (8.7 kU/l) for 2 hours at 40˚C (pH 4.8). A 50 μl aliquot of each sample was then added individually to 1 ml of a buffer (pH 7.5) containing the following: 0.3 M TEA (triethanolamine), 4 mM magnesium sulfate ($MgSO_4$), 120 mM potassium hydroxide (KOH), 1 mM adenosine triphosphate (ATP), 0.9 mM beta-nicotinamide adenosine dinucleotide phosphate (β-$NADP^+$), 700 U/l glucose-6-phosphate dehydrogenase (G-6-P DH), and hexokinase (1.3 kU/l). After 10 minutes of incubation at room temperature, fluorescent emission of the reduced form of beta-nicotinamide adenine dinucleotide phosphate (β-NADPH) was measured at 450 nm with the excitation wavelength set at 365 nm. Glycogen standards were prepared using glycogen purified from rabbit liver.

SDS-PAGE

Each sample was prepared by first diluting into 1 μg/μl SR protein with water and then adding sample buffer to 1:1 (volume:volume). Sample buffer (25 ml) was prepared by mixing 0.25 ml β-mercaptoethanol (β-MEOH), 1 g sodium dodecyl sulfate (SDS), 4 ml of 1 M Tris (pH 6.8), 5 ml glycerol, and 0.5 g bromophenol blue, and then brought to 25 ml with distilled H_2O. The samples in the sample buffer were placed in glass test tubes, boiled for 2 minutes, the tops were wrapped with parafilm, and then frozen at –80˚ C until needed. The final concentration of SR protein in the samples in the sample buffer was 0.5 μg/μl.

SDS-PAGE was performed following the method of Laemmli (1970). Gels were run on a mini-PROTEAN® II cell from Bio-Rad. The 5% acrylamide separating gel was prepared by mixing 12.3 ml H_2O, 5 ml 1.5 M Tris (pH 8.8), 0.2 ml of 10% SDS, and 2.5 ml of 40% acrylamide:bisacrylamide (37.5:1). The separating gel was placed in a vacuum for 20 minutes. 20 µl of 97% (electrophoresis grade) N,N,N',N'-tetramethylethylenediamine (TEMED) and 100 µl of 10% ammonium persulfate were added to the separating gel, gently mixed, poured into the mini-PROTEAN® II cell (1 cm below the comb), and 20% ethanol was overlaid. The separating gel was allowed to polymerize for 30 minutes. After polymerization, the ethanol was poured off, 0.1% SDS was used to wash any remaining ethanol from between the glass plates, and then filter paper was used to dry in between the glass plates. The 4% acrylamide stacking gel, prepared at the same time as the separating gel, contained 12.68 ml H_2O, 0.5 M Tris (pH 8.8), 0.02 ml of 10% SDS, and 2 ml of 40% acrylamide. After mixing the components, the stacking gel was placed in a vacuum for 20 minutes. 20 µl of 97% (electrophoresis grade) TEMED and 100 µl of 10% ammonium persulfate was added to the stacking gel, gently mixed, poured into the mini-PROTEAN® II cell, and lane combs were placed in between the two glass plates. The stacking gel was allowed to polymerize for 30 minutes. After polymerization, the combs were gently pulled out and electrode buffer (pH 8.3) containing 3 g/l Tris, 24 g/l glycine, and 3 g/l SDS was poured into the upper chamber. The lanes were washed out with the electrode buffer and then loaded with samples of SR protein in sample buffer. Electrode buffer was poured into the lower chamber and the power supply was connected. The running voltage was set at 200 V (constant), and electrophoresis was continued until the tracking dye ran off the gel. Gels were stained overnight in a solution containing 0.1% Coomassie Blue R-250, 40% methanol, and 10% acetic acid. After staining, the gels were destained for about 1 hour in a solution containing 50% H_2O, 40% methanol, and 10% acetic acid. For study 1, the bands corresponding to the molecular weight of SERCA and glycogen phosphorylase were scanned using MultiImage™ Light Cabinet from Alpha Innotech Corporation and analyzed using AlphaImager™ 2000 Documentation & Analysis System. For study 2, the bands corresponding to the molecular weight of SERCA, glycogen phosphorylase, glycogen debranching enzyme (AGL, 4-α-glucanotransferase amylo-1,6-glucosidase),

creatine kinase (CK) and pyruvate kinase (PK) were scanned using a Hewlett Packard scanjet 6200C and analyzed using NIH Image (version 1.63, from Scion Corporation). From these scanned images, the optical density of these bands was determined.

SERCA Content was determined using bovine serum albumin (BSA) standards on SDS-PAGE gels. Linear regression was used to determine SERCA content in each sample. This technique was verified using a method of calculating SERCA content described by Kandarian et al. (1994) and Wu and Lytton (1993). In this method, the percentage of SERCA in each sample was first determined by densitometry of Coomassie-stained gels. Samples (10 μg total protein) were loaded onto a large 4% stacking, 7.5% separating SDS-polyacrylamide gel (PROTEAN II Slab Cell, BioRad). They were run at 13mA until the tracking dye reached the separating gel, then the current was increased to 18mA. After Coomassie staining, a linear relationship was found between the amount of protein loaded (5-20μg) and the optical density (O.D.) associated with the SERCA band. The SERCA standards were calibrated by determining the proportion of the O.D. of an entire lane that was the O.D. of the band containing SERCA. This proportion was then used to determine the amount of SERCA loaded in each lane for a standard curve that was analyzed on the same gel as the samples. Both the use of BSA and SERCA standards, as described above, resulted in identical estimations of SERCA content in the purified SR.

Sarcoplasmic Reticulum Ca^{2+}-Uptake and Release Rates

SR Ca^{2+} handling was determined by measuring the rates of both Ca^{2+}-uptake and release at 37ºC. SR vesicles (50 μg) were placed in 1.5 ml of buffer containing 100 mM KCl, 20 mM HEPES, 1mM $MgCl_2$, 5 mM potassium phosphate (KH_2PO_4), 2 mM ATP and 250 μM antipyrylazo III (APIII) (pH. 7.0). For creatine kinase (CK) supported uptake experiments, 2 mM adenosine diphosphate (ADP) and 5 mM of creatine phosphate (CP) were included in the uptake buffer in place of the 2mM ATP. For pyruvate kinase (PK) supported uptake experiments, 2 mM ADP and 5 mM of phosphoenol-pyruvate (PEP) were included in the uptake buffer in place of the 2 mM ATP. Uptake was initiated by adding 1.2 μmol calcium chloride ($CaCl_2$) per mg SR protein and allowed to continue

until free [Ca^{2+}] in the cuvette declined to a plateau. Release was initiated by adding either 5 μM silver nitrate ($AgNO_3$) or 800 μM suramin from 100X stock solutions. A diode array spectrophotometer (Hewlett Packard 8453) was used to follow APIII absorbance at 790 nm and 710 nm. The absorbance difference was converted into [Ca^{2+}] using a standard curve (linear from 0 - 70 μM total [Ca^{2+}]).

Sarcoplasmic Reticulum Ca^{2+} ATPase Activity

Ca^{2+} ATPase activity was determined using the enzyme-linked assay described by Luckin et al. (1991). SR vesicles (50 μg) were placed in 1.5 ml of buffer (37ºC) containing 100 mM KCl, 25 mM HEPES, 10 mM $MgCl_2$, 1mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 0.2% NaN_3, 5 U/ml lactate dehydrogenase (LDH), 7.5 U/ml PK, 2μM $CaCl_2$, 0.6 mM β-nicotinamide adenine dinucleotide, reduced form (NADH), 3 mM PEP, and 2 μM ionophore A23187 (calcimysin) (pH 7.0). Basal, or Mg^{2+}-stimulated, activity was recorded for 2 minutes after the addition of 1mM ATP. Total activity was recorded for 3 minutes after adding 2 μM $CaCl_2$. Ca^{2+}-stimulated ATPase activity was determined by subtracting basal from total. A diode array spectrophotometer (Hewlett Packard 8453) was used to follow absorbance 340nm. The rate of absorbance change was converted into rate of NADH utilization using the extinction coefficient of NADH. Because rate of NADH utilization in this assay system is directly proportional to rate of ATP hydrolysis, SERCA activity can be easily calculated.

Fluorescein Isothiocyanate Binding

Fluorescein-5-isothiocyanate (FITC) maximum fluorescence emission was determined using a method modified from Lalonde et al. (1991). SR vesicles (50 μg) and 350 μl of wash buffer were added to Millipore Ultrafree-MC centrifugal filter units (30,000 normal molecular weight limit) that were pre-washed once with the wash buffer. The wash buffer contained 0.1% NaN_3, 5 mM HEPES, 0.1% Triton X-100, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 80 nM Aprotinin, 2.2 nM Leupeptin, 4 nM Bestatin, 1.5 nM Pepstatin A, and 1.4 nM E-64. The sample was

incubated at room temperature, with shaking, for 10 minutes and then centrifuged for 20 minutes at 25ºC (5,000 x g) in an Eppendorf 5417R centrifuge. The flow-through was discarded and 350µl of labeling buffer containing 0.1% NaN_3, 5 mM HEPES, 0.1% Triton X-100, 0.1 mM AEBSF, 80 nM Aprotinin, 2.2 nM Leupeptin, 4 nM Bestatin, 1.5 nM Pepstatin A, and 1.4 nM E-64 and 2.5µM FITC was added. Because FITC is light sensitive, all procedures involved in FITC preparation and labeling (including all steps following the addition of FITC) were done in the dark. The sample in the labeling buffer was incubated for 20 minutes, with shaking, at room temperature and then centrifuged for 20 minutes at 25ºC (5,000 x g). The flow-through, as well as the microcentrifuge tube, was discarded and 350µl of wash buffer was added to the spin cup and placed into a new microcentrifuge tube to wash away unbound FITC from the sample. The sample was gently vortexed and then centrifuged for 20 minutes at 25ºC (5,000 x g). The washing of unbound FITC step was repeated two additional times. 300 µl of wash buffer was added to the spin cup, vortexed gently, and 200 µl was removed and added to a 4.5-ml four-clear-sided polystyrene cuvette containing 1.3 ml of wash buffer. The cuvette was capped, gently inverted, and placed in a Perkin Elmer luminescence spectrometer LS50B and analyzed using FL WinLab software. The emission intensity of the sample was measured at 520nm with an excitation wavelength of 490 nm. The emission intensity of the sample was semi-quantitatively analyzed using FITC standards. The emission intensity of the sample was transformed into arbitrary units using linear regression of the emission intensity of free FITC standards in 1.5 ml of wash buffer (ranging from 0 to 45 nmol of free FITC).

Statistics

The effects of no-EDTA and α-amylase on SR function, glycogen concentration, protein content, and FITC binding were determined via two-way analyses of variance adjusted for repeated measures made on contra-lateral muscles taken from the same animal. Significance was set at $p \leq 0.05$.

Chapter Four: Results

Glycogen Extraction From Sarcoplasmic Reticulum

The 2 methods of glycogen extraction used in the present investigation resulted in significant decreases in measured sarcoplasmic reticulum (SR) glycogen concentration. Heavy SR (HSR, 8,000-12,000 x g) glycogen concentrations were 12.9% and 38.6% of controls in the amylase and No-EDTA groups, respectively ($p \leq 0.05$). Light SR (LSR, 12,000-49,000 x g) glycogen concentrations were 4.7% and 46.9% of control for amylase and No-EDTA groups, respectively ($p \leq 0.05$). For both HSR and LSR, glycogen concentrations were found to be higher in the No-EDTA treatment compared to amylase addition ($p \leq 0.05$) (Figure 4).

Sarcoplasmic Reticulum Protein Profile

It was hypothesized that SR glycogen extraction would result in decreased glycogen phosphorylase associated with the SR. Amylase treatment did cause a significant decrease in SR glycogen phosphorylase content. SR glycogen phosphorylase content was reduced to 37.2% and 12.2% of control in study 1 (HSR) and 2, respectively ($p \leq 0.05$). However, in the No-EDTA treatment, SR glycogen phosphorylase content was not reduced, despite a significant reduction in SR glycogen concentration (Figure 5). Representative lanes from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for each treatment highlight the discrepancy in SR glycogen phosphorylase content (Figure 6). Interestingly, glycogen debranching enzyme (AGL, 4-α-glucanotransferase amylo-1,6-glucosidase) content was also reduced in amylase-treated samples. The optical density of the band containing AGL was 54.3% of control in amylase-treated samples ($p \leq 0.05$) (Table 1).

Another interesting consequence of glycogen extraction, especially in amylase-treated samples, was the change in the SR protein profile. Increases in the content of certain proteins associated with SR Ca^{2+}-uptake accompanied the loss of glycogen phosphorylase and AGL. These increases were also observed in the No-EDTA samples where glycogen phosphorylase and AGL were not found to be different. Sarco(endo)plasmic reticulum ATPase (SERCA) content was increased by 17.4% and 19.7% in amylase-treated SR in

study 1 and 2, respectively ($p \leq 0.05$) (Figure 7). Also, creatine kinase (CK) content was increased by 41.7% and 28.4%, and pyruvate kinase (PK) content was increased by 96.1% and 17.9% in amylase-treated and No-EDTA samples, respectively ($p \leq 0.05$) (Table 1).

ATP-Supported Sarcoplasmic Reticulum Ca^{2+} Handling

Ca^{2+}-activated ATPase activity was calculated by subtracting basal (Mg^{2+}-activated) ATPase activity from total ATPase activity. Basal and Ca^{2+}-activated ATPase activities were increased in both amylase-treated and No-EDTA samples collected in study 1 ($p \leq 0.05$) (Figure 8). However, because SERCA content was increased in amylase-treated samples by 17.4%, Ca^{2+}-activated ATPase activities were normalized to SERCA content. After normalizing activities to SERCA content, only amylase-treated samples had increased basal and Ca^{2+}-activated ATPase activities ($p \leq 0.05$) (Figure 9). Also, normalizing ATPase activities to SERCA content nullified differences between No-EDTA and amylase-treated samples and control/No-EDTA and control/amylase samples.

Peak ATP-supported SR Ca^{2+}-uptake rates were increased by 28.0% and 25.2% in amylase-treated samples compared with control samples in study 1 and 2, respectively ($p \leq 0.05$). Peak ATP-supported SR Ca^{2+}-uptake rates were 29.7% higher in No-EDTA samples compared with control samples in study 1 ($p \leq 0.05$), however there was no significant difference in study 2 (Figure 10). Peak ATP-supported SR Ca^{2+}-uptake rates were normalized to SERCA content, because SERCA content was found to be higher in amylase-treated samples compared to control samples. Although not statistically significant, SERCA content was increased 9.0% and 5.4% in No-EDTA samples compared to control in study 1 and 2, respectively. Once normalized to SERCA content, only No-EDTA samples from study 1 exhibited higher peak Ca^{2+}-uptake rates than control samples ($p \leq 0.05$) (Figure 11).

Both silver nitrate- and suramin-induced SR Ca^{2+}-release rates were increased in glycogen-extracted samples compared to control samples. Amylase-treated samples

exhibited 15.6% and 44.8% higher release rates compared to control samples using silver nitrate and suramin, respectively ($p \leq 0.05$). No-EDTA samples exhibited 19.0% and 24.2% higher release rates compared to control samples using silver nitrate and suramin, respectively ($p \leq 0.05$). Also, amylase-treated samples exhibited a 16.1% higher rate compared to No-EDTA samples when under suramin-induced SR Ca^{2+}-release (Figure 12).

CP + ADP- and PEP + ADP-Supported Sarcoplasmic Reticulum Ca^{2+}-Uptake

Creatine phosphate (CP) plus adenosine diphosphate (ADP)-supported SR Ca^{2+}-uptake rates were increased under glycogen-extracted conditions. Peak Ca^{2+}-uptake rates were 23.6% and 18.9% higher than control samples in amylase-treated samples and No-EDTA samples, respectively ($p \leq 0.05$). Similarly, phosphoenol-pyruvate (PEP) and ADP-supported SR Ca^{2+}-uptake rates were also increased under glycogen-extracted conditions. Peak Ca^{2+}-uptake rates were 17.9% and 17.5% higher than control samples in amylase-treated samples and No-EDTA samples, respectively ($p \leq 0.05$) (Figure 13). However, as in ATP-supported SR Ca^{2+}-uptake, uptake rates were normalized to SERCA. After normalizing to SERCA, only No-EDTA samples were increased (16.3%) compared to control ($p \leq 0.05$). Also, No-EDTA samples and control/No-EDTA samples were increased 25.8% and 22.2% compared to amylase-treated and control/amylase samples, respectively ($p \leq 0.05$) (Figure 14).

As stated earlier, both CK and PK contents were increased under glycogen-extracted conditions (Table 1). Therefore, SR Ca^{2+}-uptake rates (normalized to SERCA) supported by endogenous CK and PK were again normalized to CK and PK contents, respectively. Amylase-treated samples were 26.3% and 50.2% lower than control samples for Ca^{2+}-uptake supported by endogenous CK and PK, respectively ($p \leq 0.05$). Although not significant, No-EDTA samples were 9.3% and 6.1% lower than control samples for Ca^{2+}-uptake supported by endogenous CK and PK, respectively. These data are interesting because these rates were significantly higher before normalizing to SERCA content and

to either CK or PK content. Also, No-EDTA samples were increased 77.0% and 167.8% compared to amylase-treated samples for Ca^{2+}-uptake supported by endogenous CK and PK, respectively ($p \leq 0.05$). In addition, control/No-EDTA samples were increased 44.0% and 42.1% compared to control/amylase samples for Ca^{2+}-uptake supported by endogenous CK and PK, respectively ($p \leq 0.05$) (Figure 15).

Sarcoplasmic Reticulum Bound Fluorescein Isothiocyanate

Fluorescein isothiocyanate (FITC) maximum fluorescence emission was not different between glycogen-extracted conditions and control samples. Even though decreases in fluorescence were not significant, FITC fluorescence emission was reduced by 26.8% and 9.7% in amylase-treated and No-EDTA samples compared with control samples when data were normalized to SERCA, respectively. Also, FITC fluorescence emissions in No-EDTA and control/No-EDTA samples were increased by 59.1% and 44.3% compared to amylase-treated and control/amylase samples when normalized to SR protein, respectively ($p \leq 0.05$). When data were normalized to SERCA content, FITC fluorescence emissions in No-EDTA and control/No-EDTA samples were increased by 159.3% and 110.2% compared to amylase-treated and control/amylase samples, respectively ($p \leq 0.05$) (Figure 16).

Summary

Figures 17 and 18 summarize the effects of normalizing Ca^{2+}-activated ATPase activity, ATP-supported SR Ca^{2+}-uptake, CP + ADP- and PEP + ADP-supported SR Ca^{2+}-uptake and FITC fluorescence emission intensity to protein content for amylase-treated samples and No-EDTA samples, respectively. These figures highlight the differences in analyses based on the criteria for normalizing the data. In many cases, amylase-treated samples and No-EDTA samples had significantly higher values when normalized to SR protein. However, when SERCA and either CK or PK contents were the basis of normalizing the data, SR Ca^{2+}-uptake rates for amylase-treated samples were significantly lower.

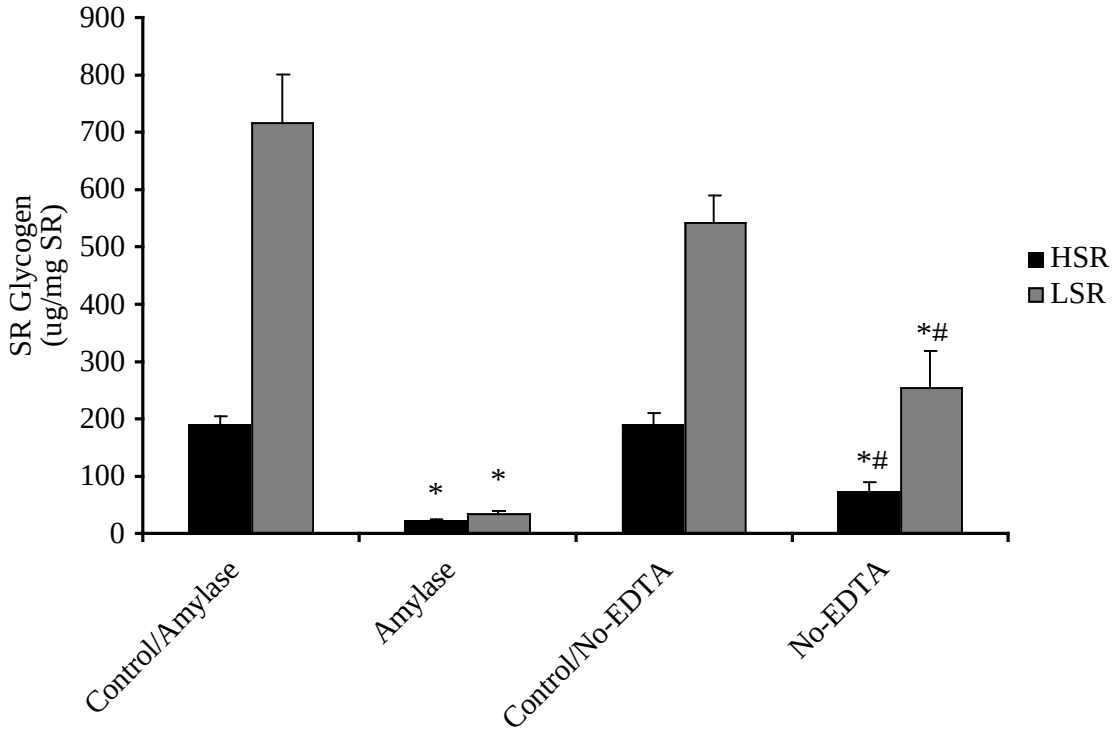

Figure 4. SR glycogen concentration
Sarcoplasmic reticulum (SR) glycogen concentrations for Control/Amylase; Amylase, α-amylase-treated samples; Control/No-EDTA; and No-EDTA, samples prepared without EDTA in the homogenizing/storage buffers. Data are expressed in μg of glycogen per mg of SR protein (error bars are SEM). Heavy SR (HSR) and light SR (LSR) fractions were purified separately in study 1. *, Significantly different from control. #, Significantly different from amylase-treated samples.

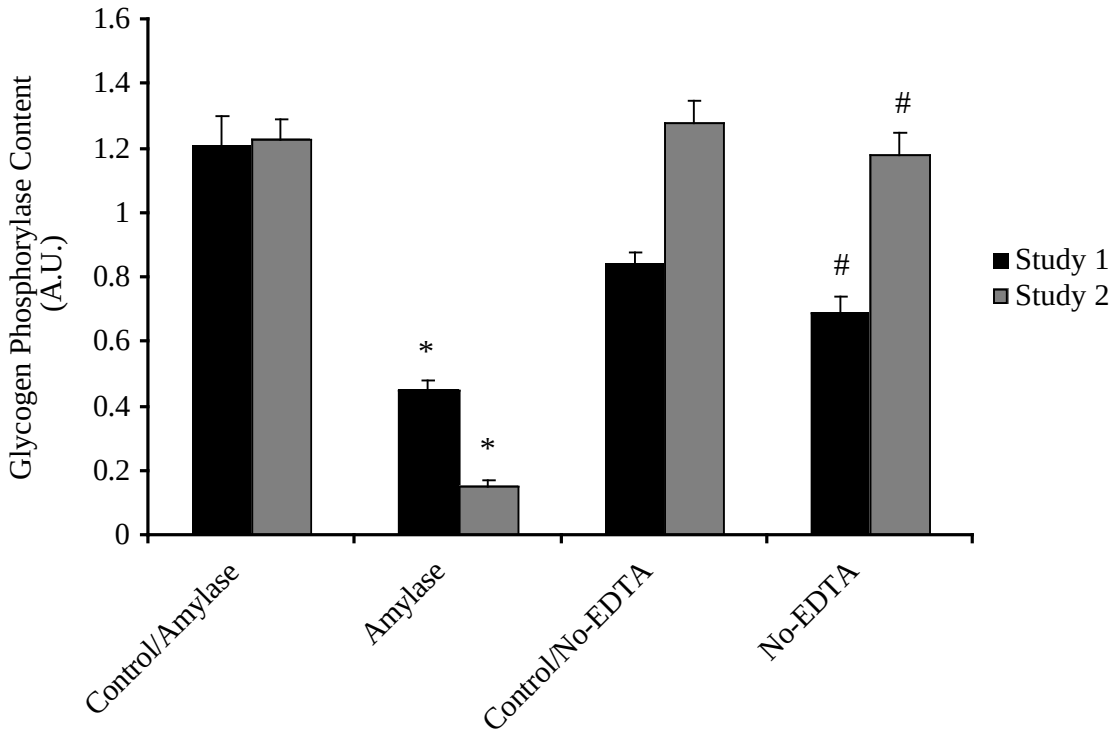

Figure 5. Glycogen phosphorylase associated with the SR
Glycogen phosphorylase content was measured in heavy sarcoplasmic reticulum for study 1 and total sarcoplasmic reticulum fraction for study 2. The optical densities of the bands containing glycogen phosphorylase were normalized to the optical density of a protein standard (error bars are SEM). *, Significantly different from control. #, Significantly different from amylase-treated samples.

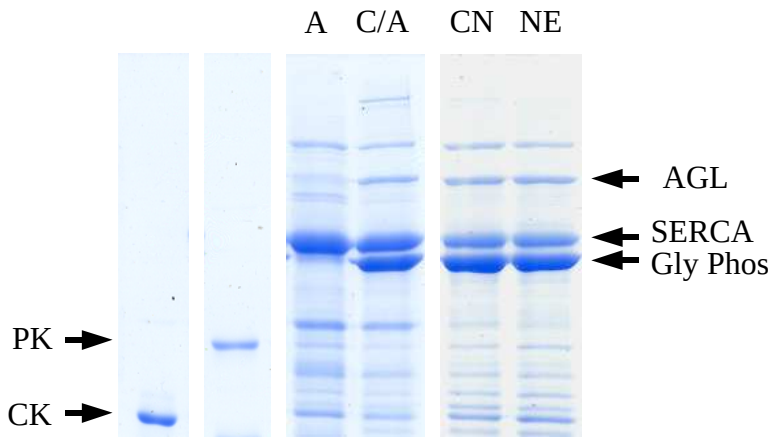

Figure 6. Representative lanes from SDS-PAGE
Representative lanes from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for each treatment. The bands containing pyruvate kinase (PK), creatine kinase (CK), glycogen debranching enzyme (AGL), sarco(endo)plasmic reticulum ATPase (SERCA) and glycogen phosphorylase (Gly Phos) are indicated with arrows. A: amylase-treated sample, C/A: control sample collected from the contra-lateral leg of the amylase sample, NE: sample without EDTA in the homogenizing and storage buffer, CN: control sample collected from the contra-lateral leg of the No-EDTA sample.

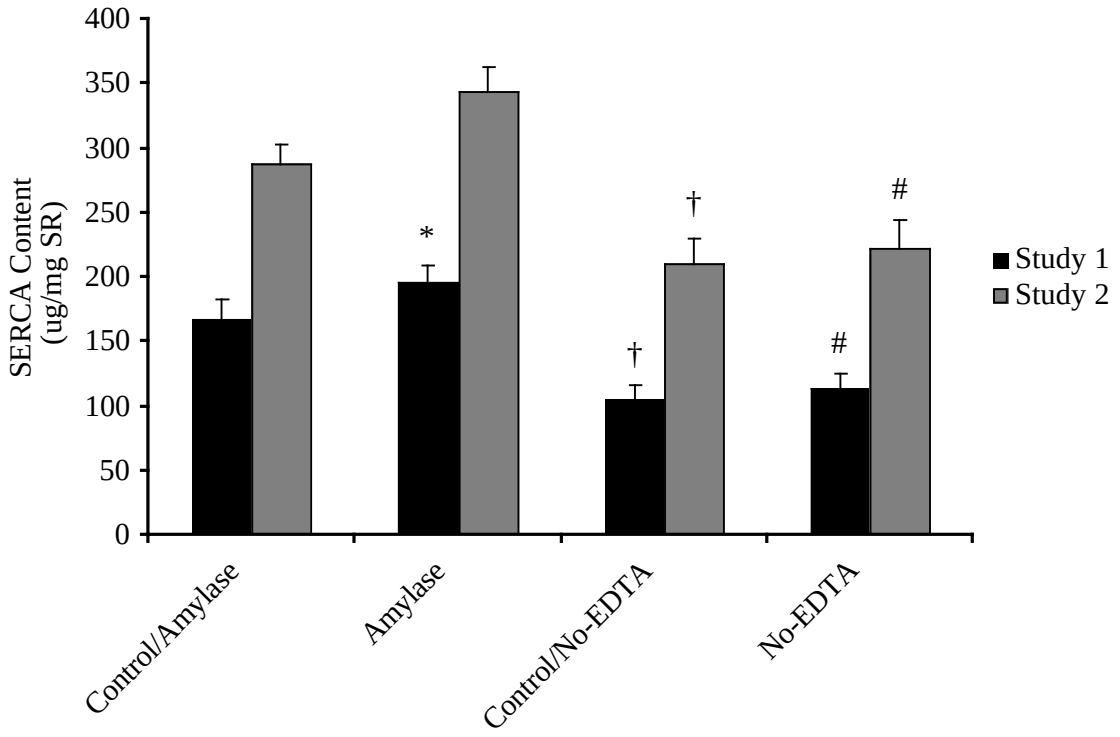

Figure 7. SERCA content
Sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) content data are expressed as μg of SERCA per mg of SR protein (error bars are SEM). Study 1: heavy sarcoplasmic reticulum (HSR) fraction was used. Study 2: total sarcoplasmic reticulum (SR) fraction was used. *, Significantly different from control. #, Significantly different from amylase. †, Significantly different from control/amylase.

	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
CK	32.36±1.91	45.73±2.85*	30.95±1.13	39.41±2.57*
PK	9.57±0.49	18.77±0.45*	8.08±0.43	9.51±0.29*
AGL	0.775±0.020	0.421±0.025*	0.799±0.048	0.794±0.040

Table 2. CK, PK and AGL content

Group mean data (±SEM) for creatine kinase (CK), pyruvate kinase (PK) and glycogen debranching enzyme (AGL) content. CK and PK content data are presented as ug/mg SR and AGL data are presented as arbitrary units (A.U.). *, Significantly different from control.

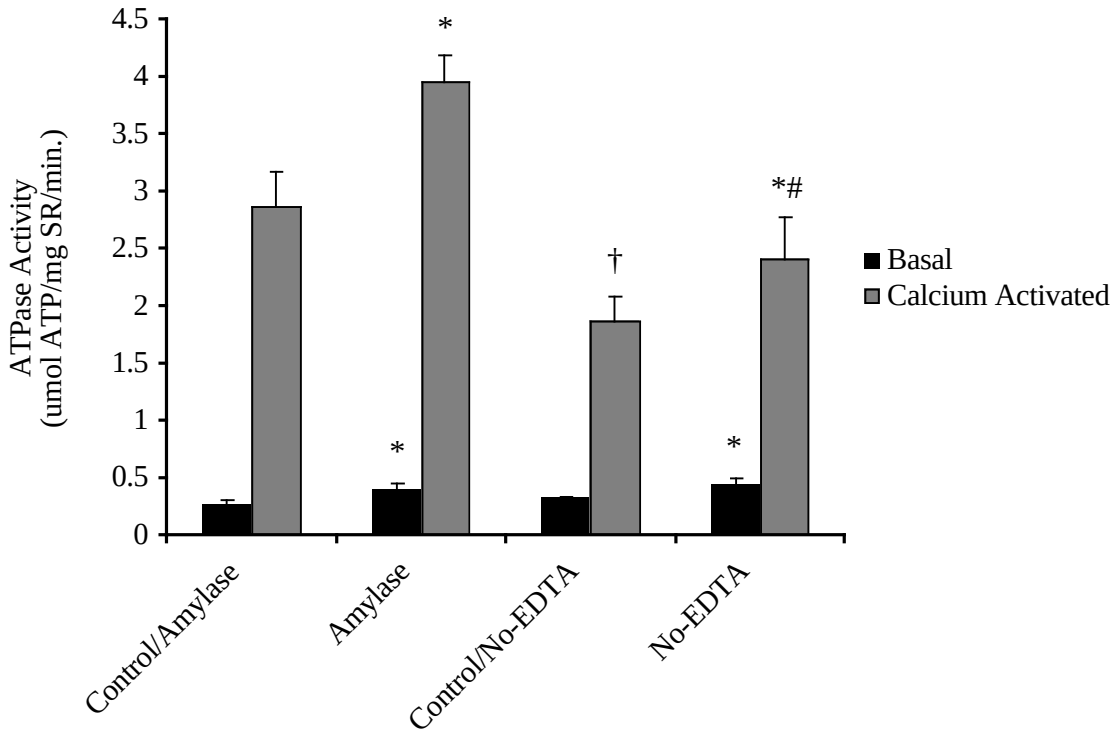

Figure 8. SR basal and Ca^{2+}-activated ATPase activities
Sarcoplasmic reticulum (SR) Ca^{2+}-activated ATPase activities were calculated by subtracting basal ATPase activity from total ATPase activity in an enzyme-linked system and expressed as μmol of ATP hydrolyzed/minute per mg SR protein (error bars are SEM). *, Significantly different from control. #, Significantly different from amylase. †, Significantly different from control/amylase.

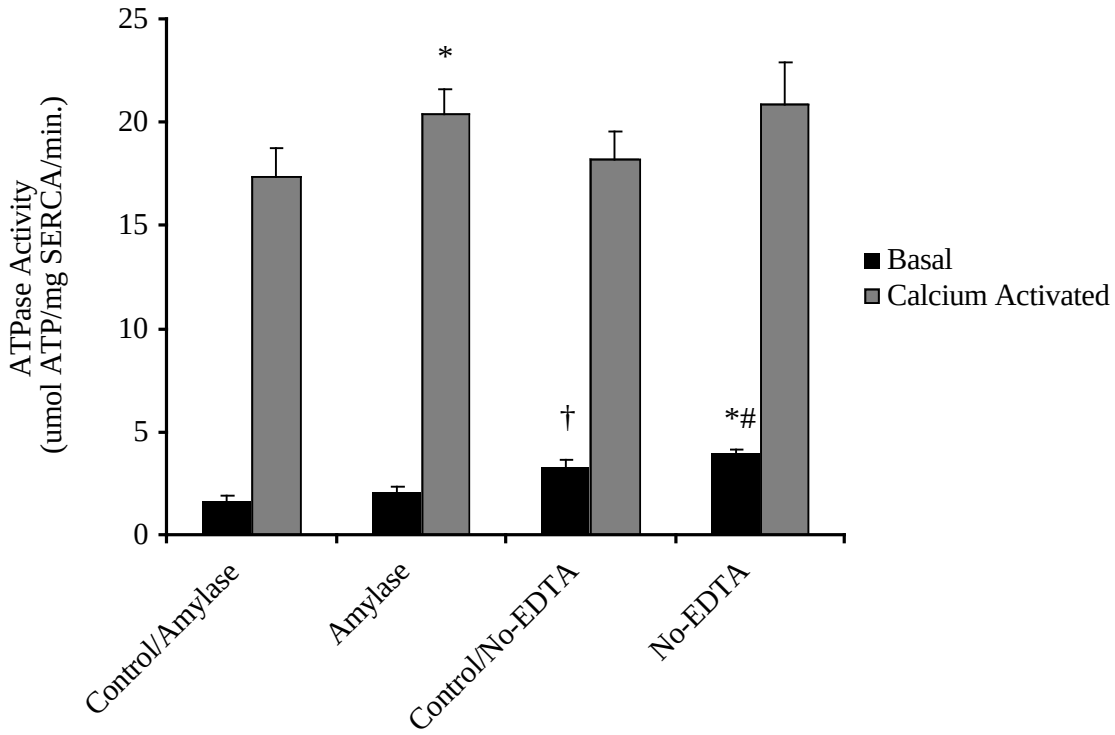

Figure 9. SR basal and Ca^{2+}-activated ATPase activities normalized to SERCA content

Sarcoplasmic reticulum (SR) Ca^{2+}-activated ATPase activities were calculated by subtracting basal ATPase activity from total ATPase activity in an enzyme-linked system, normalized to sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) content and expressed as μmol of ATP hydrolyzed/minute per mg SERCA (error bars are SEM). *, Significantly different from control. #, Significantly different from amylase. †, Significantly different from control/amylase.

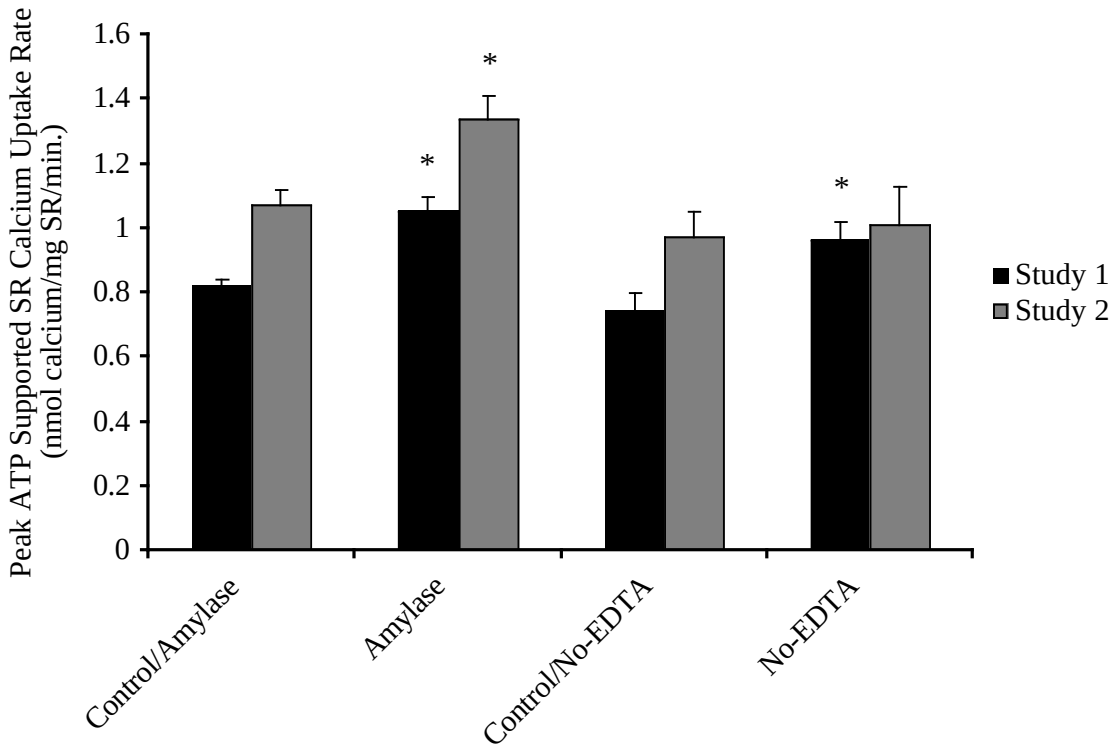

Figure 10. ATP-supported SR Ca^{2+}-uptake
Peak ATP-supported sarcoplasmic reticulum (SR) Ca^{2+}-uptake rates are expressed as nmol of Ca^{2+}/minute per mg of SR protein (error bars are SEM). Study 1: heavy sarcoplasmic reticulum (HSR) fraction was used. Study 2: total sarcoplasmic reticulum (SR) fraction was used. *, Significantly different from control.

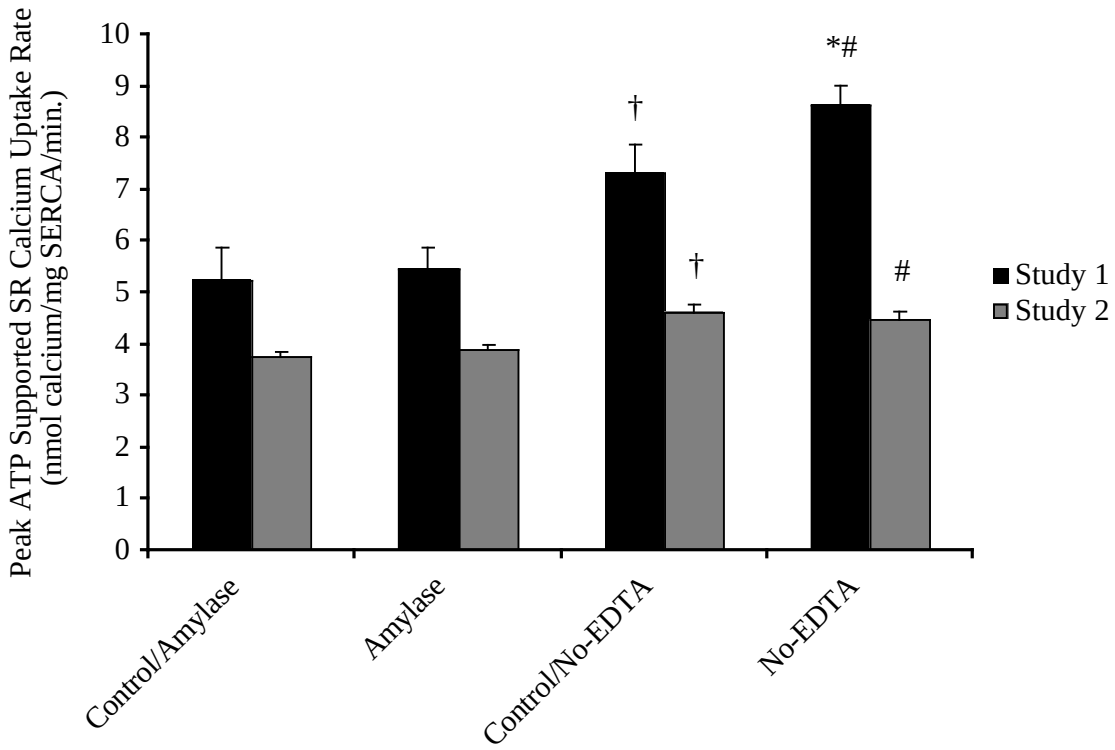

Figure 11. ATP-supported SR Ca^{2+}-uptake normalized to SERCA content
Peak ATP-supported sarcoplasmic reticulum (SR) Ca^{2+}-uptake rates normalized to sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) content are expressed as nmol of Ca^{2+}/minute per mg SERCA (error bars are SEM). Study 1: heavy sarcoplasmic reticulum (HSR) fraction was used. Study 2: total sarcoplasmic reticulum (SR) fraction was used. *, Significantly different from control. #, Significantly different from amylase. †, Significantly different from control/amylase.

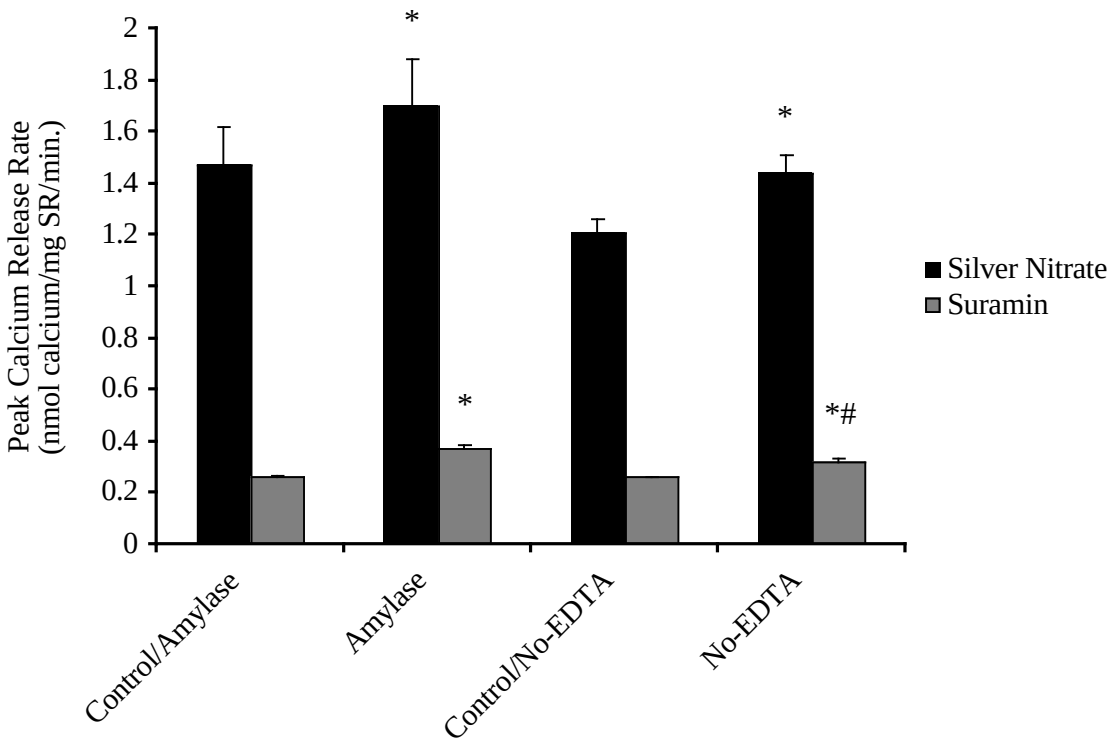

Figure 12. SR Ca^{2+}-release
Sarcoplasmic reticulum (SR) Ca^{2+}-release was induced using 2 different agents, silver nitrate and suramin. Peak SR Ca^{2+}-release rates are reported as nmol of Ca^{2+}/minute per mg of SR protein (error bars are SEM). *, Significantly different from control. #, Significantly different from amylase.

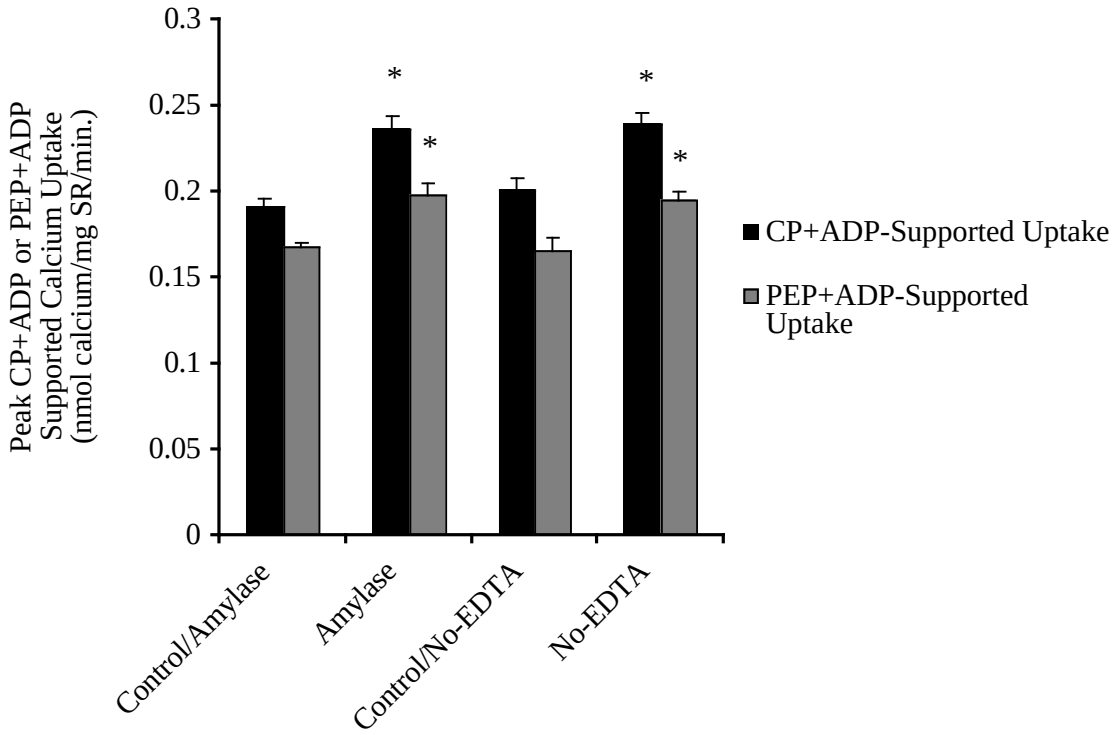

Figure 13. CK- and PK-supported SR Ca^{2+}-uptake
Creatine phosphate (CP) and adenosine diphosphate (ADP) or phosphoenol-pyruvate (PEP) and ADP-supported sarcoplasmic reticulum (SR) Ca^{2+}-uptake. Peak SR Ca^{2+}-uptake rates are reported as nmol of Ca^{2+}/minute per mg of SR protein (error bars are SEM). *, Significantly different from control.

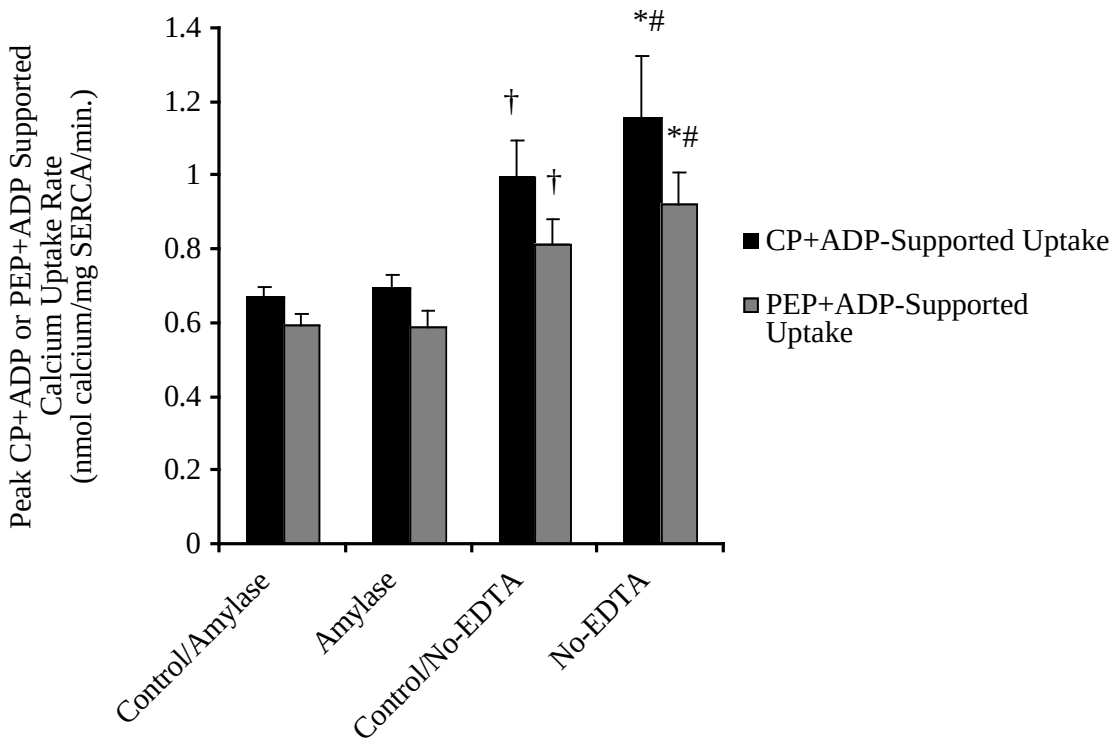

Figure 14. CK- and PK-supported SR Ca^{2+}-uptake normalized to SERCA
Creatine phosphate (CP) and adenosine diphosphate (ADP) or phosphoenol-pyruvate (PEP) and ADP-supported sarcoplasmic reticulum (SR) Ca^{2+}-uptake normalized to sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) content. Peak SR Ca^{2+}-uptake rates are reported as nmol of Ca^{2+}/minute per mg of SERCA (error bars are SEM). *, Significantly different from control. #, Significantly different from amylase. †, Significantly different from control/amylase.

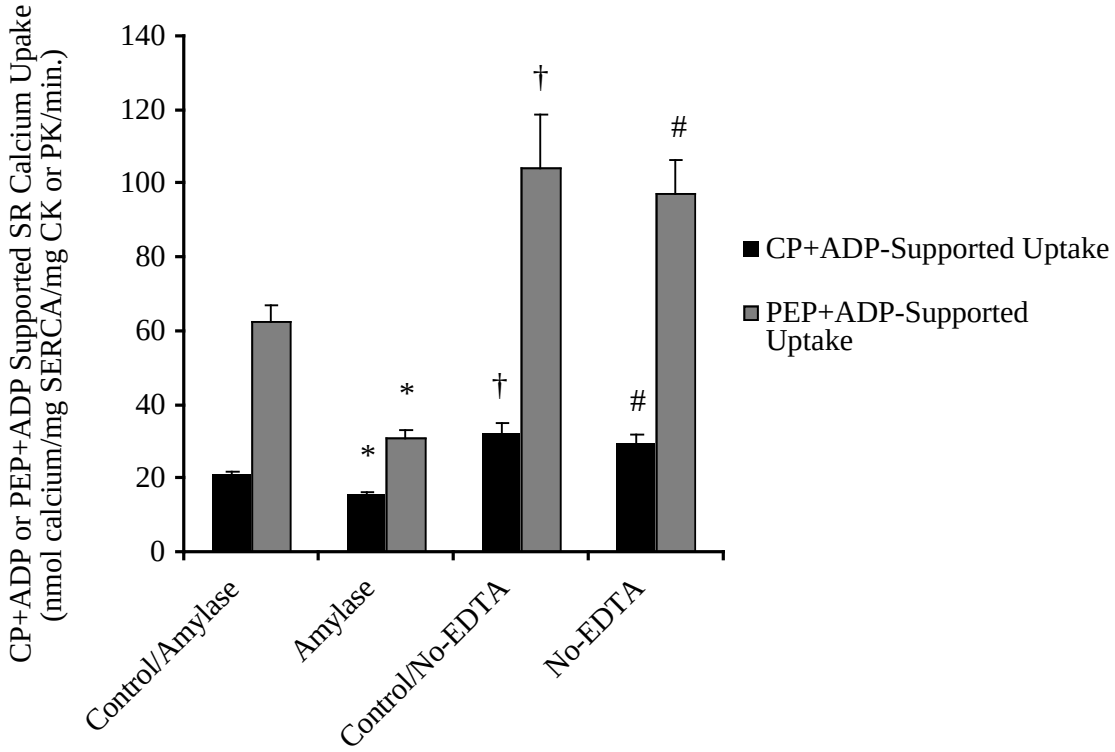

Figure 15. CK- and PK-supported SR Ca^{2+}-uptake normalized to SERCA and either CK or PK

Creatine phosphate (CP) and adenosine diphosphate (ADP) or phosphoenol-pyruvate (PEP) and ADP-supported sarcoplasmic reticulum (SR) Ca^{2+}-uptake normalized to sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) and either creatine kinase (CK) or pyruvate kinase (PK) content Peak SR Ca^{2+}-uptake rates are reported as nmol of Ca^{2+}/mg SERCA/mg CK or PK/min. (error bars are SEM). *, Significantly different from control. #, Significantly different from amylase. †, Significantly different from control/amylase.

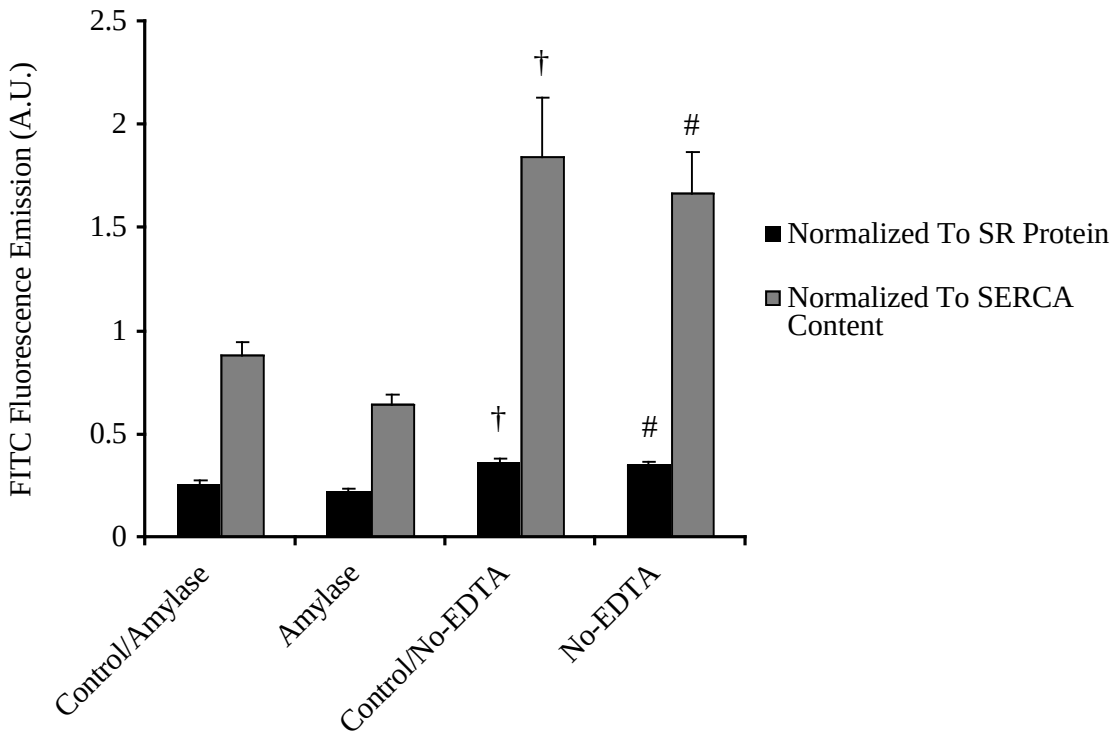

Figure 16. SR-Bound FITC
Fluorescein isothiocyanate (FITC) fluorescence emissions were normalized to SR protein and sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) content and expressed as arbitrary units (A.U.) (error bars are SEM). #, Significantly different from amylase. †, Significantly different from control/amylase.

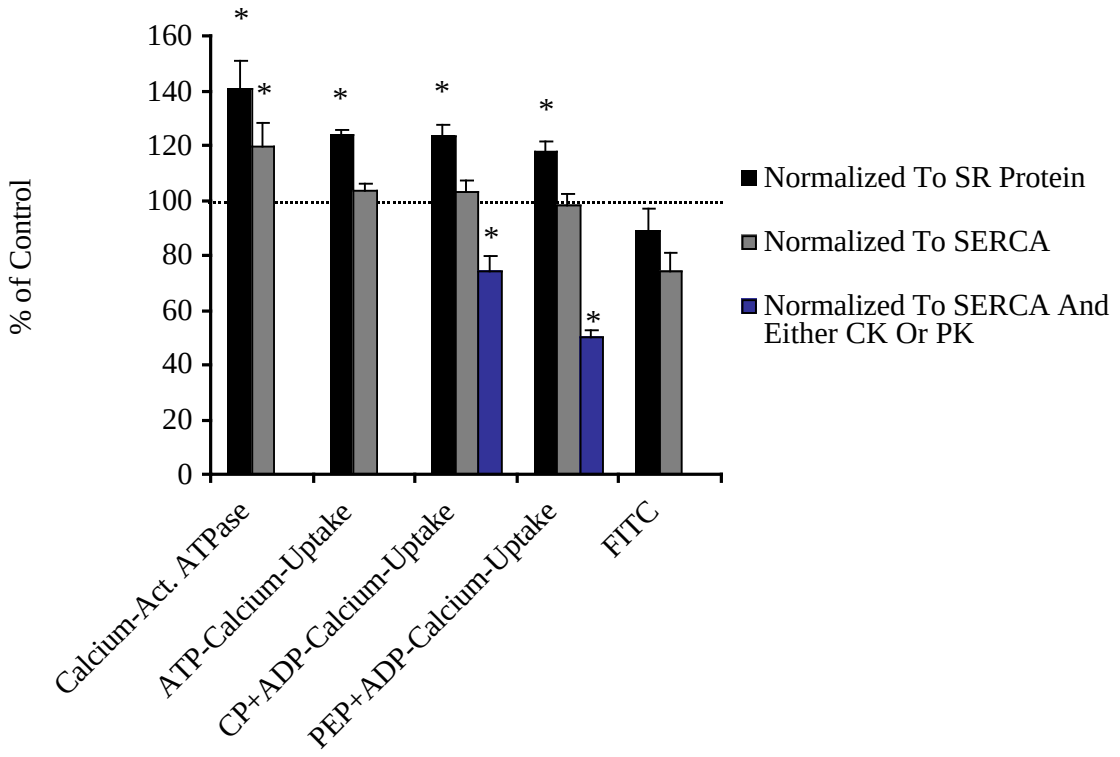

Figure 17. Summary of normalized measurements for amylase-treated samples
Summary of the measurements subjected to multiple analyses on the basis of protein content. All mean values are presented as a percentage of the mean control measurement under the same normalizing condition (error bars are in SEM). *, Significantly different from control.

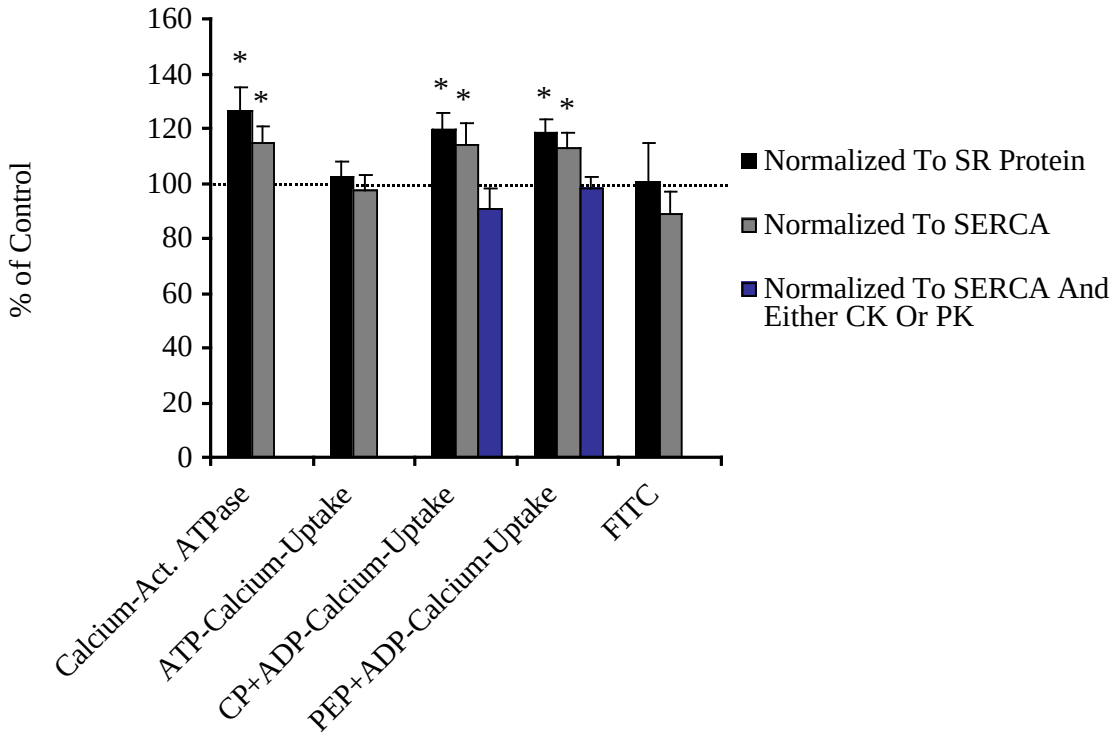

Figure 18. Summary of normalized measurements for No-EDTA samples
Summary of the measurements subjected to multiple analyses on the basis of protein content. All mean values are presented as a percentage of the mean control measurement under the same normalizing condition (error bars are in SEM). *, Significantly different from control.

Chapter Five: Discussion

Introduction

In this investigation, purified sarcoplasmic reticulum (SR) was subjected to two different biochemical glycogen-extraction protocols. The results indicate that both amylase treatment and removal of EDTA from the homogenization and storage buffers lowered the amount of glycogen associated with the SR. When the data were normalized to SR protein content, neither of these treatments impaired SR calcium (Ca^{2+}) handling under conditions where either exogenous ATP was added or endogenous ATP was synthesized and utilized for SR Ca^{2+} transport. In fact, these treatments seemed to cause a small increase in both SR Ca^{2+}-uptake and release rates under these assay conditions. As expected, glycogen phosphorylase content was decreased as a result of amylase-based glycogen extraction, however, a similar loss did not occur with the No-EDTA samples. Interestingly, the content of many other proteins differed due to glycogen extraction as well. Both treatments resulted in a greater recovery of the sarco(endo)plasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA) and a substantial loss both of glycogen phosphorylase and glycogen debranching enzyme (AGL) in amylase-treated samples. Creatine kinase (CK) and pyruvate kinase (PK) contents were increased as a result of both glycogen-extraction conditions. It was imperative to consider these altered protein contents while analyzing the data and assessing the effects of glycogen extraction on SR Ca^{2+} handling. After normalizing to SERCA and either PK or CK content, amylase-based glycogen extraction caused a lower SR Ca^{2+}-uptake rate under endogenously synthesized ATP conditions.

Glycogen, Glycogen Phosphorylase And Glycogen Debranching Enzyme Content

We previously found that fatiguing muscular activity results in a large decrease in both glycogen and glycogen phosphorylase content associated with the SR (Lees et al. 2001). The glycogen concentrations of SR vesicles prepared from stimulated muscle were found to be only 5% of control. Both No-EDTA and amylase treatments in the present investigation reduced HSR glycogen concentration to 38.6% and 12.9% and LSR glycogen concentration to 4.7% and 46.9% of control, respectively. In 1994, Cuenda et

al. compared glycogen content of SR membranes purified from animals that were starved for 48 hours to those that were fed. Although they reported an approximate 2-fold decrease in SR glycogen content (data were not provided), their control values were dramatically lower than those reported by other investigators (Entman et al. 1976; Lees et al. 2000, 2001). Cuenda et al. (1994) reported control SR glycogen content to be 32 μg of glycogen per mg of SR membrane protein, whereas Entman et al. (1976) reported a range of 300 to 700 μg of glycogen per mg of SR membrane protein isolated from dog cardiac muscle. However, Cuenda et al. (1994) purified SR from rabbit skeletal muscle and Entman et al. (1976) purified SR from dog cardiac muscle. The glycogen concentration of the control groups in the present investigation were 192 and 190 μg glycogen per mg HSR protein and 718 and 544 μg glycogen per mg LSR protein in the control/amylase and control/No-EDTA groups, respectively. Differences between those reported by Cuenda et al. (1994), those reported by Lees et al. (2000 and 2001) and the present investigation may be explained in part by a more sensitive assay (Lees et al. 2000) and the presence of sucrose in the SR preparation. The SR preparation used in Cuenda et al. (1994) had sucrose in the storage buffer, which interferes with the glycogen assay (Lees et al. 2000).

It is important to note that the magnitude of glycogen extraction was not directly proportional to the reduction of glycogen phosphorylase content. This point is emphasized when glycogen concentration is plotted against glycogen phosphorylase content. When the data from the present experiment are pooled with other data collected from our lab, there is a pronounced logarithmic relationship between SR glycogen concentration and glycogen phosphorylase content (Figure 19). Glycogen phosphorylase was significantly reduced in amylase-treated samples, but unchanged in No-EDTA samples, compared to control samples. AGL content was also reduced in amylase-treated samples, but unchanged in No-EDTA samples. These results suggest that, similarly to glycogen phosphorylase, AGL is associated with the skeletal muscle SR and is released under glycogen-depleting conditions (Appendix C).

An interesting outcome of glycogen extraction was an increase in SERCA, CK and PK contents in amylase-treated samples, compared to control. Also, CK and PK contents were higher in No-EDTA samples, compared to control. These changes were not anticipated and no physiological mechanisms are proposed. However, because glycogen phosphorylase makes up a large proportion of the total SR protein in control samples, all remaining proteins must make up a larger proportion of the total after glycogen phosphorylase is lost due to glycogen extraction (Figure 6). Certainly, it was important to account for these differences when analyzing SR Ca^{2+} handling data.

Protein Content

Determination of protein content became an integral aspect of the present investigation. This was especially true for the quantitative analysis of CK, PK and SERCA. When the CK, PK and SERCA contents were taken into account, the amylase-based glycogen extraction had a completely opposite effect on SR Ca^{2+}-uptake rate (CP + ADP- and PEP + ADP-supported) than when only total protein content was taken into account. However, it is important to understand the limitations of the method used when interpreting the results. Coomassie brilliant blue R-250 dye was used to stain the proteins after separation on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The binding mechanism, though not fully understood, involves electrostatic interactions between the sulfonic acid groups ($-SO_3^-$) of the dye and the positively-charged basic residues of the protein. A schematic representation of Coomassie brilliant blue R-250 is depicted in Figure 20. The ratio of binding between the dye and basic residues has been reported to be 1.5-3 dye molecules to every positive charge. The range reported is believed to be the result of hydrophobic binding on either side of an electrostatically bound dye molecule (Fazekas de St. Groth et al. 1963; Tal et al 1985). Bovine serum albumin (BSA) was chosen as the standard for CK, PK and SERCA quantification because it was also the standard used for the original protein estimation. The original protein estimation utilized Coomassie G-250, which is subject to the same limitations as R-250. A histogram of amino acid residues likely involved in binding of Coomassie brilliant blue R-250 for CK, PK and SERCA and BSA is shown in Figure 21. However, it is important to note that the amount of dye that binds to a protein will not be

directly proportional to the frequency of occurrence of the basic and hydrophobic amino acid residues. As for AGL and glycogen phosphorylase, semi-quantitative analyses were used, whereby the relative differences in optical density between treatments paired from the same animal were of importance. For these analyses, a linear binding relationship and the detection of optical density are of concern. Coomassie brilliant blue R-250 has a large range over which the binding of dye to protein content has a linear relationship (Tal et al. 1985). Given that BSA was already the protein standard used for SR protein quantification and the highly reproducible nature of protein staining with Coomassie brilliant blue R-250, the methods employed to measure protein content in the present investigation are justified (Fazekas de St. Groth et al. 1963).

Sarcoplasmic Reticulum Ca^{2+}-Release

Both Chin et al. (1997) and Stephenson et al. (1999) reported a decline in SR Ca^{2+}-release as a result of glycogen extraction. In 1997, Chin et al. used a muscle stimulation protocol to reduce cellular glycogen and a 60 minute recovery, without glucose, to maintain glycogen depletion. Those investigators reported a sustained depression in measured intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) during muscle contraction, when compared to fibers that were allowed to recover in the presence of glucose. These data indicate that cellular glycogen may be important for optimal SR Ca^{2+}-release. In a similar study, Stephenson et al. (1999) reduced glycogen stores from single skinned muscle fibers (using a peeled sarcolemma preparation which leaves the T-tubular system intact). These investigators reported a decline in T-system activation induced SR Ca^{2+}-release (a measure of excitation-contraction coupling).

In the present investigation, an increase in both silver nitrate- and suramin-induced SR Ca^{2+}-release from purified SR was found for both glycogen-extracted conditions. The mechanism of silver nitrate-induced SR Ca^{2+}-release involves the oxidation of sulfhydryl groups on the ryanodine receptor (RyR, Ca^{2+}-release channel) (Salama and Abramson 1984). It has been proposed that the formation of disulfide bonds between subunits of the RyR is a physiological mechanism of SR Ca^{2+}-release and that silver ions may accomplish this through the same interactions with RyR as Ca^{2+} does *in vivo* (Moutin et

al. 1989). Suramin has been shown to increase both the open probability and the conductance (the slope of the current-voltage relationship) of the RyR (Sitsapesan and Williams 1996). The mechanism of suramin-induced SR Ca^{2+}-release involves the calmodulin-binding site of the RyR. Calmodulin regulates SR Ca^{2+}-release in a biphasic manner. At low [Ca^{2+}], calmodulin is an activator of RyR whereas, at high [Ca^{2+}], calmodulin is an inhibitor of RyR. In 2002, Papineni et al. determined that suramin binds to the RyR at the calmodulin-binding site in a competitive manner. In addition, these investigators reported that suramin (at concentrations greater than 100 μM) may also displace FKBP12 (Ca^{2+}-release regulatory protein) from RyR, which may lead to increased channel activity. Studying SR Ca^{2+}-release *in vitro* is particularly difficult because the exact mechanism by which the cell mediates SR Ca^{2+}-release *in vivo* is unknown. Therefore, it is impossible to determine an optimal method that mimics the physiological mechanism. It is possible that glycogen-extracted samples had increased ryanodine receptor (RyR) content, as was the case for CK and PK. Perhaps the increased SR Ca^{2+}-release rates observed were a result of differences in the relative content of one or several other protein(s) involved in SR Ca^{2+}-release.

Another potential regulator of SR Ca^{2+}-release is the mastoparan-binding protein found to be associated with the SR (Hirata et al. 2003). In 2003, Hirata et al. (2003) investigated a mastoparan-binding protein of about 97 kDa found in heavy sarcoplasmic reticulum (HSR). Mastoparan is a wasp venom peptide that is found to cause sarcoplasmic reticulum Ca^{2+}-release (Hirai et al. 1979; Nakahata et al. 1990; Longland et al. 1998). They determined that this protein was glycogen phosphorylase through amino acid sequencing and Western blotting. This group determined that glycogen phosphorylase (active a form) inhibits both mastoparan-induced and caffeine-induced Ca^{2+}-release. Also, HSR incubated with mastoparan resulted in glycogen phosphorylase release in a concentration-dependent manner. They concluded that mastoparan induces SR Ca^{2+}-release by binding and releasing glycogen phosphorylase from the SR, thereby removing its inhibition on the RyR. Implications for the present investigation involve increased SR Ca^{2+}-release under both glycogen-extraction conditions. Although glycogen phosphorylase is reported to be associated with the SR predominantly in its b (inactive)

form (Cuenda et al. 1995), certainly some would be associated in its a (active) form. Therefore, these findings support the notion that glycogen phosphorylase release, as a result of glycogen extraction, may ameliorate SR Ca^{2+}-release rates. These data also support the notion of metabolic signals being involved in modulation of SR Ca^{2+} handling.

Sarcoplasmic Reticulum Ca^{2+}-Uptake

The most interesting finding in the present investigation is the decreased SR Ca^{2+}-uptake supported by CK and PK (normalized to protein content) as a result of glycogen extraction. There is evidence that the phospho-creatine circuit (Figure 22) in skeletal muscle is subject to regulatory modulation in response to energy demand. In 2003, Zoll et al. investigated the effects of running training in rats on skeletal muscle mitochondrial function. These investigators reported dramatic increases in CK efficacy (131% increase in superficial plantaris fibers and 75% increase in deep plantaris fibers). CK efficacy was defined as the ratio between the apparent K_m for ADP in the absence of creatine and in the presence of creatine. They concluded that this increase in CK efficacy allowed the trained animals to have better control of mitochondrial respiration by creatine, suggesting an improved linkage between energy utilization and production. Interestingly, skeletal muscle has a higher glycogen storage capacity as a result of training (see Saltin et al. 1977 for review). Based on these data, one might speculate that the phospho-creatine circuit might be sensitive to energy supply. Also, Korge and Campbell (1992) reported a greater reduction in CK-supported SR Ca^{2+}-uptake rates measured in SR purified from fatigued muscles, compared to those supported by ATP. These data suggest that impaired CK-supported SR Ca^{2+}-uptake is not solely explained by depressed SERCA function. Therefore, CK function may be under regulatory control and dictating SR Ca^{2+}-uptake *in vivo*. Although there are no data to indicate any mechanism(s) of regulation, it is interesting to speculate that energy supply, even glycogen status, may be involved. It is important to note that Korge and Campbell (1992) did not report either CK or SERCA content.

In 2002, Batts used a glycogen-depletion model to assess the effects of reduced muscle glycogen on SR Ca^{2+} handling and skeletal muscle function. Rats were fasted for 24 hours, run for 90 minutes on a treadmill, and then separated into treatment groups. High glycogen (HG) group rats were allowed standard rodent chow and a 5% sucrose solution (ad libitum) and low glycogen (LG) group rats were only allowed access to water. This model reduced whole muscle glycogen by 42% and glycogen associated with the SR by about 90% in the LG group compared to the HG group. The reduction in SR glycogen was also associated with an increase of SERCA content as a proportion of total SR protein. Interestingly, this investigator reported increased relaxation rates, a measure of SR Ca^{2+}-uptake rates, from *in situ* muscle function measurements. Also, these changes in muscle function were not accompanied by a decrease in the SR Ca^{2+}-uptake rates or Ca^{2+}-stimulated ATPase activities measured in purified SR. It is important to note that these purified SR measurements were made using exogenous ATP in the assays, whereas the *in situ* muscle function measurements are dependent on endogenous ATP synthesis.

The concept of molecular crowding should be considered when interpreting the endogenously supported SR Ca^{2+}-uptake data. Molecular crowding is a term that describes the effect of high solute concentrations on chemical reactions. Although modeling the effects of all the interactions between all the molecules present in a particular system may be impossible, volume exclusion interactions will always be present and important to consider. Volume exclusion, or molecular crowding, may act to destabilize either the reactants or products of a reaction, subsequently altering the equilibrium constants (for review see Minton 1997). It is thought that the study of enzyme systems in dilute *in vitro* conditions does not mimic the crowded cellular environment. Molecular crowding influences muscle pyruvate kinase. In 2002, Lonhienne and Winzor reported that inhibition of pyruvate kinase by phenylalanine was nullified by the addition of 0.1 M proline. The addition of proline, as a molecular crowding agent, favors the more compact, active isomeric state of pyruvate kinase. The influence of molecular crowding on isomeric state is highlighted in sedimentation studies. The presence of phenylalanine normally decreases the sedimentation coefficient of pyruvate kinase, indicating an increase in the expanded isomeric structure. In 1988,

Harris and Winzor reported the addition of 0.1 M sucrose nullified the effects of phenylalanine (up to 0.5 mM) on sedimentation coefficient.

Several researchers have investigated the effects of sugars as molecular crowding agents (Nichol et al. 1983; Harris and Winzor 1988; Bergman et al. 1989; Morar et al. 2001). In 1983, Nichol et al. investigated the effects of Dextran T70 (a polysaccharide) on the catalytic rate of reduction of pyruvate by lactate dehydrogenase. The presence of Dextran T70 increased the initial velocity (effectively maximal due to the assay condition of high concentrations of substrate) of this reaction. Although the amount of solute (~100 mM) used in the study of molecular crowding is much higher than the endogenous glycogen (~1.5 mM) present with control SR samples in the Ca^{2+}-uptake assay system, it seems plausible to consider this concept. For instance, the glycogen associated with the SR may influence molecular crowding because it is localized at the enzyme system and not freely diffusing in the assay medium. The volume of purified SR added to the assay medium is usually on the order of about 1% of the total volume. Therefore, the presence of ~1.5 mM glycogen localized at the SR may have a similar influence as ~100 mM freely diffusing macromolecular cosolute. This effect may be particularly important for the metabolic channeling involved in CP + ADP- and PEP + ADP-supported Ca^{2+}-uptake.

Sarcoplasmic Reticulum Bound Fluorescein Isothiocyanate

Fluorescein isothiocyanate (FITC) exhibits increased fluorescence intensity when bound to the ATP-binding pocket of SERCA in the E2 conformation (SERCA conformations are described in Chapter Two-Sarcoplasmic Reticulum Bound Fluorescein Isothiocyanate) (Pick and Karlish 1980; Pick 1981). There is evidence that glycogen phosphorylase has an effect on SERCA conformation. In 1991, Cuenda et al. found the addition of exogenous glycogen phosphorylase (100-150 μg/ml or ~1-1.5 μM) to purified SR (devoid of endogenous glycogen phosphorylase) resulted in an increase in FITC fluorescence emission. These data suggest that glycogen phosphorylase causes a shift in the E2/E1 equilibrium to the E2 conformation of SERCA. Also, Luckin et al. (1991) reported a 40% reduction in maximum FITC fluorescence emission along with a 40%

decrease in Ca^{2+}-activated ATPase activity in SR samples purified from fatigued muscle, compared to control. These data indicate that lowered SR Ca^{2+}-uptake rates normally found in fatigue may be related to a shift in the E2/E1 equilibrium to the E2 conformation of SERCA. In the present investigation, only amylase-treated samples displayed decreased glycogen phosphorylase content. Although not significant, FITC fluorescence emission normalized to SERCA content was reduced by 26.8% in amylase-treated samples, compared to control samples. Fittingly, FITC fluorescence emission normalized to SERCA content was only reduced by 9.7% in No-EDTA samples, compared to control samples (also, not significant). If in fact reduced FITC fluorescence emission in the amylase-treated samples was physiologically significant, even though not statistically significant, then why didn't ATP-supported measures of SERCA function reflect this? One possible explanation involves the physiological relevance of exogenously added ATP used to measure these processes. When excess amounts of energy substrate are added to measure peak enzyme activity and Ca^{2+} transport, changes in normal cellular mechanisms involved in Ca^{2+} transport (e.g., metabolic channeling and compartmentation) are not accounted for. For instance, processes involved in localized ATP synthesis in cellular compartments, as well as the channeling characteristics between enzymes are ignored when excess exogenous ATP is added to an assay.

Control/No-EDTA and No-EDTA samples exhibited higher FITC fluorescence emission than control/amylase and amylase samples, respectively. Two possible explanations for the discrepancy between FITC fluorescence emission between the control groups are: (i) variability between animals (both control treatments were not repeated in the same animal); and (ii) SERCA quantification, in that control/amylase and control/No-EDTA samples were not run on the same gels. Although these data (not statistically significant) do support the hypothesis that glycogen extraction alters the SERCA conformation, perhaps further investigation is warranted due to the trends observed.

Summary

In terms of the original specific aims, the findings are as follows:

1) Both amylase and No-EDTA treatments significantly reduced glycogen associated with the SR.

2) Glycogen phosphorylase content was significantly reduced in amylase-treated samples compared to control/amylase. Glycogen phosphorylase content was not significantly different in No-EDTA samples compared to control/No-EDTA.

3) Peak Ca^{2+}-activated ATPase activities were not significantly lower as a result of either amylase or No-EDTA treatment compared to control. In fact, after normalizing the data to SERCA content, Ca^{2+}-activated ATPase activities for amylase treated samples were significantly higher compared to control/amylase.

4) Peak ATP-supported SR Ca^{2+} uptake rates were not significantly different as a result of either amylase or No-EDTA treatment compared to control.

5) Peak CP plus ADP-supported SR Ca^{2+} uptake rates were significantly reduced in amylase-treated samples compared to control/amylase samples after the data were normalized to both SERCA and CK content. Peak CP plus ADP-supported SR Ca^{2+} uptake rates were not significantly different in No-EDTA-treated samples compared to control/No-EDTA samples after the data were normalized to both SERCA and CK content.

6) Peak PEP plus ADP-supported SR Ca^{2+} uptake rates were significantly reduced in amylase-treated samples compared to control/amylase samples after the data were normalized to both SERCA and PK content. Peak PEP plus ADP-supported SR Ca^{2+} uptake rates were not significantly different in No-EDTA-treated samples compared to control/No-EDTA samples after the data were normalized to both SERCA and PK content.

7) Peak SR Ca^{2+} release rates were not significantly different as a result of either amylase or No-EDTA treatment compared to control.

8) SR bound FITC fluorescence emission intensities were not found to be significantly different as a result of either amylase or No-EDTA treatment compared to control.

Conclusions

The results from the present investigation support the hypothesis that glycogen extraction has a detrimental effect on SR Ca^{2+}-uptake when supported by endogenously synthesized ATP and normalized to SERCA and to either PK or CK content. Samples treated with either amylase or No-EDTA resulted in two distinct degrees of glycogen extraction. The magnitude of glycogen extraction seemed to be reflected in the changes in CK- and PK-supported SR Ca^{2+}-uptake, and the loss of both glycogen phosphorylase and AGL. In these measurements, No-EDTA-based glycogen extraction did not result in any change, whereas amylase-based glycogen extraction caused significant decreases.

It is entirely possible that the SR relies heavily on glycolysis-supported ATP synthesis and the phospho-creatine circuit *in vivo*. Therefore, metabolic channeling experiments, like CP + ADP- and PEP + ADP-supported SR Ca^{2+}-uptake, may be more physiologically important than the measurement of Ca^{2+} transport supported by exogenous ATP. Altered Ca^{2+}-transport properties of the SR were only detected in the metabolic channeling systems where the SERCA utilizes endogenous ATP. Changes in endogenously supported SR Ca^{2+}-uptake due to glycogen extraction potentially affected the source of ATP synthesis (either PK or CK), the effectiveness of energy utilization for Ca^{2+}-transport (SERCA), or altered the metabolic channeling properties. This experiment highlights the importance of normalizing measurements to the enzymes of interest instead of total protein. Moreover, the measurement of physiologically important processes is vital to the understanding of altered physiological function found in fatigue.

Future Directions

Although the extent to which local ATP synthesis is needed for SR Ca^{2+}-uptake is unknown, certainly its presence alone signifies some importance. The present investigation reveals a possible link between SR glycogen and SR Ca^{2+}-uptake supported by endogenous CK and PK. Future experiments could target:

1. Additional measurements of SR Ca^{2+} handling that incorporate endogenous energy supply to better mimic actual cellular processes in future experiments;
2. Further research into glycolytic metabolic channeling involving other glycolytic enzymes;
3. Improved quantification of key proteins involved in the metabolic processes of interest; and
4. The use of other SR glycogen-extraction methods (other than amylase) that deplete glycogen concentration to the same extent as skeletal muscle fatigue.

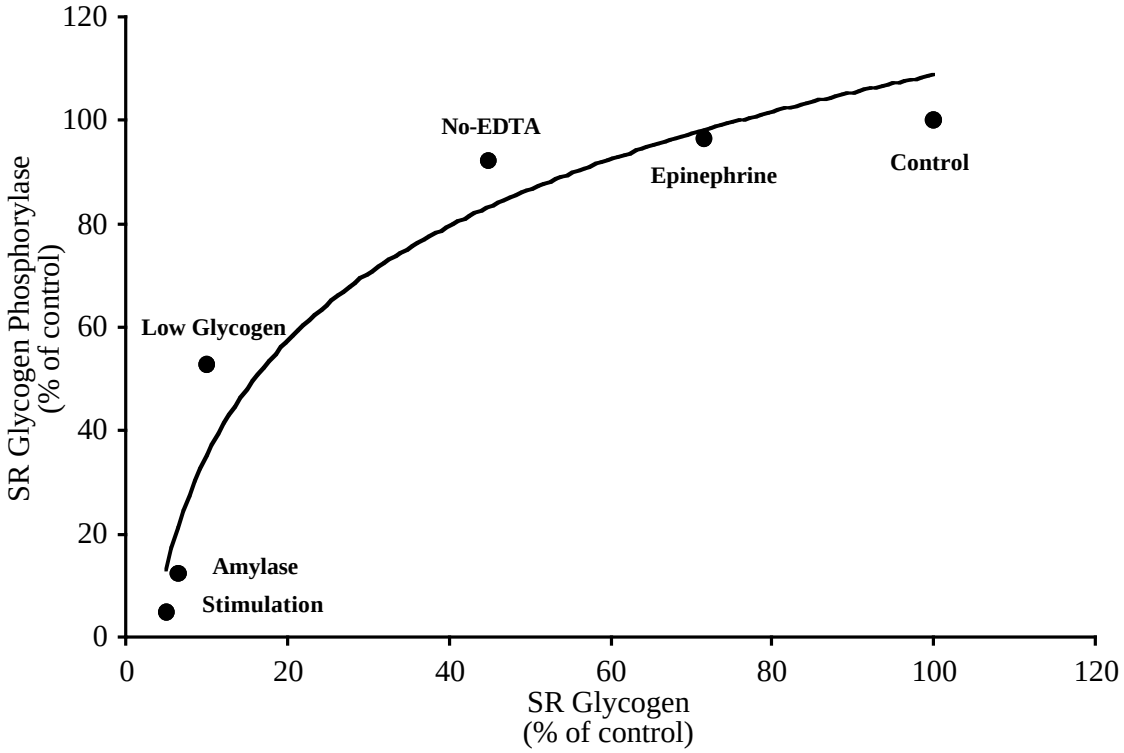

Figure 19. Relationship between sarcoplasmic reticulum (SR) glycogen and glycogen phosphorylase content

Amylase and No-EDTA data points were taken from the present investigation. Low glycogen data were taken from Batts (2002). Epinephrine data were collected from our lab. In this study, animals were initially injected with epinephrine (1 μg/g body weight: ip) and again 30 minutes later. 60 minutes after the initial injection, the animals were sacrificed and the tissue was collected. Stimulation data were taken from Lees et al. (2001). All the data are represented as a percent of control from the respective studies. The logarithmic trendline is displayed to emphasize the non-linear relationship.

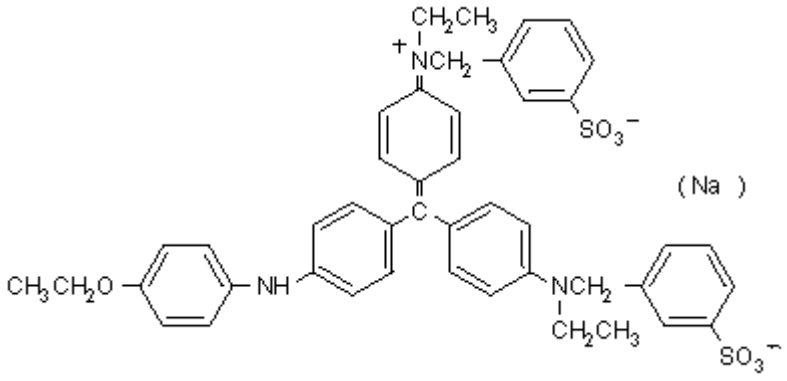

Figure 20. Structural representation of Coomassie brilliant blue R-250
The main interaction between Coomassie brilliant blue R-250 and protein is thought to be through electrostatic interaction between the sulfonic acid groups ($-SO_3^-$) of the dye and the positively charged basic amino acid residues of the protein. There also seem to be hydrophobic interactions between the dye and the protein that occur to a lesser extent (Fazekas de St. Groth et al. 1963; Tal et al. 1985).

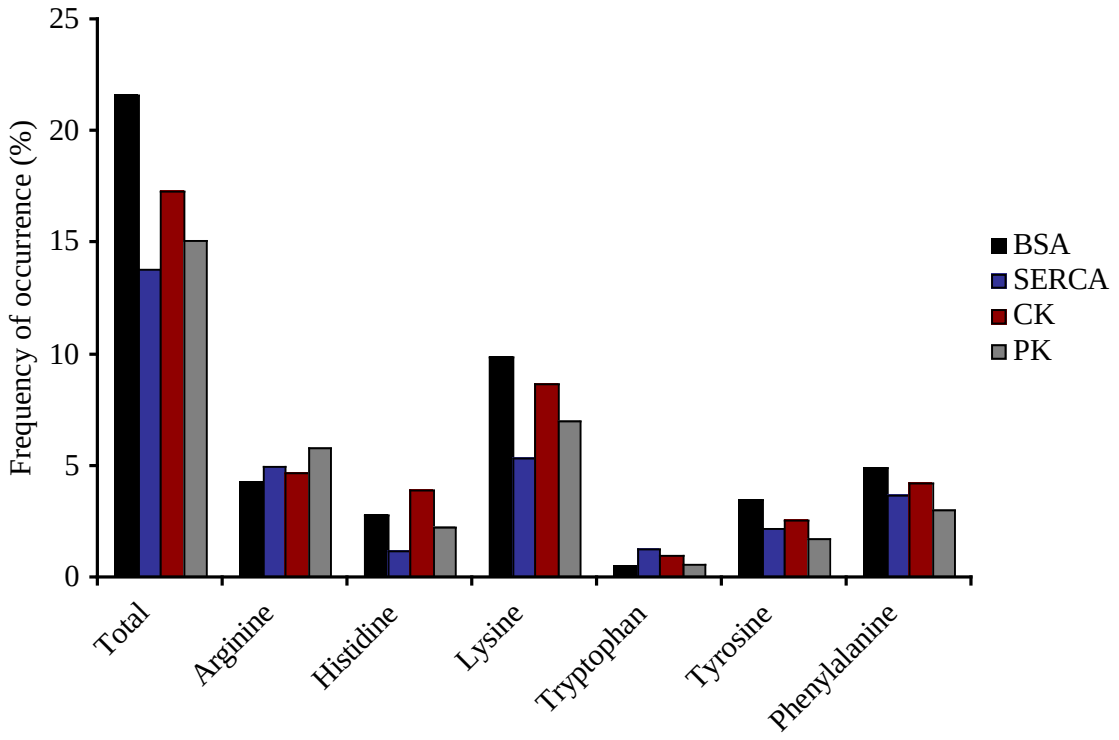

Figure 21. Histogram of basic and hydrophobic amino acids
Histogram of basic (histidine, lysine and arginine) and hydrophobic (tryptophan, tyrosine and phenylalanine) amino acids in bovine serum albumin (BSA), sarco(endo)plasmic reticulum calcium adenosine triphosphatase (SERCA), creatine kinase (CK) and pyruvate kinase (PK). The total represents the sum of all of the basic and hydrophobic amino acids. Coomassie brilliant blue R250 is thought to bind mainly to these amino acids when staining SDS-PAGE.

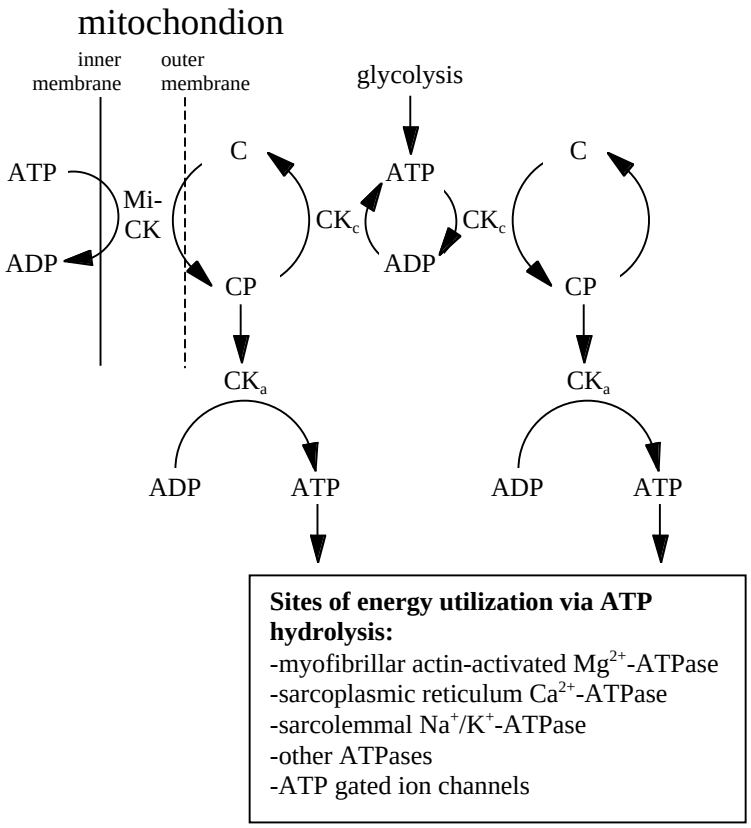

Figure 22. Schematic representation of the phospho-creatine circuit
The phospo-creatine circuit structurally and functionally couples microcompartments involving mitochondrial creatine kinase (Mi-CK), cytosolic creatine kinase (CK_c) and subcellular associated creatine kinase (CK_a). Strategically located CK_a within the muscle cell is functionally linked to metabolic processes, e.g. sarcoplasmic reticulum Ca^{2+}-ATPase. The phopho-creatine circuit serves as an energy buffer, an energy transport and channeling system, and a regulator of local ATP levels (Wallimann et al. 1989).

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Appendix A

Equation 1:

$\Delta G_p = \Delta G^{\circ\prime} + RT \ln ([ADP][P_i]/[ATP])$
Where,
ΔG_p = actual free energy
$\Delta G^{\circ\prime}$ = standard free energy, that is when all the reactants are at 1M
R = gas constant
T = temperature (K)
[ADP], $[P_i]$, and [ATP] are in moles per liter

Appendix B

Proteases

Proteolytic enzymes hydrolyze the peptide bonds in a protein. They serve to degrade either unneeded or damaged proteins in the cell.

Protease Inhibitor Cocktail

This cocktail is needed in the homogenization buffer because the cellular structures that normally package and control proteases (e.g., lysosomes) are disrupted. The addition of protease inhibitors, as well as performing all purification steps at 4º C, slows down unwanted proteolysis. The protease inhibitor cocktail (Sigma, St. Louis, MO) used has been optimized and tested for mammalian tissue use. It contains inhibitors with a broad specificity for the inhibition of serine, cysteine, aspartic and aminopeptidases.

Protease Inhibitor	Target Protease Class	Mechanism Of Action
AEBSF	Serine proteases (e.g., Thrombin)	Inhibits by acylation of the active site of the enzyme. Much less toxic than PMSF and DFP.
Aprotinin	Serine proteases (inhibits above all plasmin, kallikrein, trypsin, chymotrypsin and urokinase, but not carboxypeptidase A and B, papain, pepsin, subtilisin, thrombin and factor X)	Basic single-chain polypeptide that inhibits numerous serine proteases by binding to the active site of the enzyme, forming tight complexes.
Leupeptin	Serine and thiol proteases	Reversible competitive inhibitor.
Bestatin	Metalloprotease (e.g., leucine aminopeptidase and alanyl aminopeptidase)	Competitive and specific inhibitor.
Pepstatin A	Acid proteases or aspartyl peptidases (e.g., pepsin, cathpsin D, chymosin and renin	Reversible inhibitor. Thought to inhibit through a collected-substrate inhibition mechanism.
E-64	Cysteine and thiol proteases (e.g., calpain, papain, cathepain B and cathepsin L)	Non-competitive irreversible inhibitor. Forms a thioether bond with the sulfhydryl group in the active center of the enzyme.

Table 3. Protease Inhibitor Cocktail
Protease inhibitors included in the present investigation with the target protease class and mechanism of action. The protease inhibitor cocktail (Sigma, St. Louis, MO) used has been optimized and tested for mammalian tissue use.

Appendix C

Glycogen Debranching Enzyme Associated With The Sarcoplasmic Reticulum

This investigation sought to examine glycogen debranching enyzme (AGL, 4-α-glucanotransferase amylo-1,6-glucosidase, EC 2.4.1.25+3.2.1.33) associated with skeletal muscle SR. SDS-PAGE revealed a band of molecular weight ~170,000 Da associated with the skeletal muscle SR vesicle preparations. SR samples were also taken from muscle whose glycogen content had been reduced via stimulation of the sciatic nerve (333ms trains of 20Hz pulses delivered each second for 30 minutes). The stimulation protocol reduced whole muscle glycogen by 86% (7.4±0.4 vs 1.0±0.3 ug/mg wet mass, $p<0.05$, n=6) as well as glycogen associated with the SR by 82% (532.6±82.4 vs 95.7±6.9 ug/mg SR protein). SDS-PAGE and Western blots revealed that the content of AGL was reduced by ~53% as a result of muscle stimulation (from 1.02±0.06 to 0.48±0.03 AU and from 0.82±0.09 to 0.38±0.03 AU, respectively). In addition, AGL activity was reduced by 61% in these samples compared to non-stimulated muscles (20.33±0.95 vs 7.98±1.21 nmol/mg per min, respectively), a value consistent with reductions observed from SDS-PAGE and Western blots. These results confirm that similar to glycogen phosphorylase, AGL is associated with the skeletal muscle SR and is released under exercise conditions.

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Appendix D: Raw Data

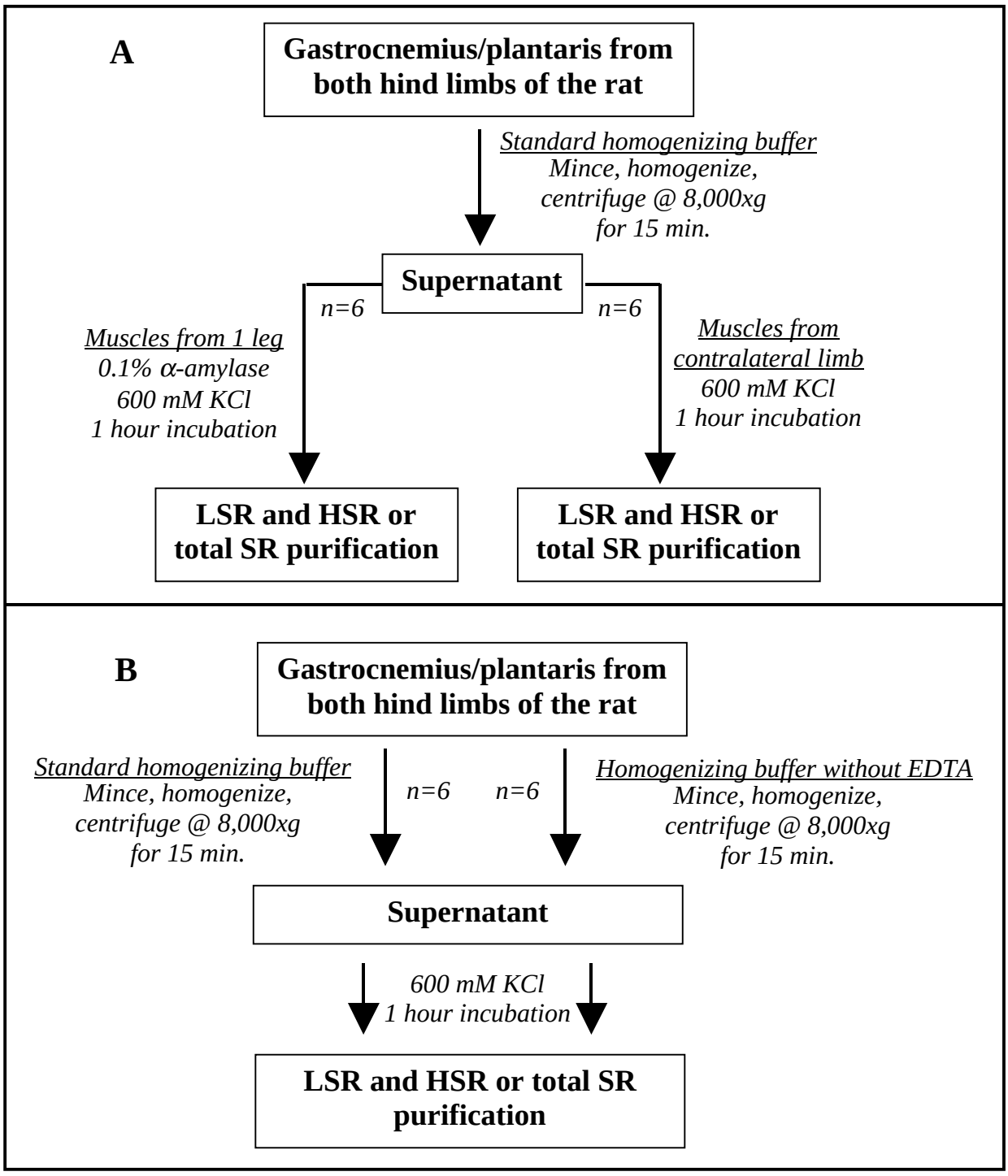

Figure 23. Flow diagrams for SR purification/glycogen extraction
A) flow diagram for amylase-treated and amylase/control SR purification. B) Flow diagram for No-EDTA and No-EDTA/control SR purification.

SR Glycogen

	Control/Amylase		Amylase	
	HSR	**LSR**	**HSR**	**LSR**
	157.6	421.3	28.1	22.0
	184.3	1107.9	26.4	43.6
	199.5	671.8	19.5	53.4
	179.9	848.6	22.5	18.8
	222.9	936.3	26.8	32.1
Mean	192.0	718.5	24.7	34.0
SEM	14.2	83.4	1.6	6.5

	Control/No-EDTA		No-EDTA	
	HSR	**LSR**	**HSR**	**LSR**
	269.4	730.6	63.2	20.5
	138.1	537.8	52.5	268.6
	184.1	493.7	52.3	262.6
	151.4	493.6	58.3	295.5
	206.9	465.1	140.9	429.1
Mean	190.0	544.2	73.4	255.3
SEM	23.2	48.0	17.0	66.0

Table 4. SR glycogen concentration
Raw data, group means and standard error of the mean (SEM) for sarcoplasmic reticulum (SR) glycogen concentration for light SR (LSR) and heavy SR (HSR) fractions. Data are presented as μg of glycogen/mg of SR protein.

SERCA Yield

	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
Study 1	106.8	170.5	131.0	161.1
	154.2	164.4	143.2	134.2
	197.7	257.9	62.9	86.9
	230.0	214.0	108.0	93.3
	163.5	192.3	80.5	94.6
	189.1	206.7	101.8	114.3
	130.1	164.0		
Study 2	317.2	408.4	230.6	243.6
	331.1	377.5	231.5	285.7
	319.2	371.1	128.6	144.2
	233.6	301.0	187.8	168.2
	258.0	306.0	219.7	241.6
	264.3	299.5	264.5	247.7
Mean	222.7	343.9	210.5	221.8
SEM	20.4	19.4	19.2	22.0

Table 5. SERCA Yield
Raw data, group means and standard error of the mean (SEM) for sarco(endo)plasmic reticulum adenosine triphosphatase (SERCA) yield. Data are presented as μg of SERCA/mg of sarcoplasmic reticulum protein. Study 1 utilized the heavy SR fraction, whereas, study 2 utilized the total SR fraction.

Glycogen Phosphorylase Content

	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
Study 1	1.447	0.334	0.969	0.837
	1.362	0.417	0.851	0.687
	1.307	0.586	0.742	0.579
	1.298	0.446	0.758	0.615
	1.141	0.496	0.857	0.725
	1.220	0.466		
	0.728	0.383		
Study 2	1.308	0.114	1.251	1.067
	1.371	0.117	1.164	1.180
	1.396	0.121	1.517	1.468
	1.111	0.191	1.427	1.260
	1.153	0.195	1.080	1.076
	1.059	0.179	1.242	1.043
Mean	1.223	0.153	1.280	1.182
SEM	0.053	0.016	0.067	0.066

Table 6. Glycogen phosphorylase content
Raw data, group means and standard error of the mean (SEM) for glycogen phosphorylase content. Data are presented as the optical density (O.D.) of the band containing glycogen phosphorylase, normalized to the O.D. of 1 μg of protein standard on SDS-PAGE. Study 1 utilized the heavy SR fraction, whereas, study 2 utilized the total SR fraction.

Glycogen Debranching Enzyme Content

	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
	0.730	0.428	0.905	0.774
	0.790	0.506	0.839	0.780
	0.766	0.386	0.876	0.978
	0.713	0.439	0.871	0.794
	0.808	0.325	0.631	0.683
	0.841	0.442	0.669	0.755
Mean	0.775	0.421	0.799	0.794
SEM	0.020	0.025	0.048	0.040

Table 7. Glycogen debranching enzyme content
Raw data, group means and standard error of the mean (SEM) for glycogen debranching enzyme (AGL, 4-α-glucanotransferase amylo-1,6-glucosidase) content. Data are presented as the optical density (O.D.) of the band containing AGL, normalized to the O.D. of 0.5 μg of protein standard on SDS-PAGE.

Creatine Kinase Content

	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
	0.114	0.165	0.150	0.144
	0.153	0.180	0.123	0.191
	0.132	0.234	0.140	0.221
	0.134	0.191	0.138	0.174
	0.136	0.188	0.137	0.135
	0.122	0.163	0.117	0.164
Mean	0.132	0.187	0.134	0.172
SEM	0.005	0.011	0.005	0.013

Table 8. Creatine kinase content
Raw data, group means and standard error of the mean (SEM) for creatine kinase content. Data are presented as the optical density (O.D.) of the band containing creatine kinase, normalized to the O.D. of 1 μg of protein standard on SDS-PAGE.

Pyruvate Kinase Content

	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
	0.254	0.555	0.335	0.353
	0.368	0.644	0.315	0.373
	0.321	0.591	0.248	0.359
	0.297	0.587	0.303	0.354
	0.278	0.569	0.277	0.323
	0.306	0.631	0.294	0.330
Mean	0.304	0.596	0.296	0.349
SEM	0.016	0.014	0.012	0.008

Table 9. Pyruvate kinase content
Raw data, group means and standard error of the mean (SEM) for pyruvate kinase content. Data are presented as the optical density (O.D.) of the band containing pyruvate kinase, normalized to the O.D. of 0.5 μg of protein standard on SDS-PAGE.

SR ATPase Activity

	Control/Amylase		Amylase	
	Basal	**Ca^{2+}-Activated**	**Basal**	**Ca^{2+}-Activated**
	0.138	2.31	0.238	4.20
	0.238	2.87	0.219	3.45
	0.197	4.06	0.286	4.30
	0.437	3.89	0.577	5.07
	0.214	1.91	0.363	3.22
	0.240	2.56	0.475	3.89
	0.421	2.49	0.598	3.51
Mean	0.269	2.87	0.394	3.95
SEM	0.043	0.306	0.060	0.240

	Control/No-EDTA		No-EDTA	
	Basal	**Ca^{2+}-Activated**	**Basal**	**Ca^{2+}-Activated**
	0.369	2.48	0.663	3.55
	0.379	2.65	0.527	3.45
	0.323	1.51	0.448	2.36
	0.298	1.62	0.331	1.79
	0.308	1.44	0.324	1.43
	0.261	1.54	0.379	1.88
Mean	0.323	1.87	0.445	2.41
SEM	0.018	0.221	0.054	0.366

Table 10. SR ATPase activity
Raw data, group means and standard error of the mean (SEM) for sarcoplasmic reticulum (SR) basal and Ca^{2+}-activated adenosine triphosphatase (ATPase) activities. Data are presented as μmol of adenosine triphosphate/minute per mg of SR protein.

ATP-Supported SR Ca^{2+}-Uptake

ATP	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
Study 1	0.840	0.970	0.838	1.138
	0.815	0.815	0.888	1.175
	0.765	1.00	0.513	0.875
	0.750	1.040	0.763	0.831
	0.810	1.130	0.781	0.819
	0.800	1.190	0.644	0.950
	0.930	1.190		
Study 2	1.134	1.428	1.140	1.145
	1.188	1.555	1.150	1.269
	1.192	1.455	0.619	0.540
	1.011	1.264	0.857	0.730
	0.933	1.157	0.970	1.168
	0.971	1.155	1.069	1.193
Mean	0.934	1.181	0.853	0.986
SEM	0.044	0.058	0.059	0.066

Table 11. SR Ca^{2+}-uptake
Raw data, group means and standard error of the mean (SEM) for peak adenosine triphosphate (ATP) supported sarcoplasmic reticulum (SR) Ca^{2+}-uptake rates. Data are presented as nmol of Ca^{2+} taken up into the SR/minute per mg of SR protein. Study 1 utilized the heavy SR fraction, whereas, study 2 utilized the total SR fraction.

Creatine Phosphate and ADP-Supported Calcium-Uptake

ADP+CP	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
	0.193	0.261	0.230	0.236
	0.195	0.238	0.196	0.222
	0.205	0.224	0.183	0.270
	0.179	0.207	0.204	0.248
	0.175	0.236	0.199	0.222
	0.197	0.249	0.192	0.233
Mean	0.191	0.236	0.201	0.239
SEM	0.005	0.008	0.007	0.007

Table 12. CP + ADP-supported SR Ca^{2+}-uptake
Raw data, group means and standard error of the mean (SEM) for peak creatine phosphate (CP) and adenosine diphosphate (ADP) supported sarcoplasmic reticulum (SR) Ca^{2+}-uptake rates. Data are presented as nmol of Ca^{2+} taken up into the SR/minute per mg of SR protein.

PEP and ADP-Supported Ca^{2+}-Uptake

ADP+PEP	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
	0.155	0.189	0.192	0.194
	0.175	0.204	0.162	0.216
	0.174	0.179	0.136	0.177
	0.166	0.186	0.174	0.195
	0.162	0.208	0.174	0.197
	0.176	0.223	0.156	0.193
Mean	0.168	0.198	0.166	0.195
SEM	0.003	0.007	0.008	0.005

Table 13. PEP + ADP-supported SR Ca^{2+}-uptake
Raw data, group means and standard error of the mean (SEM) for peak phosphoenol-pyruvate (PEP) and adenosine diphosphate (ADP) supported sarcoplasmic reticulum (SR) Ca^{2+}-uptake rates. Data are presented as nmol of Ca^{2+} taken up into the SR/minute per mg of SR protein.

SR Ca^{2+}-Release

$AgNO_3$ Release	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
	1.21	1.42	1.38	1.70
	1.21	1.27	1.29	1.63
	1.08	1.31	1.05	1.38
	1.12	1.34	1.20	1.35
	1.79	2.03	1.20	1.24
	1.86	2.32	1.11	1.34
	2.01	2.24		
Mean	1.47	1.70	1.21	1.44
SEM	0.15	0.18	0.05	0.07

Suramin Release	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
	0.206	0.372	0.251	0.365
	0.260	0.326	0.216	0.312
	0.282	0.347	0.279	0.298
	0.260	0.427	0.220	0.299
	0.233	0.381	0.264	0.301
	0.262	0.388	0.243	0.365
	0.308	0.383		
Mean	0.259	0.375	0.260	0.323
SEM	0.012	0.012	0.0010	0.013

Table 14. SR Ca^{2+}-release
Raw data, group means and standard error of the mean (SEM) for peak sarcoplasmic reticulum (SR) Ca^{2+}-release rates initiated by silver nitrate and suramin. Data are presented as nmol of Ca^{2+} taken up into the SR/minute per mg of SR protein.

SR-Bound FITC

	Control/Amylase	Amylase	Control/No-EDTA	No-EDTA
	0.372	0.258	0.419	0.298
	0.266	0.239	0.250	0.414
	0.218	0.181	0.392	0.322
	0.183	0.192	0.411	0.399
	0.222	0.262	0.330	0.357
	0.259	0.185	0.385	0.312
Mean	0.253	0.220	0.365	0.350
SEM	0.027	0.015	0.026	0.020

Table 15. SR-Bound FITC
Raw data, group means and standard error of the mean (SEM) for sarcoplasmic reticulum (SR) bound fluorescein isothiocyanate (FITC) fluorescence emission. Data are presented as arbitrary units.

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PUBLICATIONS

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Measurement of glycogen associated with skeletal muscle sarcoplasmic reticulum. Experimental Biology 2000, Skeletal Muscle Fatigue Symposium, San Diego, California.

OTHER TRAINING

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Skeletal muscle function and exercise, Skeletal muscle plasticity, Biochemistry for the life sciences, Molecular biology for the life sciences, Veterinary Physiology, Pharmacology, Laboratory animal management, Statistics for research

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Small animal handling and surgery, in vitro SR calcium uptake and release rates, Various biochemical and enzymatic assays, PCR, SDS-PAGE, Immuno-precipitation, Western blotting, Affinity precipitation, HPLC, in situ and in vitro muscle contractile measurements.