CHAPTER 1

Introduction

Introduction

Fatigue can be described as an inability to maintain an expected force output. The development of fatigue is important in athletic performance, work place productivity, and daily living. Fatigue during daily living is of special importance to the elderly and specific diseased populations as it can limit mobility and decrease the ability to accomplish everyday tasks. Both of these factors can markedly reduce the quality of life for these individuals. Thus, it is important to understand the mechanism of development of fatigue to facilitate the design of effective counter measures to prevent or reduce its occurrence.

Although the exact mechanism has yet to be elucidated, there are certain events associated with fatigue. It has been known for many years that muscle glycogen can be reduced during fatiguing events. Glycogen is one of the primary substrates needed for energy production, namely ATP, during exercise. Traditionally, it was assumed that as glycogen content declined during exercise, ATP levels were reduced and fatigue ensued. However, the decline in muscle glycogen that occurs during fatigue in not associated with noticeable reductions in muscle ATP levels (see Green, 1991). Since the reduction in muscle glycogen does not cause a concomitant reduction in energy there must be a non-metabolic link between muscle glycogen depletion and reduced force production during fatigue.

Along with the decline in the muscle glycogen during fatigue, there are reductions in the muscle's ability to regulate intracellular calcium homeostasis (Williams & Klug, 1995). The sarcoplasmic reticulum (SR) is the intracellular organelle responsible for intracellular calcium regulation, by releasing during contraction and sequestering it during relaxation. As fatigue develops, rates of calcium uptake and release are reduced by up to

50% (Williams et al., 1998, Ward et al., 1998). With a reduction in the rate of calcium release there is an obligatory reduction in force output. On the other hand, the slowed rate of relaxation that accompanies fatigue appears to be due to the decreased rate of calcium uptake(Williams & Klug, 1995, Gollnick et al., 1991).

Glycogen particles are physically associated with the SR, forming a glycogen-SR complex (Wanson & Drochmans, 1968, Meyer et al., 1970). This raises the possibility that there may be some link between the presence of glycogen and the functional attributes of the SR. In other words, the decline in muscle glycogen may directly affect calcium handling. Entman and colleagues (1980) raised the possibility that calcium uptake and release by the SR may be modulated by factors that also modulate glycogenolysis. Also in a preliminary report, Brautigan et al. (1979) showed that glycogen removal with a-amylase caused a reduction in the ability of the SR to uptake calcium. Since then it has been demonstrated that the phosphorylase b associated with the SR can stimulate calcium uptake by forming a metabolic shuttle (Cuenda et al., 1993, Nogues et al., 1996). Unfortunately, a direct link between the level of glycogen associated with the SR and its ability to release and sequester calcium has not been firmly established. Further, the notion that glycogen depletion during exercise directly affects SR function has not been investigated. These questions are critically important in determining the mechanisms that mediate fatigue during prolonged exercise. In addition, their resolution will elucidate potential links between carbohydrate metabolism, SR function, and fatigue.

Statement of problem

Reduction in muscle glycogen has been shown to occur with fatiguing exercise. The importance of intramuscular glycogen cannot be overstated, as it is the primary substrate for the production of ATP. The hydrolysis of an ATP molecule is the driving force in the contraction and relaxation of the muscle cell. An ATP molecule attaches to myosin causing the actin and myosin to shift from the strong binding state to the weak binding state and the myosin subsequently detaches from the actin. The ATP is hydrolyzed into adenosine diphosphate (ADP) and inorganic phosphate (P_I) and forms an intermediate complex with the myosin and the myosin head reattaches to the actin further down. The release of the P_I allows the (ADP) to be released and the myosin head ratchets, which is the power stroke. The cycle repeats once another ATP attaches to the myosin (see McComas, 1996).

It was once thought that the reduction in muscle glycogen during fatiguing exercise caused a reduction in available ATP, thereby causing fatigue. As previously noted, Green (1991) identified investigations that showed that there was not a noticeable decline in intramuscular ATP. That review examined studies that looked at whole muscle ATP. A reduction in localized ATP around the SR may occur causing an impairment in the function of the calcium regulatory capacity of the SR. Han and colleagues (1992) have shown that there was a compartmentalized production of ATP in the skeletal muscle triad. That observation suggests that uptake by the SR is regulated by local levels of ATP and not by whole muscle ATP levels which are relatively unaffected by fatiguing exercise. In fact, Xu et al (1995) demonstrated that the glycolytic enzymes associated with the SR could supply sufficient ATP to support Ca^{2+} uptake without exogenous ATP. However,

they did not address whether local glycogen depletion would inhibit the compartmentalized production of ATP.

As was shown by Xu and colleagues (1995), others have shown that glycolytic enzymes are intimately associated with the SR membrane (Nogues et al., 1996, Cuenda et al., 1994, Entman et al., 1980), including phosphorylase. Illustrating the importance of the membrane bound enzymes, Nogues and associates (1996) demonstrated that ATP produced by localized glycogenolysis can support calcium uptake. Cuenda et al. (1995) showed that treatment of the SR with α-amylase caused phosphorylase to dissociate from the membrane. Their results identified a link between the enzyme and glycogen associated with the membrane. These previous studies allow us to question whether fatiguing exercise causes depletion in the membrane bound glycogen, thus causing an impairment in the calcium regulatory function of the SR.

The specific purpose of this study was to determine whether removal of glycogen associated with SR, through exercise and α-amylase digestion, would impair the calcium handling of the SR. This was accomplished through a single fatiguing bout of exercise or the addition of α-amylase to the muscle preparation.

Significance of the study

It is known that there is a decrease in muscle glycogen that occurs concurrently with fatigue, but evidence has not been provided whether there is a decrease in the glycogen associated with the SR. Nor has it been shown that if there is a decrease in SR glycogen whether it will impair function. Investigating the fatigue process and the role of glycogen with regards to the SR will allow for a greater understanding of the mechanism

of fatigue development. This greater understanding may lead to ways to prevent or reduce the impact of fatigue. This would benefit the populations where fatigue is of daily importance, such as the elderly and specific diseased populations, for example congestive heart failure patients, and suffers of McArdle's disease. Scientifically, elucidation of the process would lead to a more comprehensive knowledge of the function of muscle in regards to fatigue.

Research Hypothesis

The following are the null hypothesis as tested in this investigation:

Ho_1: Prolonged exercise will not affect Ca^{2+} uptake or release rate.

Ho_2: α-Amylase treatment will not affect Ca^{2+} uptake or release rate.

Ho_3: There is no interaction between exercise and treatment with α-amylase that will affect Ca^{2+} uptake or release rate.

Delimitations

The following delimitations were set by the investigator:

1. The subjects were 10 adult, female Sprague-Dawley rats (150-200g).

2. Only one muscle, the gastrocnemius, was subjected to the treatment (amylase).

3. Calcium uptake rate was represented by the steepest negative slope of the free Ca^{2+} time curve indicating the removal of calcium from the incubation medium.

4. Calcium release rate was represented by the steepest positive slope of the free Ca^{2+} time curve indicating the reintroduction of calcium into the incubation medium.

Limitations

The following limitations were inherent in the experimental design:

1. The experiment was performed *in vitro* which only mimics a true physiological environment.

2. The experiment only included one species and gender (female Sprague-Dawley rats).

3. The experiment was limited to one type and duration of exercise (one hour treadmill run)

Basic Assumptions

The following are the basic assumptions made by the investigator:

1. The rats were disease and pathogen free.

2. The SR vesicles were undamaged by the protocol.

3. There were no underlying factors within the muscle, unknown to the investigator, that would affect normal function.

CHAPTER 2

Literature Review

Introduction

Muscle fatigue has been described in many different ways. Common to all definitions of muscular fatigue is a reduced capacity of the muscle to maintain an expected force level or production. The cause of muscular fatigue is not clearly understood, as such there are many theories as to its mechanism. Similarly, fatigue is not likely to be explained by one mechanism, since there are a cascade of events occurring during muscle contraction, there are just as likely a cascade of events leading to muscle fatigue. For a muscle cell to contract a depolarization must occur due to the stimulation from an action potential (AP). Failure of the transduction of the AP anywhere along its route from the motor cortex to the neuromuscular junction to the transverse-tubule could cause a reduction in the functional capacity of the muscle. During contraction various metabolites are produced (H^+, ADP, P_i,), leading some investigators to theorize that the presence of these metabolites exert an attenuating effect on the contractile function of the muscle. Muscle contraction requires ATP to occur and there is a reduction in energy substrates during muscular activity which could possibly be a limiting factor in the contraction process.

Along with the many events that occur during a muscle contraction there are many structures involved in the process. The sarcoplasmic reticulum (SR) is one of the major structures involved, controlling the intracellular homeostasis of calcium (Ca^{2+}). A contraction is initiated when the SR releases Ca^{2+} and the ion binds to the contracting filaments and initiating a transition from the weak to strong binding state. Relaxation occurs when the SR takes up the intracellular Ca^{2+} and the cross bridges return to the weak binding state (Williams & Klug, 1995). During fatigue the SR's ability to regulate

intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is impaired. The impairment reduces the muscle's ability to generate force. Along with the unknown mechanism of fatigue the underlying cause of the impaired SR function is also not understood.

Recently, investigators have been looking into the role of glycogen in SR function. Glycogen particles were identified as being part of the SR membrane (Wanson & Drochmans, 1968, 1972). This led authors to begin to examine the role of this glycogen-SR complex. As that complex was investigated, it was noted that there where glycogenolytic enzymes affiliated with the glycogen (Entman et al., 1977). After there was a link established between the SR-complex and glycogen metabolism, the effect of glycogen depletion (Entman et al., 1977; Brautigan et al., 1979) and the addition of metabolites from various stages of glycogen metabolism (Cuenda et al., 1993; Nogues et al., 1996; Xu et al., 1995) were examined to determine their roles in the function of the SR. These and other investigators found that there was a connection between the complex and Ca^{2+} uptake by the SR. What is not known is whether this interaction is solely metabolic or whether there is a non-metabolic link between glycogen removal and SR function. It would also be useful to know what the role of this complex during fatiguing exercise is when glycogen depletion is known to occur. There have been many studies that have looked at various causes of fatigue, including SR impairment and glycogen depletion, but none have been able to identify the underlying factor causing fatigue and whether these two are related. Therefore, this literature review will examine the idea that glycogen associated with the SR has a role in the fatigue process.

Muscle Contraction

To be able to investigate fatigue, an understanding of muscle contraction is necessary. The process initiates at the α-motor neuron residing in the gray matter of the brain stem or in the spinal cord. An AP is generated and travels down the axon to the neuromuscular junction (NMJ), or the synapse. Once the AP reaches the NMJ acetylcholine (Ach) is released and crosses the synaptic cleft where it binds to the Ach receptors (AchR) on the sarcolemma. The binding of Ach to the receptors alters the membrane permeability, allowing an influx of Na^+, bringing the membrane potential down to –15 mV from –85 mV. This membrane depolarization allows the transmittance of the AP to the sarcolemma. The AP travels into the transverse tubules (TT), invaginations of the sarcolemma. The TT are abutted by the SR on both sides within the cell. This adjacent arrangement forms a structure known as the triad. Imbedded in the SR membrane are proteins called Dihydropyridine Receptors (DHP). The DHP are voltage sensitive and respond to the depolarization by signaling another protein in the SR to release Ca^{2+} . The Ryanodine Receptor is the calcium releasing channel in the SR.

The release of Ca^{2+} causes an rise in intracellular levels and binds to troponin C on the actin myofilament. The binding of Ca^{2+} overcomes the inhibitory effect of troponin I and causes a conformational change allowing the binding of the myosin head to the actin. The myosin cross-bridge attaches to the actin forming a rigor complex, the strong binding state. An ATP molecule attaches to the myosin head allowing the cross bridge to enter the weak binding state. The myosin head detaches and moves further down the actin filament shortening the myofiber. The ATP hydrolyzes and the head reattaches. The P_i is released and the cross-bridge moves into the strong binding state and the ADP is released.

Relaxation occurs when the SR sequesters the Ca^{2+} by active uptake of the Ca^{2+} ATPase protein (McComas, 1996).

Mechanisms of Fatigue

In the study of muscle fatigue there have been many theories as to its mechanisms that have been examined. One of the possible sites of fatigue is at the central nervous system (CNS). Activation at the CNS may be impaired with repetitive exercise leading to a reduced force output by the muscle. In a review by Williams & Klug (1995), they identified a number of studies that have shown that in well motivated subjects impairment of the CNS is not an issue. It has also been demonstrated that the movement of the AP along the α-motor neuron is not impaired during repetitive muscle activity. These reports lead the authors to suggest that fatigue causes a reduction in force through a change located in the excitation-contraction coupling in the muscle fiber (Williams & Klug, 1995).

The changes in metabolites during contraction have been the focus of many studies on fatigue(Allen, 1997; Green, 1990; Korge, 1994). During fatigue pH is reduced from 7.0 to 6.2 due to a build up of H^+ (Williams & Klug, 1995). The reduction in pH causes a subsequent reduction in Ca^{2+} uptake by the SR. During the contraction process ATP is hydrolyzed into ADP and P_i, causing a build up of these metabolites. Westerblad and colleagues (1998) concluded that a reduction in the maximum shortening velocity of a muscle, during a slack test, is mainly due to a temporary increase in ADP. The other product of the hydrolysis of ATP, P_i, has been shown to decreases force production (Fryer et al., 1995) and inhibit Ca^{2+} release from the SR (Posterino and Fryer, 1998).

Glycogen is a necessary substrate to maintain the supply of ATP. In light of this necessity, one of the current theories as to the role of glycogen in muscle fatigue is the

depletion of glycogen results in lowered levels of ATP because of a decreased capacity for the production of the nucleotide(Green, 1991). The lowered levels of ATP would in turn reduce force production and the uptake of Ca^{2+} by the SR. Another theory supported by research has shown that in glycogen depleted muscles, as ATP is brokendown there is an increase in ADP levels (Maughan et al., 1997) which may affect muscle contractility. In support of that theory, Westerblad et al (1998) showed that high levels of ADP reduced the maximum velocity of shortening in single fibers.

Glycogen in Fatigue

Glycogen is the primary substrate for energy production during prolonged exercise (30-180 min, Maughan et al., 1997) at an intensity level that corresponds to 60-85% of VO_2max. The prevailing theory of glycogen's role in fatigue during submaximal exercise is the reduced substrate availability (Green, 1991). There is strong support in the literature for linking the onset of fatigue with the depletion of intramuscular stores of glycogen. In the classic study by Bergström (1967) the link was demonstrate through dietary manipulations and subsequent exercise testing. It was shown that there is a decreased work capacity with lowered intramuscular stores of glycogen.

Although there is support for linking the onset of fatigue to glycogen depletion, there is not, as Green (1991) pointed out, support for reduced ATP levels due to reduced muscle glycogen, in fact, most studies have shown that muscle ATP levels remain relatively unaffected even by fatiguing exercise. In studies using cycling as the fatiguing it was shown that ATP levels remained stable while the glycogen levels were severely depleted (see Green 1991).

Although glycogen has been shown to be reduced at the onset of fatigue a metabolic link has yet to be discovered. Because fatigue reduces the levels of glycogen there may be a non-metabolic link. Bissonnette and colleagues (1997) energy restricted rats (Hypo) for seven days and compared tetanic tension and relaxation rates of the soleus (a predominantly slow muscle) and extensor digitorum longus (EDL, predominantly fast muscle) muscles to those of control fed animals. The Hypo group had a significant decline in muscle glycogen levels in both the EDL and the soleus compared to the control fed rats ($p<0.05$). The maximum relaxation rate in both muscles was significantly decreased in the energy restricted animals ($p<0.01$). This would indicate that there was a decrease in the rate of calcium uptake by the SR due to the reduction in intramuscular glycogen levels. They found that in the Hypo rats that there was a 28% increase in fatigue of the soleus muscle ($p<0.01$) and no change in the fatigability of the EDL. Although there was a decrease in the glycogen associated with the increase in the fatigue of the soleus, the authors found no significant correlation between the events. The lack of correlation between the muscle glycogen levels and the fatigue in the soleus does not address the whether a correlation exists between the glycogen associated with the SR membrane and fatigue. The author measurement of tetanic tension did not address whether the rate of calcium release was affected. This method only identified that the amount of calcium that was necessary to elicit maximum tetanic tension was affected by the diet. There was no indication as to a correlation between the maximum rate of relaxation and glycogen levels. Direct measurements of SR function and glycogen associated with the SR would give a better indication as to the performance of the SR.

Chin and Allen (1997) suggested that there is a glycogen dependent and independent mechanism of fatigue. They electrically stimulated single flexor brevis muscle fibers from mice. The fibers underwent two fatiguing bouts of stimulation until force was reduced to 30% of maximum tetanic force. In between fatigue bouts the fibers recovered in either a buffer that contained glucose or a buffer that was glucose-free. The fibers that recovered in the glucose buffer tetanic force fully recovered, but $[Ca^{2+}]_i$ only partially recovered (82% of initial value). The fibers that recovered in the glucose free buffer force only recovered to 64% of initial value and $[Ca^{2+}]_i$ was 57%. The recovery in the glucose buffer allowed for full recovery of Ca^{2+} sensitivity and maximum Ca^{2+} activated force, whereas the fibers in the glucose free buffer only had partial recovery of Ca^{2+} activated force while the Ca^{2+} sensitivity remained depressed. They also demonstrated that glycogen availability is necessary for normal Ca^{2+} release from the SR, by showing that when muscle glycogen levels were reduced to 27% of controls, the $[Ca^{2+}]_i$ and force were reduced more rapidly in the second fatigue bout compared to the first.

The authors suggested that the partial recovery of $[Ca^{2+}]_i$ after the recovery in the glucose buffer was due to the glycogen dependent mechanism of fatigue. The $[Ca^{2+}]_i$ levels increased from 47 to 82% of the initial levels in the glucose buffer, where as the fibers in the glucose-free solution were only able to recover up to 57% of initial levels. Glycogen appears to be responsible for 25% of the recovery of the Ca^{2+} release. Given those results, the authors reasonably concluded that 25% of reduction in Ca^{2+} release during fatigue can be attributed to glycogen depletion. As for the glycogen-independent mechanism of fatigue, Chin and Allen suggested that elevated $[Ca^{2+}]_i$ or P_i are the mechanism that alter force independent of glycogen concentration. Fryer and colleagues (1995) demonstrated

that elevated P_i levels associated with fatigue inhibit calcium release. They reasoned this was due to increased P_i in the SR lumen which caused a calcium phosphate precipitate to form decreasing the calcium available for release. The elevation in $[Ca^{2+}]_i$ during fatigue may impair the function of the muscle through a calcium activated process (Chin and Allen, 1996) or through calcium activated proteases that may alter membrane integrity or the function of proteins (Williams & Klug, 1995).

While depletion of muscle glycogen has been shown to be related to fatigue and impairment in muscle function (Bergstrom et al., 1967; Chin and Allen, 1997, Bissonette at al., 1997), the mechanism behind this impairment is not known. What is also not known is whether the whole muscle glycogen depletion is the causative factor or is site specific glycogen depletion the cause of the reduced muscle performance during fatigue. Additionally, the glycogen independent mechanism may in fact be dependent on glycogen but in a non-metabolic manner. Factors that rely on glycogen may not follow the same recovery time course as glycogen.

Sarcoplasmic Reticulum and Fatigue

The SR is an organelle found in muscle cells that is a specialized version of smooth endoplasmic reticulum that is present in non-muscle cells (Tortora and Grabowski, 1993). The purpose of the SR is to regulate $[Ca^{2+}]_i$ in order to control the contraction and relaxation of the muscle cell. The SR accomplishes this primarily through two transmembrane proteins, Ca^{2+} ATPase and Ryanodine receptor (Ryr). Ca^{2+} ATPase is involved with the uptake of calcium, where as the Ryr is the calcium release channel and serves to increase cytosolic Ca^{2+}.

Through regulating the intracellular levels of the divalent calcium ion, these two proteins also control the contraction and relaxation of the cell. The SR is interrupted by the TT to form triads (McComas, 1996). The TT transmit the AP to the SR through the triad via the DHP. The DHP undergoes a voltage initiated conformational change, causing the calcium release channel to open and release Ca^{2+} from the lumen of the SR, the association between the DHP and the Ryr is not fully understood. The subsequent rise in $[Ca^{2+}]_i$ causes the crossbridges to shift from the weak binding to the strong binding state by releasing the inhibition of troponin I. Relaxation of the myofiber is initiated by the sequestering of Ca^{2+} by Ca^{2+} ATPase. For each ATP that is hydrolyzed, two Ca^{2+} ions are pumped into the SR lumen and one H^+ is pumped into the cytosol (McComas, 1996).

The role of the SR in the muscle contraction process is important to understand because of how it is affected by fatiguing exercise. Both release and uptake rates are important to the contraction-relaxation cycle of the muscle and its ability to regulate $[Ca^{2+}]_i$. When a muscle is fatigued, the performance of the SR is reduced. For example, the rate of calcium uptake has been shown to be reduced by 30-50% (Byrd et al., 1989; Chin et al., 1995) and the rate of calcium release has been reduced by 20-30% through fatiguing exercise (Favero et al., 1993). The model is relevant to the understanding of the impairment of the SR as well. For example, Allen and associates (1995) noted that in intact fibers (Westerblad and Allen, 1993) there was a greater reduction in calcium pump rate compared to the microsomal model (Byrd, 1989; 7 vs. 4 fold decrease).

As $[Ca^{2+}]_i$ levels are regulated through the uptake and release of the ion by the SR, an impairment in the Ca^{2+} ATPase would cause the concentration to increase or remain elevated. Studies by Byrd et al.(1989) and Luckin et al.(1991) have shown that prolonged

running in rats (≥45 min, 60-85% VO_2max) caused a decrease in the activity of Ca^{2+} ATPase. In their study, Byrd et al. (1989) ran rats for 20, 45 minutes, or to exhaustion (mean time 140 min) at 21 m/min and 10% grade on a treadmill. They found that 20 min or more of running caused a decrease in the activity of Ca^{2+} ATPase. They also showed that at least 45 min of running caused a decrease in the rate of Ca^{2+} uptake. Additionally, they were able to show that the reduced activity of the Ca^{2+} ATPase and the attenuation in the rate of calcium uptake was not an artifact of the procedures that were used to prepare the vesicles (Byrd, 1989).

The reduced Ca^{2+} uptake that occurs during fatigue could affect the contractile properties of the muscle by slowing the rate of relaxation. Westerblad and Allen (1994) treated single muscle fibers from mice with 2,5-di(*tert*-butyl)-1,4-benzohydroquinone, a Ca^{2+} pump inhibitor. They found that when the ability of the SR to sequester calcium was reduced there was an increase in $[Ca^{2+}]_i$ and the rate of relaxation was slowed. Similarly, Gollnick et al. (1991) found that in humans a significant ($p<0.001$) inverse relationship between calcium uptake rates and time to half relaxation after leg extensions to fatigue.

In addition to slowing the rate of relaxation, elevated $[Ca^{2+}]_i$ is important because an increased level of intracellular calcium could possibly stimulate calcium activated proteases and cause damage to the cell through degradation of important membrane proteins and phospholipids (Williams & Klug, 1995). Allen et al.(1995) noted that it has been shown that the Ryr are especially susceptible to calcium activated protease. If the Ryr or calcium release channel is altered, this could affect calcium release rates.

In 1993, Favero and colleagues ran rats on a treadmill at a 10% incline and a speed of 21 m/min until fatigued (115 min). They found that an acute fatiguing bout of exercise

caused a 20-30% reduction in the SR's ability to release calcium. It is not fully understood what effect if any this reduction in release rate has. It is possible that there may be a link with the reduced release rate and the fatigue induced reduction in force or with the maximum shortening velocity (V_{max}). Allen and associates (1995) dispute this by noting that the fall in $[Ca^{2+}]_i$ does not correspond to the fall in V_{max}. Clearly more work is needed to fully understand the mechanism behind the impairment of the SR associated with fatigue and what effects the impaired SR has on muscle function.

Glycogen and Sarcoplasmic Reticulum

In addition to glycogen being an important substrate in energy production, it is also present in the lipid bilayer of various membranes. Of particular importance to skeletal muscle, a sarcoplasmic reticulum-glycogen complex with associated glycogenolytic enzymes has been demonstrated by many investigators (Wanson and Drochmans, 1968, 1970; Michalak et al., 1977; Entman et al., 1980; Goldstein et al., 1985). They have shown that there are glycogen particles integrated in the membrane of the SR, with all of the glycogenolytic enzymes attached (Xu et al., 1995). Others have shown that there is a link between these enzymes and calcium uptake by the SR (Cuenda, 1993 ; Nogues, 1996).

Entman and colleagues (1980) showed results that suggested SR-glycogenolytic complex interacts with calcium uptake. Upon stimulation from an AP the calcium channels in the SR open to release calcium and initiate a muscle contraction. Also stimulated by the increase in Ca^{2+} is protein kinase to convert the inactive form of phosphorylase (b) to its active form (phophorylase a). It was demonstrated (Entman et al., 1980) that the uptake of Ca^{2+} by the SR inhibited phosphorylase activity by removing or reducing the calcium

available for the phosphorylation of the enzyme to its active form. This is important because it has been shown that these glycogenolytic enzymes are attached to the SR via glycogen particles (Wanson and Drochmans, 1968; Xu et al., 1995).

Of equal importance is that others have shown that supporting phosphorylase activity enhances the uptake of calcium. Cuenda et al (1993) and Nogues et al. (1996) have demonstrated that the glycogenolysis that occurs due to the enzymes attached to the SR can provide the ATP necessary to support the energy needs of Ca^{2+} ATPase in sequestering calcium.

In support of the link between the SR, glycogen, and the glycolytic enzymes, it has been demonstrated that with the removal of the glycogen associated with the SR, there is a reduction in the rate of Ca^{2+} sequestering by the vesicle. Brautigan et al (1979) treated SR vesicles with α-amylase. The addition of the enzyme digested the glycogen associated with the membrane and released the affiliated enzymes (Wanson and Drochmans 1968, 1972; Goldstein, 1985). They found that with the removal of the substrate and the enzymes the ability of the SR to take up Ca^{2+} was inhibited. It is not known whether this link is strictly a metabolic coupling or if it is a non metabolic role caused by a stearic inhibition or alteration of the membrane due to the localization of the glycogen particles.

The glycogen particles have been shown to serve as attachment sites for the glycolytic enzymes and have been shown to be able to support Ca^{2+} ATPase driven uptake (Xu, 1995, 1998). The glycolytic enzymes support Ca^{2+} uptake by the SR through a local supply of ATP. Xu and colleagues (1995) were able to demonstrate that this local endogenous supply of ATP was used preferentially over an exogenous source. Others have demonstrated that in the absence of exogenous ATP, Ca^{2+} uptake can occur with the

addition of ADP and P_i and glycolytic intermediates such as: phosphoglucomutase and hexokinase (Cuenda et al., 1993), or glucose-6-phosphate (Brautigan et al., 1980). These findings lend support to the glycolytic enzymes bound to the SR supporting calcium uptake.

It is possible that there is a non metabolic link between the SR-glycogen complex and Ca^{2+} uptake. Xu and associates (1998) have shown that the glycogen particles are located near the SR Ca^{2+} ATPase. It is possible that through removal of the glycogen in the membrane the Ca^{2+} ATPase undergoes a conformational change. The other possibility for a non-metabolic interaction could be that the removal of the glycogen alters the viscosity or integrity of the membrane, thereby inhibiting calcium uptake or making the vesicle more leaky. Clearly more work is needed to identify if indeed there is an interaction between glycogen depletion of the SR and fatigue and whether this interaction is metabolic or not.

Summary and Conclusions

The mechanism of fatigue is not clearly understood. Therefore, in an attempt to identify a mechanism we must examine what the possible roles different structures have in fatigue. The peripheral versus central fatigue debate has been pretty much decided. In well motivated individuals, the CNS is not the site of failure in fatigue (Williams & Klug, 1995). Others have just removed this issue by utilizing electrical stimulation of the muscle (Chin and Allen, 1997; Westerblad and Allen, 1994; Ward et al., 1997; Williams 1997).

After being able to attribute the site of fatigue to peripheral locations, metabolites and structures need to be examined. Many byproducts from muscle contraction have been identified as having a possible role in the fatigue process. ATP and the metabolites of the

nucleotide's hydrolysis (ADP, P_i) have been the focus of many investigations (Fryer et al., 1995; Westerblad & Lannergren, 1995; Allen et al., 1997). These authors have shown that increased levels of ADP inhibit the maximum shortening velocity in skeletal muscle and increased P_i reduced the Ca^{2+} sequestering ability of the SR. While impaired muscle function has been shown to be linked to the increase in products from the hydrolysis of ATP, it has also been noted that a decrease in the available ATP is not a likely cause of fatigue, as ATP levels remain fairly stable during fatiguing exercise (Allen, 1995).

Although current literature supports the theory that fatigue is due to a failure in a peripheral location, there is not conclusive evidence for one structure or location. One structure that is strongly supported by literature to have a role in the fatigue process, is the SR. During fatigue, the SR has been shown to have a decreased ability to regulate intracellular calcium levels. Two proteins in the membrane of the SR that regulate the uptake and release of calcium, the calcium channel and the calcium pump, have been implicated as potential sites of failure during fatigue. During fatiguing exercise it has been demonstrated that the ability of the pump to sequester Ca^{2+} is reduced (Byrd et al., 1989; Luckin et al., 1991) and the release channel is also impaired (Favero et al., 1993). Those findings suggest that during fatigue there is some alteration in either the proteins or the SR membrane that alters their ability to regulate $[Ca^{2+}]_i$.

Studies have shown that muscle glycogen depletion is related to fatigue (Bergstrom et al., 1967; Bissonnette et al., 1998; Green 1991). What is not understood is how they are related. It is known that glycogen is depleted during fatiguing exercise, but ATP remains stable and glycogen is the primary substrate for ATP production in exercise of long duration (≥45 min) and of moderate intensity (60-85% VO_2max). The relationship

may lie with an interaction between glycogen and the sarcoplasmic reticulum. A glycogenolytic-SR complex has been established (Wanson and Drochmans, 1968, 1972; Entman et al., 1980; Xu et al., 1995) that has a role with glycogen metabolism and has influence on the uptake of Ca^{2+} by the SR (Brautigan et al, 1979; Nogues et al., 1996). It is not known if exercise affect the SR-glycogenolytic complex. It is also not known whether there is a non-metabolic role of the glycogen associated with the SR membrane in addition to the metabolic interaction. Therefore, it is important to investigate the effect of glycogen removal on the glycogen-SR complex and how that removal affects the function of the SR.

CHAPTER 3

Experimental Design and Methodology

Experimental Design

In this experiment two groups of five rats were used. The first was an exercise group that underwent a single bout of prolonged treadmill running (60 min). The second was a rested group, which were not exercised. Immediately post exercise the animals were killed and the left and right gastrocnemius were removed. Both muscles were homogenized and SR vesicles prepared by differential centrifugation as described by Williams et al. (1998). During the SR vesicle preparation, glycogen was extracted from the muscle of the left limb by treating it with 0.1% α-amylase on ice for 1 hour. The right muscle was not treated. After treatment, the SR vesicles were isolated and the α-amylase was discarded. The experimental design is shown in the table below.

		GLYCOGEN REMOVAL	
		Control	**Amylase**
EXERCISE	**Rest**	Right Limb Ca^{2+} Uptake/Release n=5	Left Limb Ca^{2+} Uptake/Release n=5
	Exercised	Right Limb Ca^{2+} Uptake/Release n=5	Left Limb Ca^{2+} Uptake/Release n=5

Animals

All procedures were approved by the Animal Use and Care Committee of Virginia Tech. Ten female Sprague-Dawley were housed two per cage on a 12 hour light:dark cycle in the Virginia Tech Laboratory Animal Resource facility and had free access to food (Purina Rodent Laboratory Chow) and water.

Exercise Protocol

The exercise group ran on the treadmill for 60 minutes at a speed of 21m·min^{-1} and a 10% incline. This protocol has been shown to result in significant reductions in SR calcium uptake and release as well as a marked reductions in intramuscular glycogen (Byrd et al., 1989, Favero et al., 1993). The rest group did not perform the exercise protocol.

Tissue harvesting and SR vesicle preparation

Immediately post exercise the rats were decapitated after inhalation of CO_2. The left and right gastrocnemius muscles were quickly removed and weighed. The right muscle was placed in 10 volumes (w/v) of cold homogenization buffer containing 20 mM N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid (HEPES), 0.2% sodium azide ($NaN_{3)}$, and 0.2mM phenylmethyl sulphonic fluoride (PMSF, pH 6.8). The left gastrocnemius was placed in a similar buffer that also included 0.1% α-amylase (w/v). The tissue was minced with scissors while on ice and then homogenized for two 30 second burst using a Virtis Virtishear. The mixture remained on ice for one hour to allow the enzyme sufficient time to digest the glycogen. After incubation the crude muscle homogenates underwent a series of centrifugation steps to isolate and fractionalize the SR vesicles. The centrifugation also removed any glucose liberated from glycogen and removed all of the α-amylase from the treated samples. The first centrifugation was for 15 minutes at 8,000 x g, the resulting supernatant was filtered through four layers of gauze. The actomyosin was solubilized by adding 600mM KCl. The heavy sarcoplasmic reticulum (HSR) was separated by centrifuging the solution for 45 minutes at 12,000 x g. The supernatant was decanted off

and underwent a final spin for 60 minutes at 49,000 x g to isolate the light sarcoplasmic reticulum (LSR). The pellets were separately resuspended at a protein concentration of 0.9-3 mg/ml in storage buffer (homogenization buffer with 300mM sucrose and 150mM KCl). The HSR and LSR fractions were frozen at –80 ° C for subsequent protein determination, analysis of SR function, and glycogen determination.

Protein Concentration

The protein concentration of the purified SR was determined to allow for precise measurement of uptake and release of calcium by the SR. The concentration of the SR samples was determined using the Bradford dye-binding assay adapted by Bio-Rad with bovine serum albumin as the standard. A standard curve was generated to determine the protein concentrations of the samples .

Analysis of SR function

The ability of the SR vesicles to regulate calcium was measured fluorometrically in a Jasco CAF-110 fluorometer, with Fura-2 (2µM) used to indicate the extravesicular calcium. A 75 watt xenon pressure lamp with a monochromator that contained a 349 nm interference filter provided the excitation light. Two photomultipliers using 340 and 380 nm filters were used to detect emission fluorescence at 500 nm. The ratio of fluorescence at 340 and 380 nm was used to calculate free Ca^{2+} using the following equation: $[Ca^{2+}]_{free} = K_d \cdot ß \cdot [(R-R_{min})/(R-R_{max})]$; where K_d(the fura-2 dissociation constant) was 70 nM, ß is equal to the ratio of the fluorescence at 340 nm without calcium and the fluorescence at 340 nm with calcium, R_{min} is the ratio of the fluorescence at 340 and 380 nm in a calcium

free medium, and R_{max} is the same ratio in a calcium saturated medium. Calcium uptake and release rates were measured in 1ml of incubation buffer containing 100 mM KCl, 20 mM HEPES, 7.5 mM pyrophosphate, 1 mM $MgCl_2$, and 40mM $CaCl_2$ that was constantly stirred and maintained at 37 °C. All samples were run in triplicate.

For the uptake experiments, 50 µg of the LSR was added to 1 ml of incubation buffer, uptake was initiated by the addition of 1 mM MgATP and continued until the lumen of the SR vesicles was saturated with calcium. Release experiments utilized 50 µg of HSR. The vesicles were loaded with calcium by initiating uptake as done in the previous experiment. Silver nitrate ($AgNO_3$) was used to stimulate the ryanadine receptors of the SR causing calcium release.

Glycogen Measurement

The amount of glycogen associated with the SR from both gastrocnemius muscles from both groups was measured using a Trinder reagent kit from Sigma Chemicals. Heavy and light sarcoplasmic reticulum vesicle preparations underwent incubation with α-amylase to digest the glycogen to allow for measurement of glucose. Samples contained 300 µg of protein and were brought up to 300 or 475 µl using storage buffer. The samples were incubated at 37 °C for 60 minutes with 25 µl of 0.1% α-amylase. After incubation, samples were centrifuged for 15 minutes at 16,000 x g. The supernatant was transferred to a 1000 µl cuvette and 1000 µl of Trinder was added. After 15 minutes of incubation the absorbence was measured at a wavelength on 505 nm on a Milton Roy Spectronic 1001 Plus spectrophotometer. To account for the sucrose in the storage buffer, 300 or 475 µl of buffer underwent the same procedures performed on the SR samples. The absorbence of

the buffer alone was subtracted from the absorbence of the sample and glycogen concentration was calculated from a standard curve.

Statistical Analysis

All data were collected by computer for subsequent analysis. Data were analyzed statistically using a two way ANOVA for repeated measures by the Sigma Stat package, significance was set at $p<0.05$.

CHAPTER 4

Results

The effect of amylase treatment and exercise on uptake rate of SR fractions

Figure 1 shows the uptake rates of the LSR fractions from the gastrocnemius muscle from both groups of animals, rested and exercise, and control and treated muscles. There was no effect of exercise on the ability of the LSR to take up calcium. Amylase treatment caused a 22.89% decrease in the ability of the LSR to take up calcium ($p<0.05$). There was no interaction between the amylase treatment and the exercise.

The uptake rates of the HSR fractions of both groups and control and treated muscles are shown in Figure 2. As with the LSR fraction, amylase treatment caused HSR calcium uptake to be significantly reduced compared to the control by 25.22% ($p<0.05$). Again similar to the LSR, there was no interaction between amylase treatment and exercise.

The effect of amylase treatment and exercise on release rate of HSR fraction

The release rates of the HSR fraction from both groups and the control and amylase treated animals are illustrated in Figure 3. There was no effect of either exercise or amylase treatment on the ability of the HSR fraction to release calcium. There was no interaction between amylase and exercise.

The effect of amylase treatment and exercise on glycogen concentration of SR fractions

The glycogen concentration of the LSR fractions is shown in Figure 4. The rested control LSR glycogen concentration was 0.022 ± 0.001 $\mu mol \cdot mg^{-1}$ with the amylase treated concentration being no different at 0.021 ± 0.002 $\mu mol \cdot mg^{-1}$. The exercise group also showed no difference between control and treatment with respective concentrations of 0.019 ± 0.002 and 0.019 ± 0.001 $\mu mol \cdot mg^{-1}$.

Figure 5 illustrates the glycogen associated with the HSR fractions. As was demonstrated in the LSR there was no difference between the control and the amylase treatment in the rested group, 0.020±0.001 and 0.018±0.001 μmol·mg^{-1} respectively. The exercise control HSR had a glycogen concentration of 0.019±0.002 μmol·mg^{-1} with the treated SR being no different at 0.018±0.002 μmol·mg^{-1}.

Correlation between glycogen concentration and SR function

Figure 6 shows the rate of calcium uptake plotted against the concentration of glycogen associated with the LSR fraction. The correlation coefficient was –0.223 and was not significant.

The HSR uptake and release were plotted against the glycogen concentration in Figures 7 and 8 accordingly. Neither correlation was significant. The correlation coefficient for uptake and glycogen was 0.214 and the coefficient for release and glycogen was 0.183.

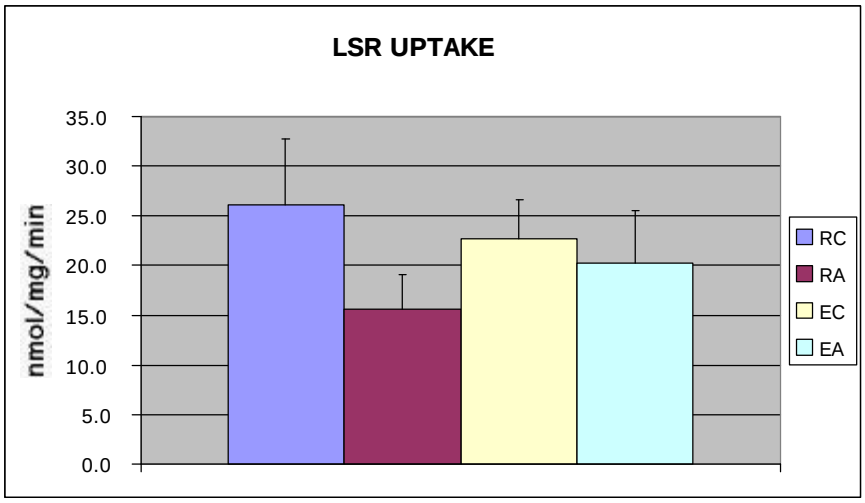

Fig. 1 Rate of calcium uptake by LSR fraction in $nmol \cdot mg^{-1} \cdot min^{-1}$.
(rested control, RC; rested amylase, RA; exercise control, EC; exercise amylase, EA)

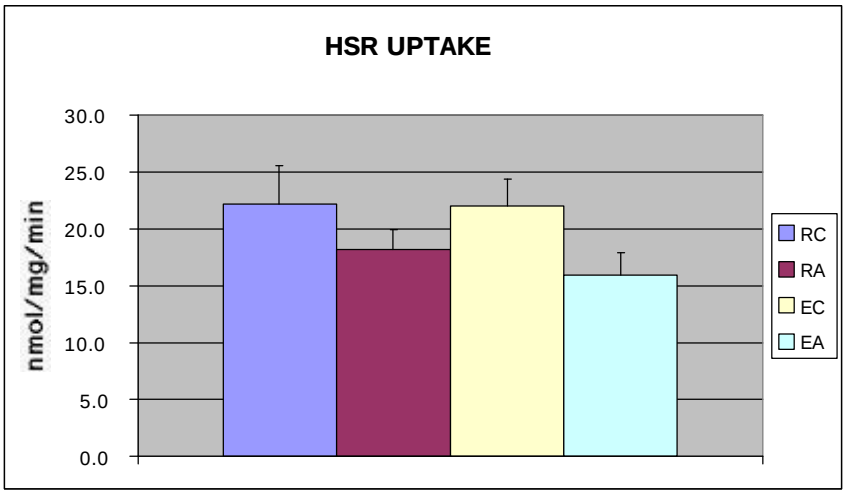

Fig. 2 Rate of calcium uptake by HSR fraction in nmol·mg^{-1}·min^{-1}.
(rested control, RC; rested amylase, RA; exercise control, EC; exercise amylase, EA)

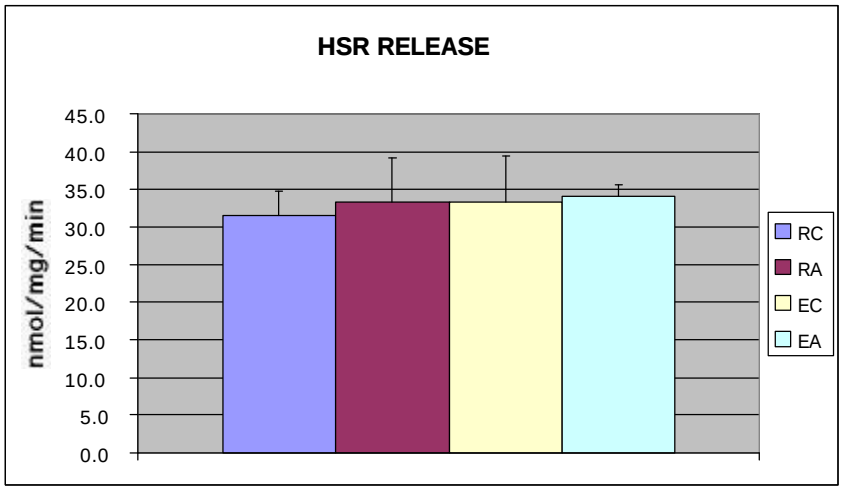

Fig. 3 Rate of calcium release by HSR fraction in nmol·mg^{-1}·min^{-1}.
(rested control, RC; rested amylase, RA; exercise control, EC; exercise amylase, EA)

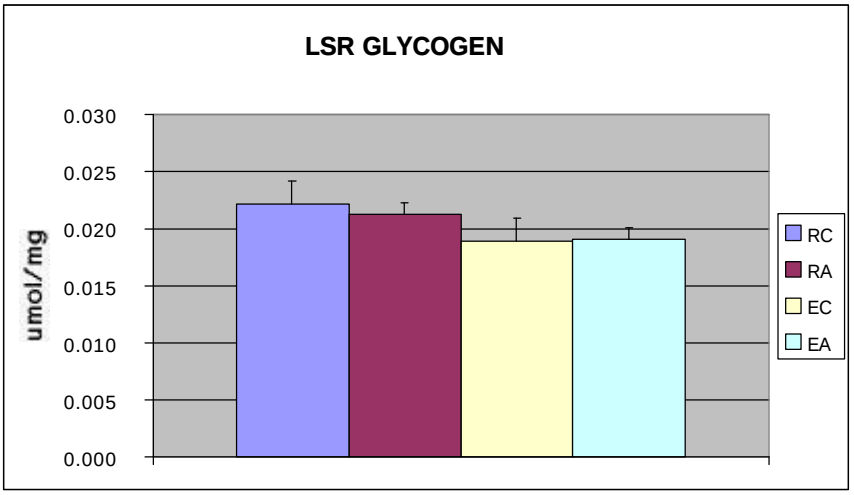

Fig. 4 Concentration of glycogen associated with LSR fraction in μmol·mg^{-1} protein. (rested control, RC; rested amylase, RA; exercise control, EC; exercise amylase, EA)

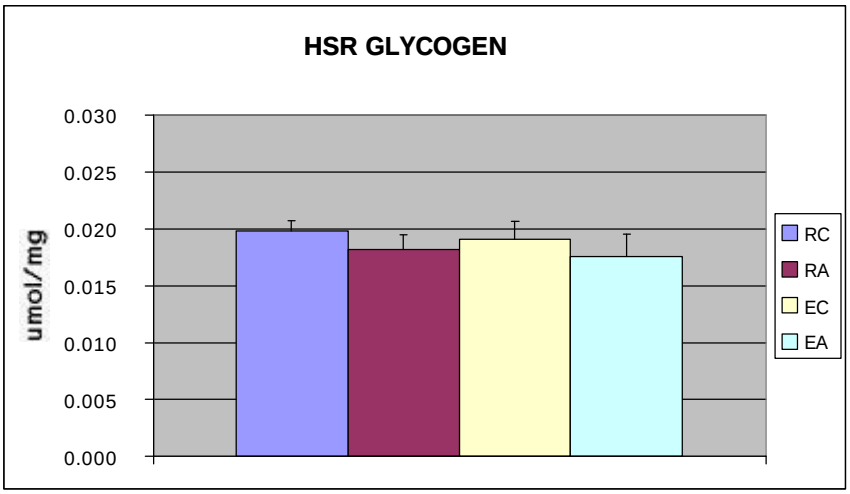

Fig. 5 Concentration of glycogen associated with HSR fraction in μmol·mg^{-1} protein. (rested control, RC; rested amylase, RA; exercise control, EC; exercise amylase, EA)

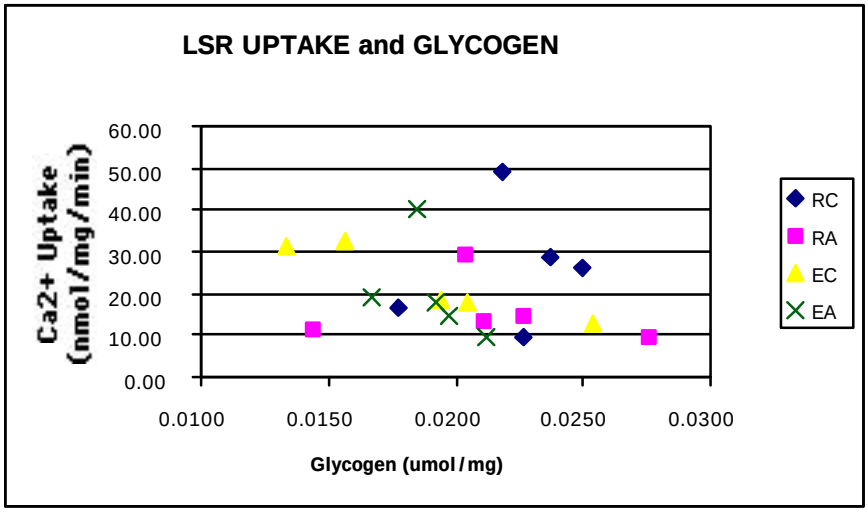

Fig. 6 Correlation between rate of calcium uptake by LSR ($nmol \cdot mg^{-1} \cdot min^{-1}$) and concentration of glycogen ($\mu mol \cdot mg^{-1}$)
(rested control, RC; rested amylase, RA; exercise control, EC; exercise amylase, EA)

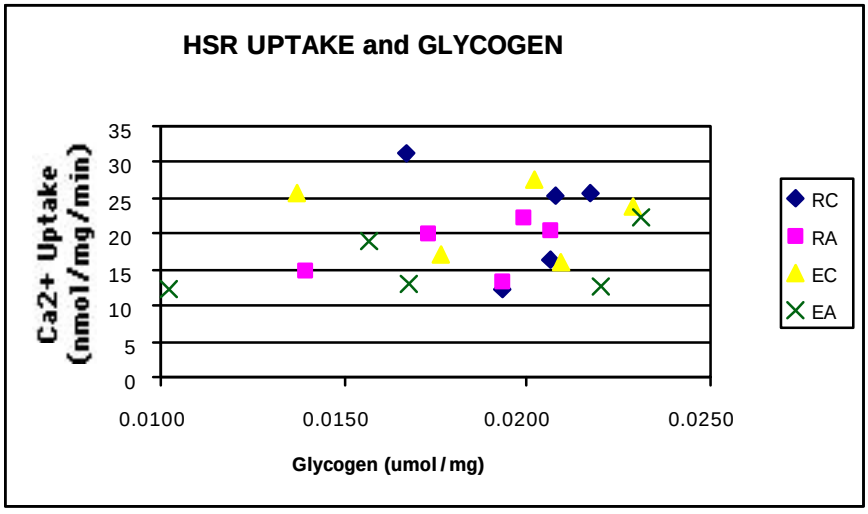

Fig. 7 Correlation between rate of calcium uptake by HSR ($nmol \cdot mg^{-1} \cdot min^{-1}$) and concentration of glycogen ($\mu mol \cdot mg^{-1}$)
(rested control, RC; rested amylase, RA; exercise control, EC; exercise amylase, EA)

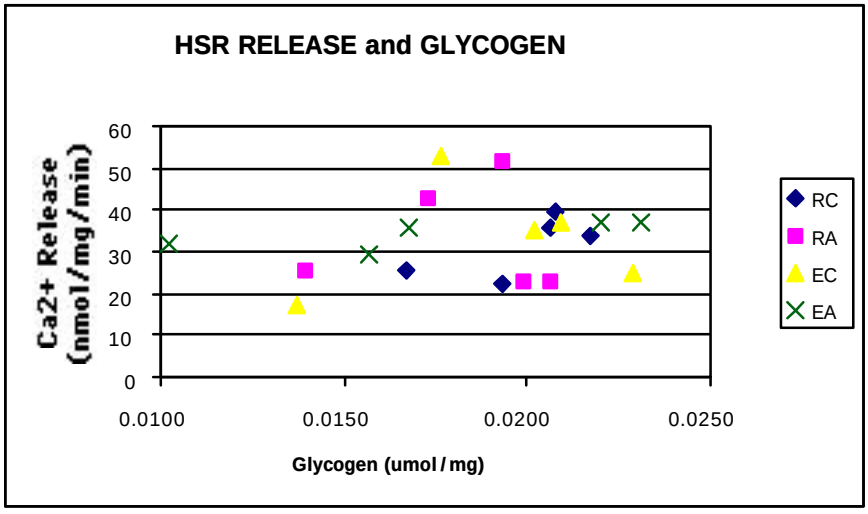

Fig. 8 Correlation between rate of calcium release by HSR (nmol·mg^{-1}·min^{-1}) and concentration of glycogen (μmol·mg^{-1})
(rested control, RC; rested amylase, RA; exercise control, EC; exercise amylase, EA)

CHAPTER 5

Discussion

Discussion

These data show that there was no effect of treadmill running (60 min, 10% incline, 21 m/min) on the ability of the SR to uptake and release calcium. This is in contrast to what Byrd et al. (1989) showed with rats that ran at the same speed and incline for 15 minutes less. In their study, the rate of Ca^{2+} uptake was ~60% of the control after 45 min of running. Also, Luckin and colleagues (1991) showed that there was a 40% decrease in Ca^{2+} ATPase activity, the protein that is involved with calcium uptake, after running to exhaustion with the same grade and speed used in this study. After running rats to exhaustion using the same protocol as Luckin et al.(1991), Favero and associates (1993) were able to demonstrate that the Ca^{2+} release rate was depressed by 20-30% compared to controls after release was initiated with varying concentrations of $AgNO_3$, a known releasing agent. Calcium release rate was unaffected in this study, possibly because the duration was not long enough. In the present study rats ran for only 60 min, whereas Favero et al.(1993) ran their rats to fatigue (mean run time=115 min). Sixty minutes may not have been sufficient to fatigue the rats to the extent where depressions in release rate would occur. The run duration may also not have been sufficient to induce changes in the rate of Ca^{2+} uptake either, but Byrd et al. (1989) was able to show depression in the rate after only 45 min. It may have been that the rats in this study where more reluctant to run and were more willing to sit on the electric grid for longer periods of time and resist other means of encouragement thereby getting sufficient rest as to not fatigue in 60 min. Other differences include the body mass of the animals, procedures used to isolate the SR and techniques used to measure SR Ca^{2+} handling.

The results of this study also suggest that there is a significant effect of α-amylase on the ability of the SR to take up Ca^{2+}. In the LSR fraction, the rate of calcium uptake was depressed by 22.89% due to α-amylase treatment when compared to controls. The HSR uptake rate was also depressed due to the treatment but only by 25.22% in comparison to the controls. In a preliminary report, Brautigan et al. (1979) noted that glycogen digestion by α-amylase suppressed the activity of Ca^{2+} ATPase, which would reduce the rate of calcium uptake into the SR. Calcium release was unaffected by the treatment in both fraction. Of the studies that have looked at the glycogenolytic-SR complex, a number have connected the complex with calcium uptake (Cuenda et al., 1993; Nogues et al., 1996) yet none have demonstrated any effect on the ability of the SR to release calcium into the myoplasm.

It has been speculated that the decreased ability of the SR to sequester calcium during fatigue is due to energy depletion, but this has been refuted by Allen (1995) noting that a number of studies have shown that ATP levels remain stable even during fatiguing exercise. Others have qualified the energy depletion theory by saying that it may be a local depletion of ATP around the Ca^{2+} ATPase versus a whole muscle effect (Han et al., 1992; Korge et al., 1994; Korge, 1995). This is supported by the findings of others that have added glycolytic intermediates to SR vesicle preparations in the absence of exogenous ATP and Ca^{2+} uptake has occurred (Montero-Lomeli & de Meis, 1992; Cuenda et al., 1993; Xu et al., 1995; Nogues et al., 1996). All of this information suggests that the local ATP concentration around the calcium pump is more important that the total cell ATP equilibrium. This is especially important since the a depression in the local concentration may occur and reduce Ca^{2+} uptake while there is no significant effect on the total

concentration. While this may be important *in situ*, it is not a possibility in the preparation used here, because during the protocol ATP in excess is added to assure an energy supply is not limiting. Therefore, the effect of α-amylase is not due to energy depletion, but may be due to the removal of the glycogen from the SR membrane or another unknown effect of the enzyme.

Although it was demonstrated that there was an effect of α-amylase on the ability of the SR to sequester calcium, there was no significant difference between treatment and control SR preparation in the glycogen concentration. Alpha-amylase is known to digest glycogen and has been used before to digest the glycogen associated with the SR membrane (Wanson & Drochmans, 1972; Brautigan et al., 1979; Goldstein et al ., 1985). Cuenda et al. (1994) showed that treatment of isolated SR vesicles with α-amylase removed 95% of initial phosphorylase bound to the membrane, most likely through removal of the glycogen particles. Most studies that have utilized α-amylase to digest glycogen associated with the SR have incubated their preparation with 0.1% (w/v) of the enzyme for 2 h at 37°C. This temperature is too high to maintain the integrity of the SR membranes which is vital to the calcium uptake and release experiments. Others that have looked at the coupling between the complex and the ability of the SR to regulate $[Ca^{2+}]_i$ have looked at the Ca^{2+} ATPase, which is unaffected by this temperature.

While this physiological temperature is commonly used, α-amylase has been shown to be active at temperatures well below 0°C (Hiranpradit, 1974). With respect to complete glycogen removal, ample time for the enzyme to digest the substrate may not have been provided. Although it was shown to remain active below 0°C, Hiranpradit (1974) noted that extended time was necessary for the enzyme to be as effective compared to higher

temperatures. Also of importance was that the α-amylase in this study was added to the homogenate before the first step of the differential centrifugation. Goldstein et al. (1985) as well as Wanson & Drochmans (1972) added the enzyme to the SR fractions itself. There may have been too much glycogen in the homogenate for the α-amylase to digest at the lower temperature during the reduced time.

In spite of the reduced ability of the treated SR fractions to take up calcium, no difference was shown in glycogen concentration between the groups, which might indicate that the assay utilized was not sensitive enough for the amount of glycogen that remained after the exercise and treatment with α-amylase. The results of the Trinder assay calculated the average glycogen concentration of all of the groups to be 0.021 $\mu mol \cdot mg^{-1}$ protein. Michalak et al. (1977) found the range of glycogen in the HSR fraction to be 0.5-0.6 $\mu g \cdot mg^{-1}$ of muscle and in the LSR the range was found to be higher, 0.8-1.0 $\mu g \cdot mg^{-1}$. The assay may not be able to accurately assess the small amount of glycogen associated with the membrane of the SR, therefore it would be able to identify a change due to exercise or enzymatic treatment.

Although there was no difference between treatments or activities, there still might have been a change in the concentration in glycogen associated with the membrane, but the assay was unable to detect the variation. The loss of the glycogen from the membrane may have disrupted the SR's ability to sequester calcium. It has been shown that glycogen particles are more densely associated with the terminal cisternae of the SR where most of the Ca^{2+} pumps are located (Friden et al., 1989). In support of this, Xu et al. (1998) used electron microscopy and immunogold labeling to locate glycolytic enzymes in close proximity to Ca^{2+} ATPase. It is known that glycolytic enzymes are bound to the SR

membrane through glycogen particles (Xu et al., 1995; Cuenda et al., 1994; Wanson & Drochmans, 1968). Therefore, a loss of glycogen closely associated with Ca^{2+} ATPase may cause a conformational change of the protein thereby inhibiting uptake. In keeping with a non-metabolic association of the glycogen with the calcium sequestering protein, the carbohydrate may be structural in purpose and its removal may disrupt membrane integrity which may allow the protein to rotate or otherwise be rendered ineffective. Another reason for the inhibition of Ca^{2+} uptake, it may be possible for the glycogen to act as an anchor for other protein that are involved with the sequestering of calcium, such as sarcolypin. Removal of the glycogen particle may cause the protein to be released and thereby not be able to participate in the reduction of $[Ca^{2+}]_i$.

More research is necessary to accurately identify the role of the glycogen-SR complex in the maintenance of calcium homeostasis by the SR vesicle. This study was able to show a decrease in calcium uptake by the SR after treatment with α-amylase, but there was no link identified with the removal of the glycogen particles because of the poor resolution of the assay. This study was also unable to show an effect of exercise, which has previously been shown to reduce the calcium handling abilities of the SR (Byrd et al. 1989; Favero et al., 1993). Therefore, the following are suggestions to improve upon this study for future application:

1. Increase the duration of the run protocol to fatigue versus 60 min. This would allow for comparison of exercise induced glycogen depletion and chemical glycogen extraction.
2. Add α-amylase directly to the vesicle preparations as was done by Goldstein et al. (1985) and Wanson & Drochmans (1972). This would insure that the

enzyme would be able to work directly on the SR membrane and not be inhibited by other structures still in the homogenate.

3. Increase the duration of incubation with α-amylase. Hiranpradit (1974) demonstrated that α-amylase is active at temperatures well below 0°C, but more time was necessary to accomplish similar digestion as at the higher temperatures. Even at 37°C, others have incubated for two hours versus one (Goldstein et al., 1985; Wanson & Drochmans, 1972).
4. Utilize a glycogen assay that is more sensitive. The small sample sizes due to the small amount of tissue and the differential centrifugation necessitate use of an assay that has a higher resolution. Pooling of samples may also be of benefit.
5. Using all of the previous suggestions, the study could be furthered by repeating the measurements after adding glycogen back into the preparation. This would serve to possibly shed light onto the incorporation of exogenous glycogen into the SR membrane

The results of this study indicate that treatment of both HSR and LSR fraction with α-amylase reduces the ability of the fractions to sequester calcium ions. Most likely this attenuation in function is due to the removal of glycogen particles associated with the membrane, although this was not shown by the glycogen assay. More investigation is necessary in the relationship between glycogen and the SR to fully elucidate the function of this complex.

Appendix A

Table Two-Way (Activity X Condition) repeated measures ANOVA for LSR uptake

Source of Variation	DF	SS	MS	F	P
Activity	1	7.405	7.405	0.191	0.673
Col 1 (Activity)	8	309.504	38.688		
Condition	1	128.069	128.069	6.096	0.039
Activity X Condition	1	5.886	5.886	0.280	0.611
Residual	8	168.077	21.010		
Total	19	618.941	32.576		

Power of performed test with alpha=0.0500: for Activity: 0.0500
Power of performed test with alpha=0.0500: for Condition: 0.505
Power of performed test with alpha=0.0500: for Activity x Condition: 0.0500

Table Two-Way (Activity X Condition) repeated measures ANOVA for HSR uptake

Source of Variation	DF	SS	MS	F	P
Activity	1	2.106	2.106	.00949	0.925
Col 1 (Activity)	8	1775.491	221.936		
Condition	1	208.077	208.077	7.579	0.025
Activity X Condition	1	82.947	82.947	3.021	0.120
Residual	8	219.650	27.456		
Total	19	2288.270	120.435		

Power of performed test with alpha=0.0500: for Activity: 0.0500
Power of performed test with alpha=0.0500: for Condition: 0.615
Power of performed test with alpha=0.0500: for Activity x Condition: 0..231

Table Two-Way (Activity X Condition) repeated measures ANOVA for HSR release

Source of Variation	DF	SS	MS	F	P
Activity	1	9.072	9.072	0.0637	0.807
Col 1 (Activity)	8	1138.601	142.325		
Condition	1	7.676	7.676	0.112	0.746
Activity X Condition	1	0.912	0.912	0.0133	0.911
Residual	8	548.137	68.517		
Total	19	1704.398	89.705		

Power of performed test with alpha=0.0500: for Activity: 0.0500
Power of performed test with alpha=0.0500: for Condition: 0.0500
Power of performed test with alpha=0.0500: for Activity x Condition: 0.0500

Table Two-Way (Activity X Condition) repeated measures ANOVA for LSR glycogen concentration

Source of Variation	DF	SS	MS	F	P
Activity	1	0.00000252	0.00000252	0.123	0.735
Col 1 (Activity)	8	0.000164	0.0000206		
Condition	1	0.0000120	0.0000120		
Activity X Condition	1	0.0000000125	0.0000000125	0.00264	0.960
Residual	8	0.0000379	0.00000473		
Total	19	0.000217	0.0000114		

Power of performed test with alpha=0.0500: for Activity: 0.0500
Power of performed test with alpha=0.0500: for Condition: 0.186
Power of performed test with alpha=0.0500: for Activity x Condition: 0.0500

Table Two-Way (Activity X Condition) repeated measures ANOVA for HSR glycogen concentration

Source of Variation	DF	SS	MS	F	P
Activity	1	0.0000381	0.0000381	1.755	0.222
Col 1 (Activity)	8	0.000174	0.0000217		
Condition	1	0.00000080	0.00000080	0.146	0.713
Activity X Condition	1	0.00000168	0.00000168	0.306	0.595
Residual	8	0.0000439	0.00000549		
Total	19	0.000258	0.0000136		

Power of performed test with alpha=0.0500: for Activity: 0.115
Power of performed test with alpha=0.0500: for Condition: 0.0500
Power of performed test with alpha=0.0500: for Activity x Condition: 0.0500

RAW DATA

RAT	ACTIVITY	CONDITION	CONC GLYCOGEN umol/mg pro	HSR UPTAKE nmol/mg pro/min	HSR RELEASE nmol/mg pro/min
5LH	R	A	0.0206	20.35	23.22
5RH	R	C	0.0167	31.1	25.76
6LH	E	A	0.0231	22.27	36.74
6RH	E	C	0.0229	23.83	24.67
7LH	R	A	0.0199	22.21	22.78
7RH	R	C	0.0206	16.39	35.97
8LH	E	A	0.0220	12.81	37.07
8RH	E	C	0.0209	16.11	36.79
9LH	R	A	0.0193	13.49	51.69
9RH	R	C	0.0208	25.17	39.71
10LH	E	A	0.0168	12.89	35.45
10RH	E	C	0.0176	17.04	52.92
11LH	R	A	0.0173	20.02	42.64
11RH	R	C	0.0217	25.81	34.02
12LH	E	A	0.0157	19.16	29.51
12RH	E	C	0.0202	27.58	35.17
13LH	R	A	0.0140	14.91	25.77
13RH	R	C	0.0193	12.39	22.31
14LH	E	A	0.0102	12.34	31.93
14RH	E	C	0.0137	25.64	17.09
RAT	ACTIVITY	CONDITION	CONCENTRATION	LSR UPTAKE	
5LL	R	A	0.0204	29.07	
5RL	R	C	0.0219	49.01	
6LL	E	A	0.0185	40.23	
6RL	E	C	0.0157	32.46	
7LL	R	A	0.0276	9.29	
7RL	R	C	0.0227	9.64	
8LL	E	A	0.0212	9.27	
8RL	E	C	0.0254	12.80	
9LL	R	A	0.0227	14.65	
9RL	R	C	0.0237	28.72	
10LL	E	A	0.0197	14.94	
10RL	E	C	0.0195	18.69	
11LL	R	A	0.0211	13.42	
11RL	R	C	0.0250	26.19	
12LL	E	A	0.0192	17.65	
12RL	E	C	0.0205	17.97	
13LL	R	A	0.0144	11.40	

13RL	R	C	0.0178	16.89	
14LL	E	A	0.0167	19.35	
14RL	E	C	0.0133	31.41	

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VITA

Benjamin James Toderico was born in South Portland Maine. Through the sports that he was involved in throughout middle and high school, he developed his interest in the body specifically exercise science.

After graduating from South Portland High School, Ben went on to enroll in University Studies at Virginia Tech, still not sure of the degree he wanted to pursue. During his sophomore year Ben transferred over to what was then Exercise Science under the Physical Education Department.

In the Spring of 1997 after graduating with Honors, the opportunity to attend graduate school at Virginia Tech presented an assistantship in the Basic Instruction Program. Ben spent two years in the pursuit of his Master's degree all the while looking for what he would spend his life doing.