# Effects of Ischemia and Ischemia-Reperfusion on Sarcoplasmic Reticulum Structure and Function in Rat Skeletal Muscle

by

Allan Russell Tupling

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**Abstract:**

The primary objective for this thesis was to investigate the changes that occur in the regulation of intracellular $Ca^{2+}$ ($[Ca^{2+}]_f$) in skeletal muscle by the sarcoplasmic reticulum (SR) during ischemia (I) and ischemia-reperfusion (IR). In particular, the effects of I and IR on SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase activity, measured in homogenates and isolated SR vesicles in vitro, were determined. In addition, the fluorescent probes, fluorescein isothiocyanate (FITC) and [(N-cyclohexyl-N'-dimethylamino-α-napthyl) carbodiimide] (NCD-4), were used to measure structural alterations to the ATP binding site and the $Ca^{2+}$ binding sites of the $Ca^{2+}$-ATPase, respectively, with I and IR.

In the first study, an in vivo model of prolonged I in rat skeletal muscle was employed. To induce total I, a tourniquet was placed around the upper hindlimb in 30 female Sprague-Dawley rats, weighing 256 ± 6.7g (mean ± SE), and inflated to 350 mmHg for 4 hours. In the second study, using 40 female Sprague-Dawley rats, weighing 290 ± 3.5g (mean ± SE), the tourniquet was deflated and removed for a period of 1 hour (reperfusion), following the 4 hour ischemic period. In half of the animals, a single dose of superoxide dismutase (SOD) + catalase (CAT) was administered just prior to reperfusion (R). In both studies, the contralateral limb served as control (C). Immediately following the experimental protocols, mixed gastrocnemius and tibialis anterior muscle were sampled from both limbs, and both homogenates and SR vesicles were prepared. In another 10 control animals (CC), muscle was sampled and prepared exactly the same way, but immediately following anesthetization.

Results from the first study indicated that sub-maximal $Ca^{2+}$ uptake, measured with Indo-1, was lower ($p<0.05$) with I compared with CC and C, by 25 and 45% in homogenates and SR vesicles, respectively. Silver nitrate ($AgNO_3$)-induced $Ca^{2+}$ release, which occurred in 2 phases, was also lower ($p<0.05$) with I compared with CC and C, in both muscle homogenates and SR vesicles. With I, Phase 1 peak $Ca^{2+}$ release was 26% lower in SR vesicles only. For Phase 2, peak $Ca^{2+}$ release was 54 and 24% lower in SR vesicles and homogenates, respectively. In the presence of cyclopiazonic acid (CPA), an inhibitor of SR $Ca^{2+}$-ATPase activity, Phase 1 $Ca^{2+}$ release rates were higher ($p<0.05$) and Phase 2 $Ca^{2+}$ release rates were lower ($p<0.05$) in all groups. However, the decrease in Phase 2 $Ca^{2+}$ release with CPA was blunted ($p<0.05$) in I compared with CC and C. Maximal $Ca^{2+}$-

ATPase activity was depressed by 34 and 27% ($p<0.05$) with I compared with CC and C, respectively, but only in isolated SR vesicles. No differences were found between groups in either the Hill coefficient or the $[Ca^{2+}]_f$ at half-maximal activity ($Ca_{50}$). The fluorescent probes, FITC and NCD-4, only indicated reductions ($p<0.05$) in FITC binding capacity (26% lower with I as compared with both CC and C). Total SERCA1 protein, as determined by SDS-PAGE and Western blot analysis, was not affected by I, however, evidence for greater protein aggregation with I was observed.

In the second study of ischemia-reperfusion (IR), $Ca^{2+}$ uptake was lower ($p<0.05$) with IR compared with CC, by 35% in purified SR vesicles. There were no differences between CC and C. In muscle homogenates, $Ca^{2+}$ uptake although 20-25% lower ($p<0.05$) in C, compared with CC, was not different in the IR muscle. $Ca^{2+}$ release, in muscle homogenates, using $AgNO_3$-induced $Ca^{2+}$ release, were not different between any of the groups for either Phase 1 or Phase 2. However, in purified SR vesicles, maximal $Ca^{2+}$ release was 31% higher ($p<0.05$) with IR, compared with CC for Phase 1, with no difference between CC and C. No differences existed between any of the groups for Phase 2 in purified SR vesicles. For $Ca^{2+}$-ATPase activity, there were no differences between groups in either homogenates or isolated SR vesicles. SOD + CAT treatment protected against reductions in homogenate $Ca^{2+}$ uptake in C, compared with CC ($p<0.05$) but had no effect on any other measure, including mechanical function.

It is concluded that prolonged I alone reduces SR $Ca^{2+}$ uptake and $Ca^{2+}$ release measured in vitro in both muscle homogenates and SR vesicles, and that reductions in maximal $Ca^{2+}$-ATPase activity with I are implicated in the impaired SR $Ca^{2+}$ transport that occurs, but only in SR vesicles. Furthermore, the reduction in maximal SR $Ca^{2+}$-ATPase activity in SR vesicles with I is related to structural modification of the nucleotide binding domain, probably as a result of free-radical induced oxidation of one or more cysteines. Secondly, a short period of R following prolonged I, largely restores SR function when assessed in vitro, but may affect SR function in contralateral control skeletal muscle. With IR, SOD + CAT treatment was without effect on both SR and mechanical function in ischemic muscle, but did prevent alterations in SR $Ca^{2+}$ uptake from occurring in contralateral control muscle. However, the results depend on whether SR function was

assessed in homogenates or isolated SR vesicles, suggesting that a differential yield of SR vesicles is obtained during isolation following IR compared with control.

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**Dedication**

This thesis is dedicated to my beautiful wife, Susan Tupling (Grant). Undoubtedly, our marriage and the birth of our first child, Olivia Leslie Tupling, were my greatest accomplishments in the past 3 years. Thanks for your wisdom, support and understanding – you are my rock! Together, we will pass on the love of learning to our children.

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## List of Abbreviations

ADP – adenosine diphosphate

$AgNO_3$ – silver nitrate

AMP – adenosine monophosphate

ATP – adenosine triphosphate

C – contralateral control limb group

$[Ca^{2+}]_f$ – intracellular free calcium concentration

$Ca_{50}$ – the $[Ca^{2+}]_f$ at which half-maximal $Ca^{2+}$-ATPase activity occurs

CAT – catalase

CC – control animal, control control group

CPA – cyclopiazonic acid

Cr – creatine

CRC – SR $Ca^{2+}$ release channel

CT – control treatment limb

DSC – differential scanning calorimetry

DTT – dithiothreitol

E – experimental animal

EDL – extensor digitorum longus muscle

ET – experimental treatment animal

FITC – fluorescein isothiocyanate

G – gastrocnemius muscle

HSP – heat shock protein

I – ischemia, ischemic group

IMP – inosine monophosphate

IR – ischemia-reperfusion

IT – ischemic treatment limb

$L_o$ – optimum muscle length for maximum twitch force

NCD-4 – (N-cyclohexyl-N'-dimethylamino-α-napthyl) carbodiimide

$O_2$ – oxygen

PCa – the negative logarithm of $[Ca^{2+}]_f$

PCr – phosphocreatine

Pi – inorganic phosphate

$P_t$ – peak twitch tension

R – reperfusion

SH – sulfhydryl

SOD – superoxide dismutase

SR – sarcoplasmic reticulum

TA – tibialis anterior muscle

$T_m$ – thermal denaturation temperature

# CHAPTER I

## INTRODUCTION, REVIEW OF THE LITERATURE AND STATEMENT OF THE PROBLEM

## INTRODUCTION

There are many clinical situations in which skeletal muscles are subjected to ischemia (I) and I followed by reperfusion (R). The biochemical mechanisms underlying the ischemia-reperfusion (IR) induced damage that occurs has been the subject of a number of recent studies. Muscle cells subjected to I are characterized by a loss in mechanical function first, followed by progressive structural damage and possible necrosis upon R, with minimal or no functional recovery depending on the length of the ischemic period (Blaisdell, 1989; Rubin *et al.*, 1992; Carvalho *et al.*, 1997).

Prolonged muscle I leads to large increases in lactate, reductions in glycogen and pH (Carvalho *et al.*, 1995a; Harris *et al.*, 1986; Welsh and Lindinger, 1993), depletion of phosphocreatine (PCr) with near stoichiometric increases in inorganic phosphate (Pi) and creatine (Cr) (Carvalho *et al.*, 1995a; Harris *et al.*, 1986; Idström *et al.*, 1990; Rubin *et al.*, 1992; Soussi *et al.*, 1993; Welsh and Lindinger, 1993) and reductions in total adenine nucleotides (ATP + ADP + AMP + IMP) with concomitant increases in dephosphorylated nucleosides and bases (Carvalho *et al.*, 1995a; Idström *et al.*, 1990; Rubin *et al.*, 1992; Soussi *et al.*, 1993).

Metabolic recovery during R is highly dependent on the length of the previous ischemic insult (Carvalho *et al.*, 1995b: Harris *et al.*, 1986; Idström *et al.*, 1990; Soussi *et al.*, 1993). During R following prolonged I (i.e., 2-4 hours), there is little or no recovery of PCr (Harris *et al.*, 1986; Idström *et al.*, 1990), even further reductions in total adenine nucleotides (Harris *et al.*, 1986; Idström *et al.*, 1990; Rubin *et al.*, 1992) and no recovery in glycogen (Harris *et al.*, 1986). However, lactate returns to resting levels even after 7 hours of I and 3 hours R (Harris *et al.*, 1986).

When ischemic muscles are reperfused, damage may occur due to a) a net loss of phosphorylated adenine nucleotides and associated elevations in selected metabolic by-products b) production of oxygen ($O_2$) free radicals or c) intracellular calcium ($[Ca^{2+}]_f$) overload (Rubin *et al.*, 1996). Since the sarcoplasmic reticulum (SR) is the primary organelle responsible for maintaining $Ca^{2+}$ homeostasis within the cell, which is an energy dependent process, and since the SR may be a principal site of attack by free radicals (Kukreja *et al.*, 1988), studying the effects of I and IR on SR function and the possible role of the SR in IR-induced muscle damage would appear appropriate.

Numerous studies have reported increased levels of $[Ca^{2+}]_f$ during I and IR in both cardiac and skeletal muscle (Jones *et al.*, 1983; Klenerman *et al.*, 1995; Welsh and Lindinger, 1996). Evidence has also been provided for beneficial effects of preventing the rise in $[Ca^{2+}]_f$ (Jones *et al.*, 1984; Klenerman *et al.*, 1995; Welsh and Lindinger, 1996). It has been proposed that calcium may cause IR cell membrane damage indirectly by activating phospholipase A2 (Jackson *et al.*, 1984; Klenerman *et al.*, 1995). Calcium may also indirectly lead to protein damage due to its activation of the neutral protease, calpain (Belcastro *et al.*, 1996). Since the SR may be susceptible to both types of $Ca^{2+}$-induced damage, the ability of the SR to actively sequester $Ca^{2+}$ may be compromised during conditions of sustained elevations in $[Ca^{2+}]_f$.

In the face of raised $[Ca^{2+}]_f$, mitochondria are forced to take up calcium to try to maintain intracellular $Ca^{2+}$ homeostasis (Miller and Tormey, 1995; Siegmund *et al.*, 1993). This may be counterproductive for the muscle cell because mitochondrial calcium overload leads to an inability of mitochondria to produce ATP (Benzi and Lerch, 1992; Carafoli, 1987; Ferrari *et al.*, 1982; Peng *et al.*, 1980). Indeed, it has been shown that ruthenium red,

an inhibitor of mitochondrial calcium uptake, improves mitochondrial energy production and protects against IR injury (Benzi and Lerch, 1992; Ferrari *et al.*, 1982; Peng *et al.*, 1980). However, reduced mitochondrial calcium uptake may be secondary to reductions in calcium release from the SR, since ruthenium red also blocks SR calcium release (Melzer *et al.*, 1995). The effects of ruthenium red on mitochondrial calcium uptake and SR calcium release can not be differentiated unless $[Ca^{2+}]_f$ is monitored.

As mentioned, the SR is primarily responsible for maintaining calcium homeostasis within the muscle cell. It has been shown repeatedly that I and IR leads to a reduction in SR $Ca^{2+}$-ATPase activity and $Ca^{2+}$ uptake, when measured in vitro (Green *et al.*, 1997; Kaplan *et al.*, 1992; Meno *et al.*, 1984). These results imply that the rate of active $Ca^{2+}$ uptake is reduced during I and IR, probably due to structural alterations to the $Ca^{2+}$-ATPase and/or the SR membrane. It is also widely accepted that the accumulation of various metabolic by-products such as ADP, $P_i$ and $H^+$ directly impair SR $Ca^{2+}$-transport function in vivo (Inesi and Hill, 1983; Lamb *et al.*, 1992; Zhu and Nosek, 1991). Therefore, the early metabolic disturbances that occur with I (Carvalho *et al.*, 1995a; Harris *et al.*, 1986; Idström *et al.*, 1990) also probably impair SR function and contribute to the rise in $[Ca^{2+}]_f$ that is observed with I.

In the face of a depressed SR function, the cell is not only unable to regulate the intracellular stores of calcium, but must contend with greater amounts of extracellular calcium which enters the cell during I as well (Jones *et al.*, 1984; Meno *et al.*, 1984; Welsh and Lindinger, 1996). The contribution of extracellular calcium to IR injury is illustrated by the protective effects of calcium antagonist agents such as verapamil (Ruth *et al.*, 1988; Watts *et al.*, 1980), diltiazem (Meno *et al.*, 1984; Welsh and Lindinger, 1996) and 3,4-

dichlorobenzamil (Welsh and Lindinger, 1996). Extracellular calcium may enter the cell through the L-type $Ca^{2+}$ channel and via the $Na^{+}/Ca^{2+}$ exchanger, both of which contribute to the rise in intracellular calcium that occurs in skeletal muscle during IR (Welsh and Lindinger, 1996).

During skeletal muscle I and IR, where the muscle is quiescent, the major energy utilizing processes are involved in maintaining ionic homeostasis in the cell, such as $Ca^{2+}$, $Na^{+}$ and $K^{+}$ homeostasis. Since $Ca^{2+}$-ATPase activity is a function of $[Ca^{2+}]_f$ (Simonides and van Hardeveld, 1990), rises in $[Ca^{2+}]_f$ would increase the rate of ATP utilization by the ischemic cell. However, with an accelerated ATP utilization rate, ATP levels fall and glycolytic activity increases resulting in increased levels of metabolites that are known to inhibit $Ca^{2+}$-ATPase activity. This in turn would exacerbate the increase in $[Ca^{2+}]_f$. Figure 1.1 provides an overall model for possible factors that may affect calcium homeostasis within the muscle cell.

In skeletal muscle, most of the $Ca^{2+}$ is stored primarily bound to calsequestrin within the terminal cisternae of the SR (for review, see Pozzan *et al.*, 1994) and released in response to muscle stimulation, through the SR $Ca^{2+}$-release channel (CRC), to initiate contraction. Therefore, the SR CRC, known as the ryanodine receptor due to its high affinity for the neutral plant alkaloid, ryanodine (Fleischer *et al.*, 1985), plays a very important role in regulating $[Ca^{2+}]_f$ in skeletal muscle.

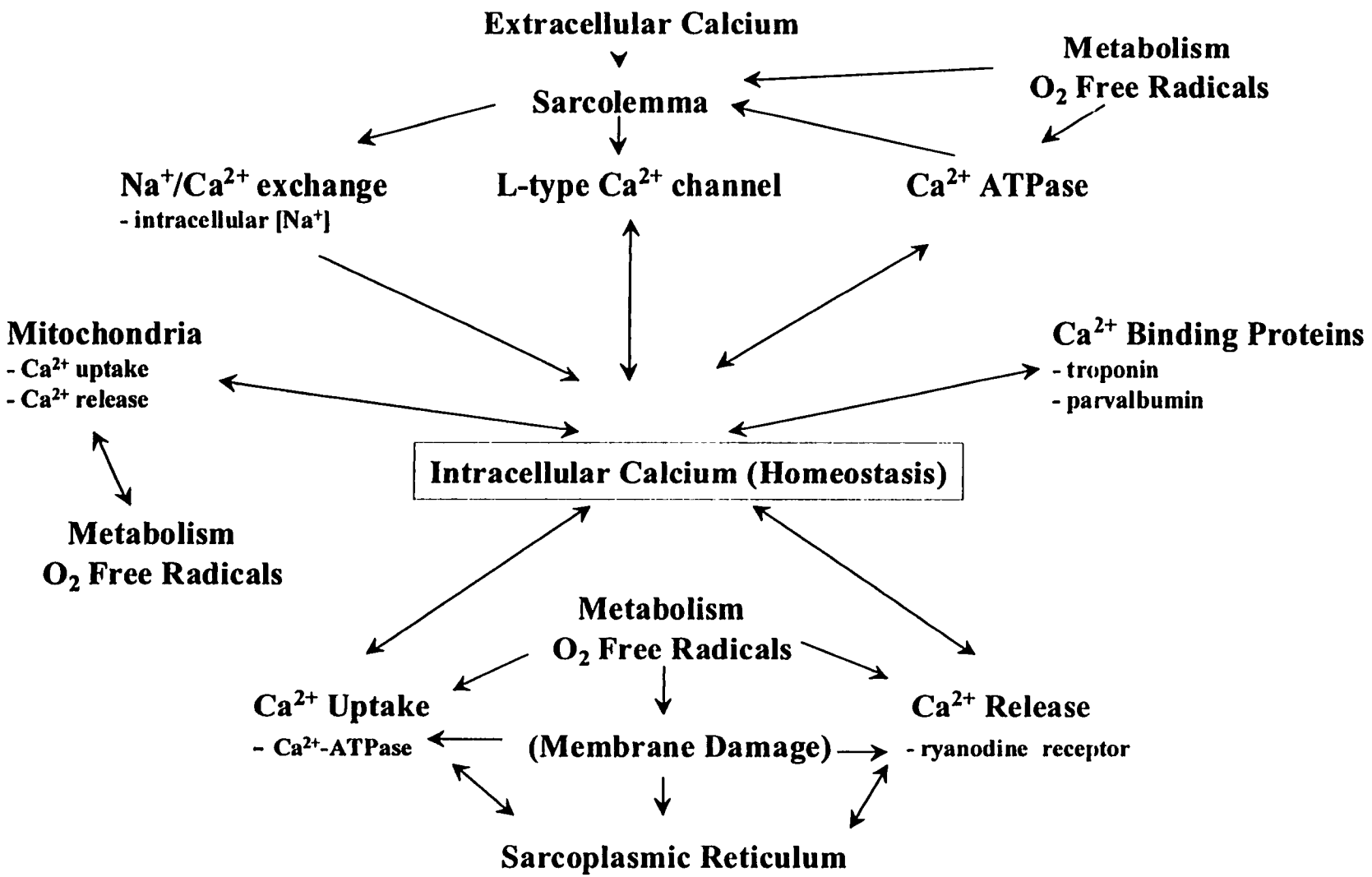



**Figure 1.1. A schematic diagram illustrating the factors which regulate intracellular calcium homeostasis in the muscle cell. Notice the double headed arrows which demonstrate how calcium itself may affect the regulation of its intracellular levels.**

The skeletal muscle CRC is a large homotetramer containing four sub-units. Cloning and sequencing of the rabbit muscle CRC and electron microscopic studies have revealed a primary structure consisting of 5037 amino acids, resulting in a calculated relative molecular mass of about 565 kDa (Takeshima *et al.*, 1989). The primary structure of the CRC predicts potential regulatory binding sites for cytoplasmic $Ca^{2+}$, calmodulin and adenine nucleotides as well as phosphorylation sites (for review, see Melzer *et al.*, 1995).

Under physiological conditions, the CRC is initially activated by a conformational change of the dihydropyridine receptor in the T-tubule membrane, leading via a yet unexplained mechanism to a further conformational change in the CRC protein (Melzer, 1995). In the isolated system, the channel is ligand-activated with a wide spectrum of compounds known to affect SR $Ca^{2+}$ release. For a comprehensive list of known activators and inhibitors of the SR CRC, see Favero *et al.* (1999).

It is becoming increasingly apparent that free radicals can trigger rapid $Ca^{2+}$ release from the SR by modifying critical sulfhydryls (SH) in the CRC and increasing the open probability of the channel (for review see, Abramson and Salama, 1989; Favero, 1999). If this occurs with IR, elevations in $Ca^{2+}$ release through the CRC at rest, may contribute to the rise in $[Ca^{2+}]_f$ and $Ca^{2+}$ overload that occurs with IR. In the heart, there is some evidence to suggest that increased CRC activity and excessive $Ca^{2+}$ leakage at rest, may occur with IR (Davis *et al.*, 1992; O'Brien *et al.*, 1997).

On the other hand, as discussed above for the SR $Ca^{2+}$-ATPase, it is well known that altered muscle metabolite levels simulating I and IR conditions, such as increases in $H^+$, $Mg^{2+}$ and lactate and decreases in adenine nucleotides, impair SR $Ca^{2+}$ release (Favero *et al.*, 1995; Xu *et al.*, 1996). It has also been shown that maximal $Ca^{2+}$ release is depressed in

vitro, following myocardial IR in isolated rat hearts (Osada *et al.*, 1998; Temsah *et al.*, 2000). Osada *et al.* (1998) and others (Holmberg and Williams, 1992; Zucchi *et al.*, 1994), also reported decreases in the number of functioning CRCs, assessed by $^{3}$H-labeled ryanodine ([$^{3}$H]ryanodine) binding, in ischemic myocardium compared with control.

In contrast to the heart, no study has apparantly examined the effects of I or IR on maximal SR $Ca^{2+}$ release in vitro in skeletal muscle. However, silver nitrate ($AgNO_3$), a SH oxidizing agent that activates the CRC, has been used extensively for measurement of SR $Ca^{2+}$ release in vitro, following fatiguing contractile activity in rat and frog skeletal muscle (Favero *et al.*, 1993; Ward *et al.*, 1998; Williams *et al.*, 1998). Similar to the SR $Ca^{2+}$-ATPase and $Ca^{2+}$ uptake, changes in SR $Ca^{2+}$ release measured in vitro following I and IR, must be interpreted as structural alterations in either the CRC itself or the SR membrane, that occur during I and IR and persist throughout sample preparation and measurement of SR $Ca^{2+}$ release in vitro.

In work completed to-date, we have been investigating the effect of I (Green *et al.*, 1996) and IR (Green *et al.*, 1997) on SR function in vitro, and specifically changes in $Ca^{2+}$-ATPase activity in fibre specific types. Unexpectedly, we have found in both slow-twitch (rat soleus) and fast-twitch (rat EDL) muscle, that I produces elevations in $Ca^{2+}$-ATPase activity while IR produces depressions in $Ca^{2+}$-ATPase activity, both measured in muscle homogenates. Both the molecular mechanisms involved in inducing the alterations in $Ca^{2+}$-ATPase activity and the intracellular stimuli promoting the change remain in question.

The $Ca^{2+}$-ATPase is a 110-kDa protein with known amino acid sequence and structure. The enzyme consists of basepiece, stalk and headpiece components (MacLennan *et al.*, 1985; Brandl *et al.*, 1986). The large cytoplasmic extramembrane portion of the

enzyme (headpiece) contains a phosphorylation domain, a nucleotide domain for the binding of MgATP and a transduction domain or β-strand domain (MacLennan *et al.*, 1985).

The competitive inhibitory effect of fluorescein isothiocyanate (FITC), bound to the Lys 515 residue, on ATP binding, illustrates the close relation of this domain with the nucleotide binding site (Champeil *et al.*, 1988). Mutation of this residue was found to reduce $Ca^{2+}$ transport, but not phosphoenzyme formation (Maruyama *et al.*, 1989). Lys 515 appears to be important for energy transduction following phosphorylation (Clarke *et al.*, 1990b). The actual ATP-binding site is located around the first tryptic cleavage site (Arg 505) (Taylor and Green, 1989).

The five transmembrane helices toward the N terminus of the enzyme continue into a narrow stalk segment, connecting the extramembrane domains. This stalk region is very rich in negatively charged glutamate residues, suggesting a possible localization for the $Ca^{2+}$-binding site (MacLennan *et al.*, 1985; Brandl *et al.*, 1986). Clarke *et al.* (1989) confirmed that the charged residues in the transmembrane helices 4, 5, 6 and 8 were necessary for normal calcium transport function and that these 4 helices were the most likely candidates for the formation of the $Ca^{2+}$-binding site and the hydrophilic channel across the membrane.

The basepiece is made up of four and six transmembrane helices in the $NH_2$ and COOH termini, respectively (MacLennan *et al.*, 1985). These helices, being buried in the phospholipid membrane, are made up of a high number of hydrophobic residues compared to the cytoplasmic domains.

The $Ca^{2+}$-ATPase contains 24 cysteine residues (Brandl *et al.*, 1986). Consequently, the SR $Ca^{2+}$-ATPase may be a principal target for modulation of muscle function by reactive oxygen species (Kukreja *et al.*, 1988). Inactivation of the SR $Ca^{2+}$-ATPase as a result of free

radical formation, has been demonstrated in numerous in vitro studies (Morris and Sulakhe, 1997; Senisterra *et al.*, 1997; Viner *et al.*, 1999; Xu *et al.*, 1997). Pretreatment with oxygen free radical scavengers, superoxide dismutase and catalase, has been shown to maintain higher $Ca^{2+}$ uptake by the SR of skeletal muscle, following 3 hours of I and 19 hours of R in rat hindlimb (Lee *et al.*, 1987). Thus, free radical formation is likely a mechanism for impaired SR function with IR.

Any alterations in $Ca^{2+}$-ATPase activity that occur with I or IR, when measured in vitro under optimal conditions, must be due to either changes in protein structure or concentration, or structural alterations in the SR membrane which would affect $Ca^{2+}$-ATPase function. Where changes in $Ca^{2+}$-ATPase activity do occur and a change in enzyme structure is expected, it is possible to probe various domains and amino acid residues to detect which site(s) is precisely altered structurally. For example, both FITC and NCD-4 [(N-cyclohexyl-N'-dimethylamino-α-napthyl) carbodiimide] can be used to probe for alterations in the nucleotide binding site and the $Ca^{2+}$-binding site of the enzyme, respectively (Lalonde *et al.*, 1991). These measurements must be made on isolated SR vesicles as opposed to crude muscle homogenates.

Reductions in FITC or NCD-4 binding would indicate structural alterations in the regions of the protein responsible for nucleotide binding and calcium binding, respectively. It has been shown that an acute bout of low-intensity exercise reduces $Ca^{2+}$-ATPase activity measured in vitro, which is accompanied by reductions in FITC binding (Luckin *et al.*, 1991). The reduction in FITC binding could potentially explain the observed decrease in $Ca^{2+}$-ATPase activity.

As mentioned above, and emphasized again here in summary, impaired SR function would be expected in vivo with I and IR, given the severe metabolic disturbance and altered intracellular environment that results (Zhu and Nosek, 1991). However, when muscle is sampled following I and IR in vivo, and prepared for measurement of SR function in vitro, measurements are made under supposedly optimal conditions. Therefore, alterations in SR function in vitro, are assumed to reflect structural alterations to the SR that occurred in vivo, and that persist during preparation and measurement of SR function in vitro. Alterations to specific structural regions of the SR, and in particular, the SR $Ca^{2+}$-ATPase, following I and IR in skeletal muscle, have not been directly assessed.

**STATEMENT OF THE PROBLEM:**

The primary objective for this thesis is to investigate the changes that occur in the regulation of $[Ca^{2+}]_f$ in skeletal muscle by the SR during I and IR.

Specific goals include:

1. Determination of the effects of I and IR on SR $Ca^{2+}$-transport function, by measuring both $Ca^{2+}$ uptake and $Ca^{2+}$ release.
2. Determination of the effects of I and IR on SR $Ca^{2+}$-ATPase activity, including maximal activity, the Hill coefficient and the $[Ca^{2+}]_f$ at which half-maximal activity occurs ($Ca_{50}$).
3. Examination of the possible structural alterations to the nucleotide binding domain and the $Ca^{2+}$ binding sites of the $Ca^{2+}$-ATPase protein, by measuring FITC and NCD-4 binding, respectively.
4. Determining if the effects of I and IR on measurements of SR function in vitro, are similar, in both crude muscle homogenates and isolated SR fractions.
5. Assessment of the role of $O_2$ free radicals in the IR-induced changes in muscle mechanical function (twitch tension, $P_t$), and SR function in vitro, by administering a single dose of the enzymes, superoxide dismutase (SOD) plus catalase (CAT), in one group of rats just prior to R.

The specific hypotheses are:

1. Both SR $Ca^{2+}$ uptake and $Ca^{2+}$ release will be decreased following prolonged I and decreased even further with IR.
2. Reductions in maximal SR $Ca^{2+}$-ATPase activity in SR vesicles will occur with prolonged I. These reductions in SR $Ca^{2+}$-ATPase activity are expected based on pilot work completed prior to starting this thesis. Further reductions in maximal SR $Ca^{2+}$-

ATPase activity will occur with IR. The Hill coefficient and the $Ca_{50}$ will also be reduced with I and IR.

3. Reductions in SR $Ca^{2+}$-ATPase activity will be accompanied by similar reductions in FITC binding capacity with I and IR.
4. Changes in SR $Ca^{2+}$-ATPase activity, $Ca^{2+}$ uptake and $Ca^{2+}$ release measured in vitro, following both I and IR, will not be different between crude muscle homogenates and isolated SR fractions.
5. SOD + CAT treatment will protect against reductions in SR $Ca^{2+}$-ATPase activity, $Ca^{2+}$ uptake and $Ca^{2+}$ release. This will also be reflected in higher FITC binding.
6. $P_t$ of the gastrocnemius-plantaris-soleus complex, will be reduced with I, and remain depressed during recovery with R. However, recovery of $P_t$ in the group of rats that received SOD + CAT treatment will be improved compared to the group of animals that did not receive SOD + CAT treatment.

# CHAPTER II

## EFFECTS OF ISCHEMIA ON SARCOPLASMIC RETICULUM STRUCTURE AND FUNCTION IN RAT SKELETAL MUSCLE

## Abstract

In this study, an in vivo model of prolonged I in rat skeletal muscle was employed, to determine the effects of prolonged I on SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase structure and function in vitro. To induce total I, a tourniquet was placed around the upper hindlimb in 30 female Sprague-Dawley rats weighing 256 ± 6.7 g (mean ± SE), and inflated to 350 mmHg for 4 hours. The contralateral limb served as control (C). Immediately following I, mixed gastrocnemius and tibialis anterior muscles were sampled from both limbs, and both crude muscle homogenates and SR vesicles were prepared. In another 10 control animals (CC), muscle was sampled and prepared exactly the same way, but immediately following anesthetization. $Ca^{2+}$ uptake and $Ca^{2+}$ release were measured in vitro with Indo-1 on both homogenates and SR vesicles. As hypothesized, sub-maximal $Ca^{2+}$ uptake was lower ($p<0.05$) in I compared with CC and C, by 25 and 45% in homogenates and SR vesicles, respectively. Silver nitrate ($AgNO_3$)-induced $Ca^{2+}$ release, which occurred in 2 phases (Phase 1 and Phase 2), was also lower ($p<0.05$) in I compared with CC and C, in both muscle homogenates and SR vesicles. With I, Phase 1 peak $Ca^{2+}$ release was 26% lower in SR vesicles only. For Phase 2, peak $Ca^{2+}$ release was 54 and 24% lower in SR vesicles and homogenates, respectively. Maximal $Ca^{2+}$-ATPase activity was depressed by 34 and 27% ($P<0.05$) in I compared with CC and C, respectively, only in SR vesicles. No differences were found between groups in either the Hill coefficient or the $Ca_{50}$. The fluorescent probes, FITC and NCD-4, used to assess structural alterations to the ATP binding site and the $Ca^{2+}$ binding sites of the $Ca^{2+}$-ATPase, respectively, only indicated reductions ($p<0.05$) in FITC binding capacity (26% lower in I as compared with both CC and C). Total SERCA1 protein, as determined by SDS-PAGE and Western blot analysis,

was not affected by I, however, evidence for greater protein aggregation with I was observed. These results demonstrate that prolonged skeletal muscle I leads to a reduced SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and maximal $Ca^{2+}$-ATPase activity in vitro. The reduction in maximal SR $Ca^{2+}$-ATPase activity with I is related to structural modification of the nucleotide binding domain, probably as a result of free-radical induced oxidation of one or more cysteines.

## Introduction

Sarcoplasmic reticulum (SR) is a membranous structure in skeletal muscle which plays a critical role in the regulation of intracellular free calcium ($[Ca^{2+}]_f$). It acts both as a site of $Ca^{2+}$ storage for subsequent release in response to muscle excitation, and as a sink for removal of $Ca^{2+}$ from the cytoplasm when excitation stops. In skeletal muscle, $Ca^{2+}$ is stored primarily within the terminal cisternae of the SR, released through the CRC to initiate contraction and resequestered into the lumen of the SR by the SR $Ca^{2+}$-ATPase pump to allow relaxation (Lytton and MacLennan, 1991).

In addition to its role in muscle contraction, it is well known that $Ca^{2+}$ regulates numerous physiological cellular phenomena as a second messenger (Whitaker and Patel, 1990), as well as triggering pathological events such as cell injury and death (Gupta and Pushkala, 1999). Therefore, a normal functioning SR, particularly in skeletal muscle, is critical for normal cellular function and cellular survival. In fact, numerous investigations have implicated abnormal SR function in the etiology of various pathological conditions, such as malignant hyperthermia and central core disease (Loke and MacLennan, 1998), as well as with acute traumatic conditions, such as cellular I (Mubagwa, 1995).

In the heart, alterations in $Ca^{2+}$ cycling with I and IR are well documented (Mubagwa, 1995). It has been shown repeatedly that I and I-R leads to a reduction in SR $Ca^{2+}$ uptake, when measured in vitro (Meno *et al.*, 1984; Kaplan *et al.*, 1992; Osada *et al.*, 1998). Generally, reductions in $Ca^{2+}$ uptake with myocardial I are attributed to parallel reductions in $Ca^{2+}$-ATPase activity, however, there is some evidence to suggest that increased CRC activity and excessive $Ca^{2+}$ leakage, may also be involved (Davis *et al.*, 1992; O'Brien *et al.*, 1997).

It has also been shown that maximal $Ca^{2+}$ release is depressed in vitro, following myocardial IR in isolated rat hearts (Osada *et al.*, 1998). In that study and others (Holmberg and Williams, 1992; Zucchi *et al.*, 1994), there were decreases in the number of functioning CRCs, assessed by $^3$H-labeled ryanodine ([$^3$H]ryanodine) binding, in ischemic myocardium compared with control.

The $Ca^{2+}$-ATPase is a 110-kDa polypeptide chain with known sequence and structure of 997 amino acid residues (MacLennan *et al.*, 1985; Toyoshima *et al.*, 1993). The large cytoplasmic extramembrane portion of the enzyme (headpiece) contains the phosphate acceptor Asp-351 residue (MacLennan *et al.*, 1985; Brandl *et al.*, 1986) and the binding site for ATP (Taylor and Green, 1989). Other functionally important residues for binding and translocation of $Ca^{2+}$ are oriented in two sites among transmembrane helices M4, M5 and M6 (Rice *et al.*, 1997). The $Ca^{2+}$-ATPase contains 24 cysteine residues (Brandl *et al.*, 1986). Consequently, the SR $Ca^{2+}$-ATPase may be a principal target for modulation of muscle function by reactive oxygen species (Kukreja *et al.*, 1988). Inactivation of the SR $Ca^{2+}$-ATPase as a result of free radical formation, has been demonstrated in numerous in vitro studies (Morris and Sulakhe, 1997; Senisterra *et al.*, 1997; Viner *et al.*, 1999; Xu *et al.*, 1997). Free radicals may cause protein denaturation and damage involving the ATP binding site (Xu *et al.*, 1997), enhanced lipid peroxidation (Morris and Sulakhe, 1997; Kourie, 1998) and increased protein oxidation, leading to both a reduced SH group content and increased protein aggregation, (Morris and Sulakhe, 1997; Senisterra *et al.*, 1997; Viner *et al.*, 1999).

It is well established that free radical formation plays a primary role in the etiology of I-induced damage in skeletal muscle (Rubin *et al*, 1996). With prolonged muscle I, a significant loss of ATP and total adenine nucleotides occurs (Idström *et al.*, 1990; Soussi *et*

*al.*, 1993; Rubin *et al.*, 1992; Carvalho *et al.*, 1997). This leads to elevations in substrates for the enzyme xanthine oxidase, which catalyzes the reaction where both superoxide radicals and hydrogen peroxide are formed in muscle (Parks and Granger, 1986).

Studies of IR in cardiac muscle, and more recently in skeletal muscle, have shown that impaired SR function and $Ca^{2+}$ homeostasis may also be involved in the etiology of IR injury (Lee *et al.*, 1987; Kaplan *et al.*, 1992; Green *et al.*, 1997). In one study, pretreatment with oxygen free radical scavengers, superoxide dismutase and catalase, maintained higher $Ca^{2+}$ uptake by the SR of skeletal muscle, following 3 hours of I and 19 hours of R in rat hindlimb (Lee *et al.*, 1987). Thus, free radical formation is likely a mechanism for impaired SR function with IR.

Only a few studies have examined the effects of I on SR $Ca^{2+}$-sequestering function in skeletal muscle and no study could be found that examined the effects of I on maximal SR $Ca^{2+}$ release in skeletal muscle. Muscle cells subjected to I are characterized by a loss in function first, followed by progressive structural damage and possible necrosis upon R, with minimal or no functional recovery depending on the length of the ischemic period. Current evidence suggests that $[Ca^{2+}]_f$ overload may contribute to postischemic injury in skeletal muscle (Rubin *et al.*, 1996). Based on studies in the heart, and given the important role of the SR in maintaining cellular $Ca^{2+}$ homeostasis in skeletal muscle, it would be expected that prolonged I may impair SR function in skeletal muscle.

However, it remains unclear whether the disturbance in $Ca^{2+}$-regulation occurs during the ischemic period per se or whether I simply predisposes the muscle, with the actual changes becoming manifest during R. Unexpectedly, we have found a time-dependent increase in maximal $Ca^{2+}$-ATPase activity with I in both rat soleus and EDL muscles (Green

*et al.*, 1996). Since the effects were only demonstrated in homogenate preparations, it remains unclear what is happening at the level of enriched SR vesicle preparations, which are free from the potential contaminating effects of other proteins and cellular ATPases.

Moreover, since measurement of SR function in these studies is done in vitro, under supposedly ideal conditions, I-induced reductions in $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase activity are probably due to structural alterations to the $Ca^{2+}$-ATPase and CRC and/or the SR membrane. However, the nature or precise location of altered structure on the SR $Ca^{2+}$-ATPase, has not been determined in skeletal muscle samples harvested from tissue that was made ischemic in vivo.

In this study, a 4 hour hindlimb I model in rats was employed to characterize the alterations in both SR $Ca^{2+}$ handling and $Ca^{2+}$-ATPase activity and structure that occur with prolonged periods of skeletal muscle I. In mixed fast twitch muscle from both ischemic and control hindlimbs, we measured oxalate-supported $Ca^{2+}$ uptake and silver nitrate ($AgNO_3$)-induced $Ca^{2+}$ release, using the $Ca^{2+}$-fluorescent dye, Indo-1, and $Ca^{2+}$-ATPase activity in both muscle homogenates and purified SR vesicles. To assess the structural alterations to the $Ca^{2+}$-ATPase, the fluorescent probes, FITC and NCD-4 were used to measure structural alterations to the ATP binding site and the $Ca^{2+}$ binding sites of the $Ca^{2+}$-ATPase, respectively. It was hypothesized that the $Ca^{2+}$-ATPase activity would be lower in ischemic SR compared to control and that the lower $Ca^{2+}$-ATPase would be accompanied by a reduction in FITC binding, indicative of structural alterations to the ATP binding site.

## Methods

**Animal description and care.** Adult female Sprague-Dawley rats weighing 256 ± 6.7 g (mean ± SE), were housed in an environmentally controlled room (temperature 22-24°C, 40-60% relative humidity) with reversed light/dark cycles. Animals were fed ad libitum on laboratory chow and water until the time of the experiment. All experiments were initiated at approximately the same time each day to avoid large diurnal variations in muscle glycogen (Conlee *et al.*, 1976). Experimental protocols were approved by the Animal Care Committee of the University of Waterloo.

**Experimental Protocol.** To investigate the effects of complete I on SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase structure and function, animals were randomly assigned to control (CC) (n = 10) and experimental (E) (n = 30) groups. For each E animal, the experimental condition, 4 hours of total I, was randomly assigned to one hindlimb (I) while the contralateral limb served as a control limb (C). Due to tissue requirements for the isolation procedure used to obtain SR vesicles, experiments were conducted on one CC and 3 E animals each day. I was induced by placing a tourniquet around the upper hindlimb and proximal to the knee joint. To ensure total occlusion of blood flow to the hindlimb, a 350-mmHg pressure was employed (Walker *et al.*, 1989; Fish *et al.*, 1993). Total I was confirmed based on almost total depletion of muscle PCr and ATP following 4 hours of I (see Table 2.1).

Before the induction of I, the rats were weighed and anesthetized. Anesthesia was initially accomplished using an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body wt) and then was maintained using supplementary intraperitoneal injections, as required. To prevent dehydration, experimental rats were administered 2 ml of saline by

injection just under the skin, prior to the induction of I. Throughout the ischemic periods, body temperature was maintained between 37 and 39 °C, by having the rats lay in a prone position on a warm heating pad. At the end of each of the ischemic periods, a small piece of white gastrocnemius (G) muscle was rapidly sampled from each of the I and C limbs, and frozen in liquid nitrogen for later analysis of muscle metabolites. The remainder of the G muscle (both red and white portions) along with the entire tibialis anterior (TA) muscle were excised and placed in ice cold buffer, to be used for SR isolation by differential centrifugation. The hindlimbs were not reperfused prior to muscle sampling. The G and TA muscles from the CC animal were sampled and excised in the same manner, immediately following anesthetization.

**Analytic Procedures**

**Muscle metabolite analysis.** To determine the effects of I on muscle metabolic behaviour, metabolites of the high-energy phosphate system [PCr, Cr, $P_i$, glycogen and lactate] were assessed. Metabolites were measured in freeze-dried tissue after perchloric acid extraction by fluorometric procedures as previously reported (Green *et al.*, 1987). All concentrations were corrected for total creatine content (Sabina *et al.*, 1983).

**Sample preparation for SR assessment in vitro.** Ischemic and control muscles were prepared according to Heilmann *et al.* (1977). Mixed G and TA muscles were diluted approximately 1:5 (w/v) in homogenizing buffer containing (in mM) 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.5), 250 sucrose, 0.2% sodium azide and 0.2 phenylmethylsulfonyl fluoride [(no dithiothreitol (DTT)] and mechanically homogenized with a polytron homogenizer (PT 3100) at 16500 rpm, for 2x30sec bursts. Aliquots of muscle homogenate were quick frozen in liquid nitrogen and stored at –70 to -

80°C for later analysis of SR function. To obtain an enriched SR membrane fraction, a combination of two SR isolation protocols were used (Eletr and Inesi, 1972; Heilmann *et al.*, 1977). The homogenate was centrifuged at 5500 g for 10 min to remove cellular debris, and the supernatant was filtered through 4 layers of gauze to remove as much fat as possible. The supernatant was then transferred to clean tubes and centrifuged at 12500 g for 18 min. These pellets were discarded and the spin was repeated. Again, the supernatant was transferred to clean tubes and centrifuged at 50000 g for 52 min. These pellets were resuspended in 10 ml homogenizing buffer plus 600 mM KCl, and allowed to incubate at 4°C for 30 min. This suspension was then centrifuged at 15000 g for 10 min to pellet all the mitochondria. The supernatant was centrifuged at 50000 g for 52 min. The final pellet, enriched in SR membranes (no sucrose cushion), was resuspended in homogenizing buffer at a protein concentration of 2-6 mg/ml. SR isolation was carried out by differential centrifugation using a Beckmann Ultracentrifuge with a 70.1 Ti fixed-angle rotor.

**SR $Ca^{2+}$ uptake measurements.** Oxalate supported $Ca^{2+}$ uptake was measured in muscle homogenates and purified SR using the $Ca^{2+}$ flourescent dye Indo-1 according to methods of O'Brien (O'Brien, 1990; O'Brien *et al.*, 1991) with minor modifications. Fluorescence measurements were made on a spectrofluorometer (RatioMaster™ system, Photon Technology International) equipped with dual emission monochromators. The measurement of $[Ca^{2+}]_f$ using the indo-1 procedure is based on the difference in the maximal emission wavelengths between the $Ca^{2+}$-bound form of indo-1 and the $Ca^{2+}$-free form. The excitation wavelength was 355 nm and the emission maxima were 485 and 405 nm for $Ca^{2+}$-free (G) and $Ca^{2+}$-bound (F) Indo-1, respectively. Photon counts per second were recorded simultaneously for both emission wavelengths. The $Ca^{2+}$-

independent (background) fluorescence was measured in the reaction medium (without Indo-1) at each emission wavelength before starting the experiment. Background fluorescence was automatically corrected prior to starting each assay using the Felix software (Photon Technology International).

The reaction buffer for muscle homogenates (SR in parentheses) contained 200 (100) mM KCl, 20 mM HEPES, 10 mM $NaN_3$, 5 μM TPEN, 5 mM oxalate, 15 (10) mM $MgCl_2$ and 10 mM PEP, pH 7.0. Prior to collecting emission spectra, 18 U/ml each of LDH and PK and 1.5 μM Indo-1 were added to a cuvette containing 2 ml of reaction buffer. Also, 3 μl of $CaCl_2$ (10 mM) was added each trial to achieve an initial $[Ca^{2+}]_f$, prior to starting the reaction, of approximately 3.0 μM. Immediately after data collection was initiated, 40 μl of homogenate (20 μl SR) was added to the cuvette. Shortly after the addition of homogenate or SR, 5 mM ATP was added to initiate $Ca^{2+}$ uptake.

As $Ca^{2+}$ decreases because of active SR $Ca^{2+}$ uptake, F decreases, G increases, and the ratio of F to G decreases. The ratio (R) is used to calculate $[Ca^{2+}]_f$. With the use of Felix software, the ionized $Ca^{2+}$ concentration was calculated by the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_f = K_d * (G_{max}/G_{min}) (R - R_{min}) / (R_{max} - R) \qquad (1)$$

where $K_d$ is the equilibrium constant for the interaction between $Ca^{2+}$ and Indo-1, $R_{min}$ is the minimum value of R at addition of 250 μM EGTA, $G_{max}$ is the maximum value of G at addition of 250 μM EGTA, $G_{min}$ is the minimum value of G at addition of 1 mM $CaCl_2$ and $R_{max}$ is the maximum value of R at addition of 1 mM $CaCl_2$. The $K_d$ value for the $Ca^{2+}$-dye complex is 250 and 135 nM for muscle homogenates and purified SR vesicles, respectively

(Grynkiewicz *et al.*, 1985). For all $Ca^{2+}$ uptake trials, $R_{min}$ and $R_{max}$ were not determined until $Ca^{2+}$ uptake had plateaued, which occurs at approximately 100 nM $[Ca^{2+}]_f$.

Before the rate of $Ca^{2+}$ uptake was calculated, the generated curve from **equation 1**, $[Ca^{2+}]_f$ versus time, was smoothed over 21 points using the Savitsky-Golay algorithm. The rate of $Ca^{2+}$ uptake was then analyzed at 4 separate free calcium concentrations (0.5, 1.0, 1.5 and 2.0 μM) for both muscle homogenates and SR. First, linear regression was done on a range of values 100 nM above and below the desired $[Ca^{2+}]_f$. The rate of $Ca^{2+}$ uptake was then determined by differentiating the linear-fit curve and expressed in μmoles·g protein$^{-1}$·min$^{-1}$.

In a subset of homogenate samples (n=5), $Ca^{2+}$ uptake assays were run with and without 300 μM ryanodine to assess the possible role of the CRC channel in altering $Ca^{2+}$ uptake with I. There is some evidence to suggest that increased CRC activity and excessive $Ca^{2+}$ leakage at rest, may contribute to $[Ca^{2+}]_f$, producing erroneous values for $Ca^{2+}$ uptake with I (Davis *et al.*, 1992; O'Brien *et al.*, 1997).

**SR $Ca^{2+}$ release measurements.** $Ca^{2+}$ release was measured on both muscle homogenates and SR vesicles according to the methods of Ruell (Ruell *et al.*, 1995), with minor modifications. $Ca^{2+}$ release assays were conducted as for $Ca^{2+}$ uptake, except that when the $[Ca^{2+}]_f$ declined to a plateau, 3 μl $AgNO_3$ was added to give a final concentration of 141 μM. The reaction was then allowed to proceed for approximately 3 min. In muscle homogenates only, we also measured $Ca^{2+}$ release in the presence of 40 μM cyclopiazonic acid (CPA), which completely inhibits SR $Ca^{2+}$-ATPase activity and $Ca^{2+}$ uptake (Seidler *et al.*, 1989). In the trials with CPA, 40 μM CPA was added following active loading of the SR with $Ca^{2+}$ and just prior to the addition of $AgNO_3$. This was done to assess the effects of I on

$Ca^{2+}$ release independent of changes in $Ca^{2+}$ uptake that may occur with I. In a subset of muscle homogenate samples (n = 5), in the absence and presence of CPA, $Ca^{2+}$ release was allowed to proceed until a plateau was reached, in order to measure total $Ca^{2+}$ release in each group. This procedure was intended to assess the amount of loading of calcium in the SR, prior to the initiation of release.

With the addition of $AgNO_3$, $Ca^{2+}$ release consistently proceeded in 2 distinct phases. There was an initial rapid rate of release (Phase 1) followed by a slower, more prolonged rate of release (phase 2) (see Fig. 2.1). As with $Ca^{2+}$ uptake, the generated curve from **equation 1** was smoothed over 21 points and differentiated. The maximal rate of $Ca^{2+}$ release was calculated by taking the maximum positive derivative for each phase and expressed in $\mu moles \cdot g\ protein^{-1} \cdot min^{-1}$. For both $Ca^{2+}$ uptake and $Ca^{2+}$ release, protein was determined by the method of Lowry as modified by Schacterle and Pollock (Schacterle and Pollock, 1973). On a given day, an equal number of samples from each condition and for a given variable were analyzed in duplicate.

**SR $Ca^{2+}$-ATPase activity measurements.** Spectrophotometric (Schimadzu UV 160U) measurement of SR $Ca^{2+}$-ATPase activity was performed on homogenates using procedures developed by Simonides and van Hardeveld (1990) and SR samples according to Leberer *et al.* (1987) with minor modifications. Total ($Mg^{2+}$-activated)-ATPase activity was measured in the presence of the $Ca^{2+}$ ionophore A-23187, across a range of $CaCl_2$ concentrations. Basal activity was measured in the presence of 40 μM CPA, which completely inhibits SR $Ca^{2+}$-ATPase activity (Seidler *et al.*, 1989). The difference between total and basal activities represents the $Ca^{2+}$-activated ATPase activity. Maximal activity and the $Ca^{2+}$ dependency of $Ca^{2+}$-ATPase activity were assessed by adding 1-11 μl of 100 mM

$CaCl_2$ in 0.5 µl additions. $Ca^{2+}$-ATPase activity increases with $[Ca^{2+}]_f$ until a plateau occurs, once maximal activity is reached. The $[Ca^{2+}]_f$ corresponding to each $CaCl_2$ addition was assessed separately, on a different SR aliquot, using dual-wavelength spectrofluorometry and the $Ca^{2+}$ fluorescent dye, Indo-1. $Ca^{2+}$-ATPase activity was then plotted against the negative logarithm of $[Ca^{2+}]_f$ (pCa) and the Hill coefficient along with the $Ca_{50}$ were determined. These properties were determined through non-linear regression with computer software (GraphPad Software Inc.) using the following sigmoidal dose-response equation

$$Y = Y_{bot} + (Y_{top} - Y_{bot})/(1+10^{(LogCa_{50} - X)} \bullet n_H)$$

where $Y_{bot}$ is the value at the bottom of the plateau, $Y_{top}$ is the value at the top of the plateau, $LogCa_{50}$ is the logarithm of $Ca_{50}$, the concentration that gives a response halfway between $Y_{bot}$ and $Y_{top}$ and $n_H$ is the Hill coefficient. For calculation of these properties, only a portion of the curve, which corresponded with between 20 and 80% of maximal activity was used. Protein was determined by the method of Lowry as modified by Schacterle and Pollock (Schacterle and Pollock, 1973). On a given analytical day, all samples were analyzed in duplicate.

**SDS-PAGE and Western blotting.** A suspension of 0.5 mg/ml SR protein in either 40 µl reducing buffer (1.25 M sucrose, 0.1 M DTT, 0.25 M Tris/HCl, pH 6.8, 5% SDS, 0.01% bromophenol) or non-reducing buffer (1.25 M sucrose, 0.25 M Tris/HCl, pH 6.8, 5% SDS, 0.01% bromophenol) brought to 200 µl by distilled water, was heated for 10 min at 100 °C, and solid DTT added to the sample in reducing buffer to raise the final concentration of DTT to 100 mM. All samples were then sonicated for 10 s in a probe sonicator, and 5 µg of each sample was analyzed in duplicate on separate 7% polyacrylamide SDS gels (BIO-RAD Mini-PROTEAN II), with a 3.75% stacking gel.

After SDS-PAGE and a 15 min equilibration in cold transfer buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol), the proteins were transferred to a polyvinylidene difluoride membrane (PVDF membrane, Bio-Rad) by placing the gel in transfer buffer and applying a high voltage (100 V) for 45 min (Trans-Blot Cell, Bio-Rad). Non-specific binding sites were blocked with 10% skim milk powder in Tris-buffered saline (pH 7.5), applied overnight at room temperature. Immunoblotting was performed using the primary monoclonal antibody IIH11 specific for rat (Affinity Bioreagents), for determination of SERCA1 protein and the aggregation state of SERCA1. Incubation with the primary antibodies was performed for 60 min at room temperature. Following washing, a secondary antibody (anti-mouse IgG1 conjugated to horseradish peroxidase) was applied for 60 min at room temperature. Protein quantification was performed using densitometry and an enhanced chemiluminescence immunodetection procedure (Amersham-ECL-RPN2106P1). After exposure to photographic film (Kodak Hyperfilm-ECL), the blot was developed for 90 sec in Kodak GBX developing solution and fixed in Kodak GBX fixer. Relative SERCA1 protein levels were determined by scanning densitormetry and values were expressed as a % of the CC value.

**Fluorescence measurements.** Fluorescence measurements were made on an SLM-4800S spectrofluorometer (SLM Instruments, Urbana, IL) according to Lalonde *et al.* (1991). FITC (Sigma) and NCD-4 (Molecular Probe) were stored at a concentration of 5 mM in ethanol at –20 °C. FITC emission spectra (500 to 550 nm) were collected by exciting samples at 490 nm (see Fig. 5A). FITC labeling was done by washing the SR samples once in wash buffer with no DTT, then resuspending the samples in FITC labeling buffer (wash buffer plus 2.5 μM FITC, pH 8.8), and vortexing gently in darkness for 20 min at 25 °C. The

SR samples were then washed again in ordinary wash buffer to remove unbound label. NCD-4 emission spectra were collected by exciting samples at 340 nm and scanning the emission intensity from 400 to 430 nm at 1 nm increments. NCD-4 labeling was done by washing the SR samples once in wash buffer with no DTT, then resuspending in NCD-4 labeling buffer (wash buffer plus 150 μM NCD-4, pH 6.2) and incubating in darkness for 3 hours at 25 °C. As before, the sample was washed to remove unbound label.

**Statistical Analysis.** For $Ca^{2+}$ release measurements in muscle homogenates, a two-way ANOVA was used to discriminate between differences due to assay (with vs. without CPA) and group (CC vs. C vs. I). Where an overall interaction between assay and group was found, post hoc analyses (Tukey's) were performed to determine specific assay and group effects. For all other measurements, a one-way ANOVA was used to test for differences between means. Where significant differences were found, Tukey's post hoc tests were used to compare specific means. For all comparisons, statistical significance was accepted at $p < 0.05$. All data are expressed as means ± SE.

## Results

**Muscle metabolites.** Muscle metabolites for each group are shown in table 2.1. With the exception of $P_i$, there were no differences between CC and C for any of the metabolites measured. Muscle ATP and PCr were nearly fully depleted in I, both reaching levels below 2 mmol/kg dry wt. Compared with CC and C, muscle Cr and $P_i$ were 1.9- and 3.9-fold higher ($p<0.05$) in I, respectively. Lactate was 10.6-fold higher ($p<0.05$) and glycogen was 88% lower ($p<0.05$) in I, compared with CC and C.

**Table 2.1.** *Muscle metabolite concentrations for ischemic and control groups.*

| | CC | C | E |
|---|---|---|---|
| ATP | 28.3 ± 0.5 | 27.5 ± 0.3 | 1.1 ± 0.3 * |
| PCr | 77.1 ± 2.1 | 77.2 ± 3.0 | 1.3 ± 0.4 * |
| Cr | 84.9 ± 2.0 | 78.5 ± 2.5 | 150.0 ± 2.3 * |
| Pi | 46.8 ± 1.5 | 37.6 ± 2.0 ⁺ | 163.7 ± 3.4 * |
| Lactate | 22.4 ± 1.0 | 24.5 ± 2.7 | 249.0 ± 5.6 * |
| Glycogen | 117.3 ± 2.6 | 118.8 ± 3.7 | 14.1 ± 1.0 * |

*Values are means ± SE in μmol/g dry wt with the exception of Glycogen, which is in μmol glucosyl units/g dry wt (N=10 per group). CC, control control, C, control, I, Ischemic, PCr, phosphocreatine, Cr, creatine, Pi, inorganic phosphate. *Significantly different (p<0.05) from CC and C. ⁺Significantly different (p<0.05) from CC.*

**$Ca^{2+}$ uptake.** $Ca^{2+}$ uptake was assessed at 4 different $[Ca^{2+}]_f$ in both muscle homogenates and purified SR vesicles. In muscle homogenates, $Ca^{2+}$ uptake was 25–28% lower ($p<0.05$) in I, compared with CC and C, across all $[Ca^{2+}]_f$ (Fig. 2.2). The addition of 300 μM ryanodine in the homogenate $Ca^{2+}$ uptake buffer (n=5), to inhibit $Ca^{2+}$ release channel opening, had no effect on $Ca^{2+}$ uptake in any of the groups, at either 500 or 1000 nM $[Ca^{2+}]_f$ (Fig. 2.3). However, a main effect ($p<0.05$) of ryanodine was observed such that $Ca^{2+}$ uptake was generally lower in the presence of ryanodine at 500 nM compared to 1000 nM $[Ca^{2+}]_f$. In purified SR vesicles, $Ca^{2+}$ uptake was also lower ($p<0.05$) in I by 40-46% across all $[Ca^{2+}]_f$, compared with CC and C (Fig. 2.4). There were no differences between CC and C in either muscle homogenates or purified SR vesicles.

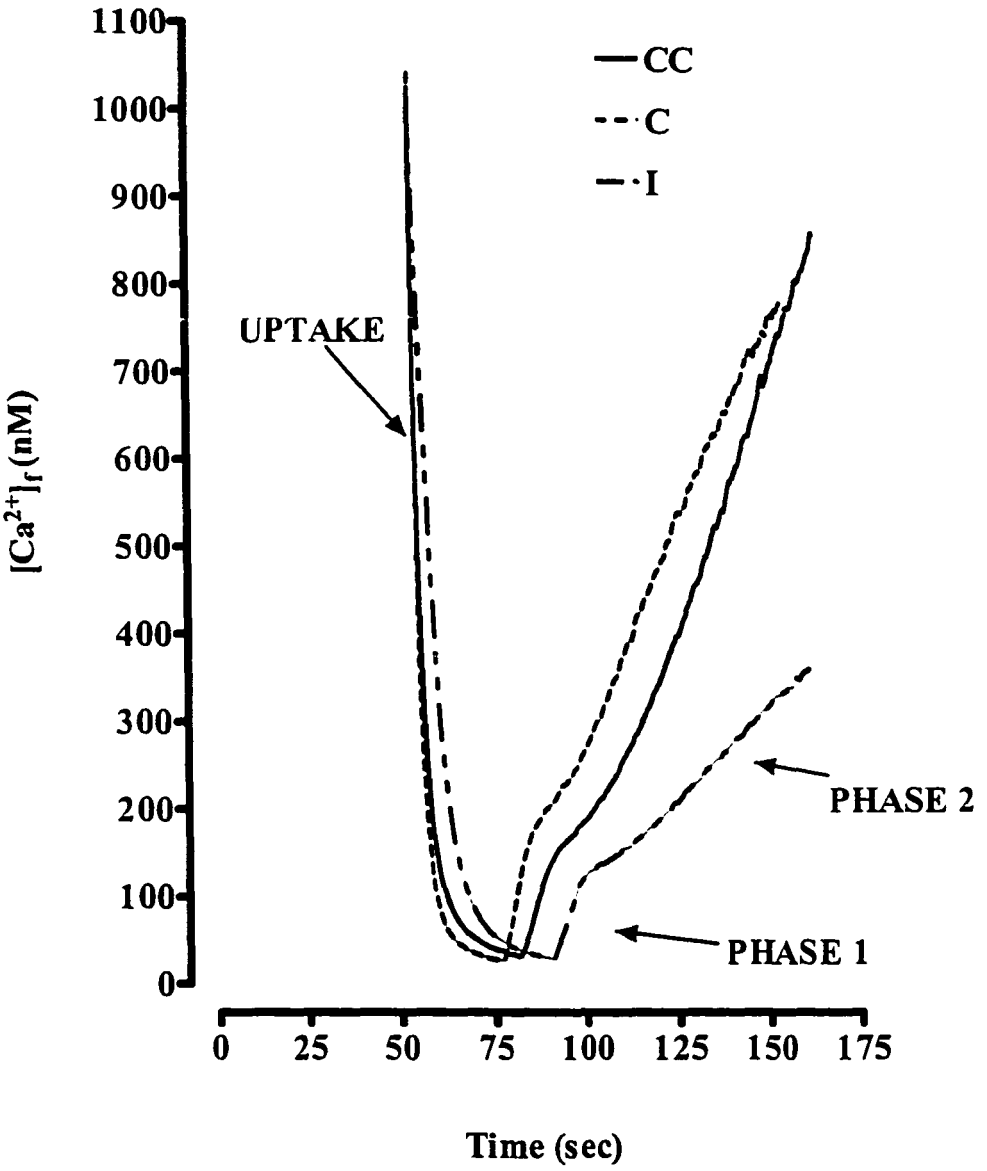



**Figure 2.1.** *A sample trace showing $[Ca^{2+}]_f$ kinetic changes for each group, measured with Indo-1, during active loading of SR vesicles with $Ca^{2+}$ (Uptake), and during $AgNO_3$-induced $Ca^{2+}$ release. The $Ca^{2+}$ release response was biphasic in nature. There was an initial rapid rate of release (Phase 1) followed by a slower more prolonged rate of release (Phase 2). Each phase was analyzed separately and the peak rate of $Ca^{2+}$ release corresponding to each phase is reported in the Results. CC, control control, C, control, I, ischemic.*

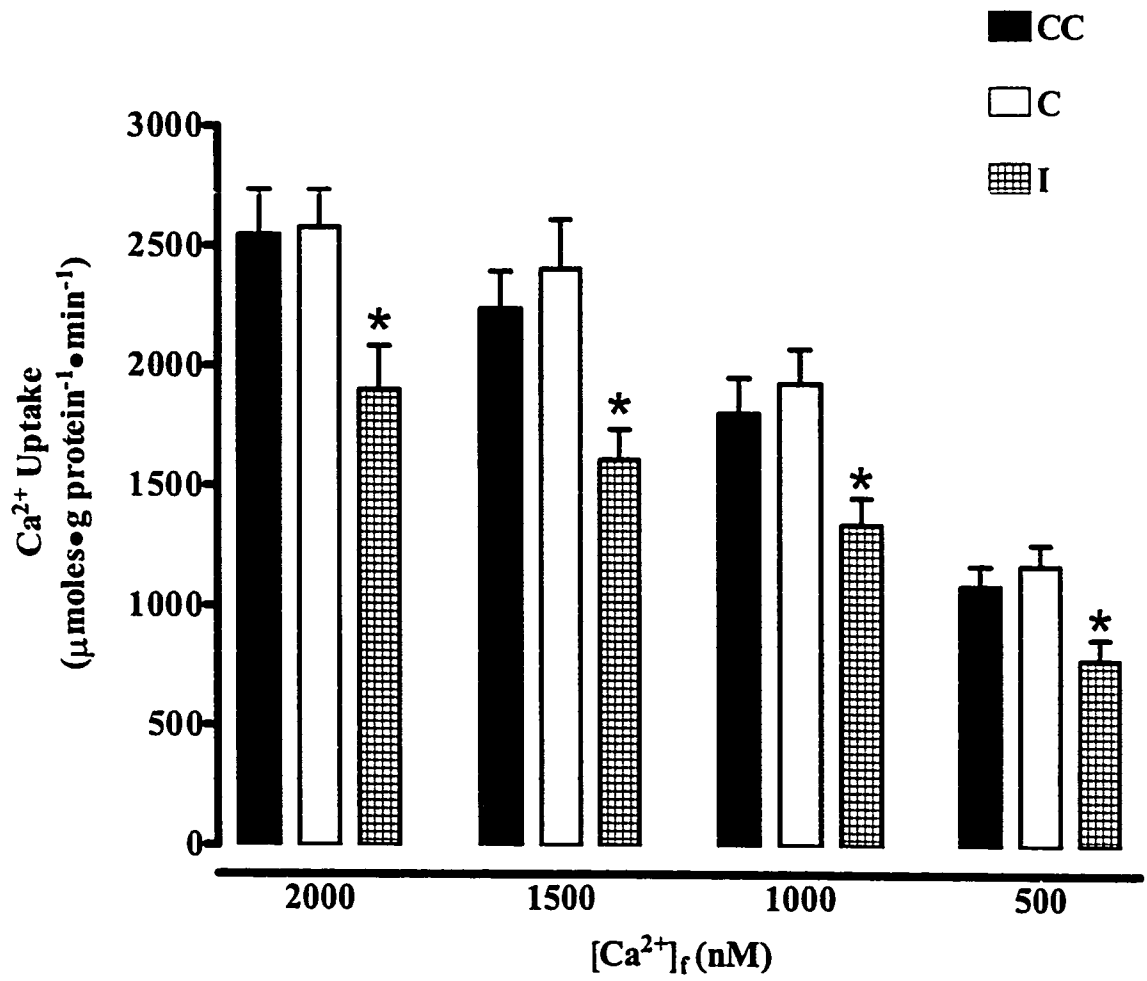



**Figure 2.2.** *Calcium uptake of CC, C and I homogenates prepared from mixed gastrocnemius and tibialis anterior muscles. $Ca^{2+}$ uptake was measured using Indo-1 and at 4 sub-maximal $[Ca^{2+}]_f$ (2000, 1500, 1000 and 500 nM). Values are means ± SE; n = 9. CC, control control, C, control, I, ischemic. * Significantly different from CC and C.*

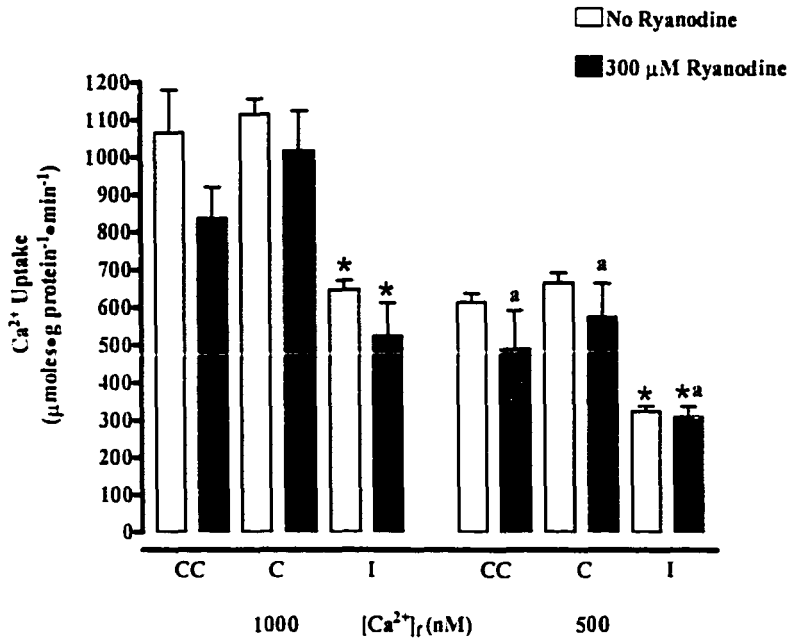



**Figure 2.3.** *Calcium uptake of CC, C and I homogenates prepared from mixed gastrocnemius and tibialis anterior muscles, measured with and without 300 µM ryanodine. $Ca^{2+}$ uptake was measured using Indo-1 and at 2 sub-maximal $[Ca^{2+}]_f$ (1000 and 500 nM). Values are means ± SE; n = 5. CC. control control, C, control, I, ischemic. a Significant main effect for ryanodine at 500 nM $[Ca^{2+}]_f$, with uptake being lower in the presence of ryanodine. * Significantly different from CC and C in the same condition (with or without ryanodine).*

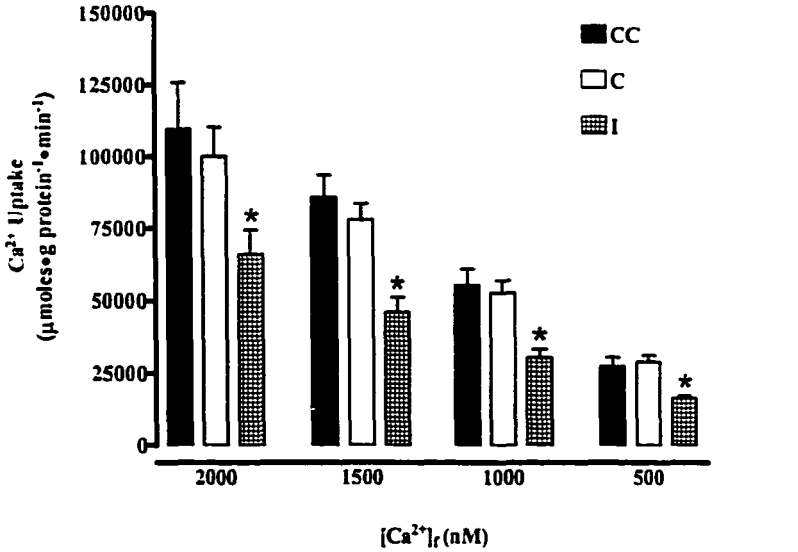



**Figure 2.4.** *Calcium uptake of CC, C and I SR vesicles prepared from mixed gastrocnemius and tibialis anterior muscles. $Ca^{2+}$ uptake was measured using Indo-1 and at 4 sub-maximal $[Ca^{2+}]_f$ (2000, 1500, 1000 and 500 nM). Values are means ± SE; n = 9. CC, control control, C, control, I, ischemic. * Significantly different from CC and C.*

**$Ca^{2+}$ release.** Due to the biphasic response of $Ca^{2+}$ release in this study, we obtained two measures of maximal $Ca^{2+}$ release, corresponding to each phase, for each assay (Fig. 2.1). In muscle homogenates, both with and without CPA, maximal $Ca^{2+}$ release for Phase 1 was not different between groups (Fig. 2.5). For phase 1, maximal release was higher ($p<0.05$) in the presence of CPA than without CPA in all groups. On the other hand, for Phase 2, in the absence of CPA, maximal $Ca^{2+}$ release was 24% lower ($p<0.05$) in I, compared with CC and C. However, there were no differences between groups for phase 2, when $Ca^{2+}$ release was measured in the presence of CPA. Unlike phase 1, maximal $Ca^{2+}$ release for phase 2, was lower ($p<0.05$) in all groups when measured in the presence of CPA, compared with no CPA. There were no differences between CC and C in any condition. In purified SR vesicles, maximal $Ca^{2+}$ release was 26 and 54% lower ($p<0.05$) in I, compared with CC and C, for Phase 1 and Phase 2, respectively (Fig. 2.6). No differences existed between CC and C for either phase. Total $Ca^{2+}$ release was assessed in a subset of muscle homogenate samples (n=5) to ensure that SR $Ca^{2+}$ loading was similar in all groups, prior to the initiation of $Ca^{2+}$ release. There were no differences in total $Ca^{2+}$ release between groups, regardless of whether CPA was present or not (Fig. 2.7). However, there was a main effect ($p<0.05$) for CPA, where total release was lower with CPA than without CPA.

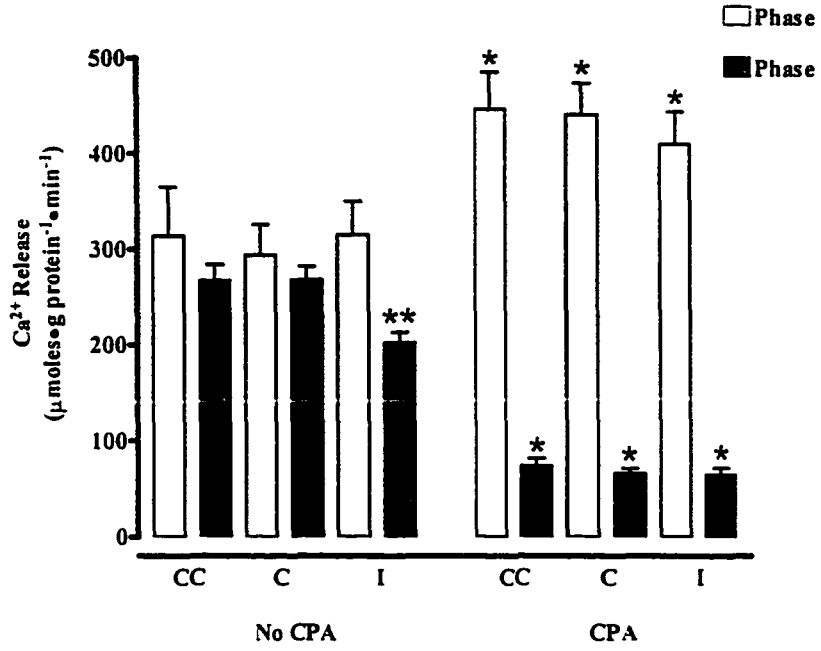



**Figure 2.5.** *Calcium release of CC, C and I homogenates prepared from mixed gastrocnemius and tibialis anterior muscles. $Ca^{2+}$ release was induced using $AgNO_3$ and was measured using Indo-1. $Ca^{2+}$ release was measured with and without CPA to block $Ca^{2+}$ uptake. The peak rates of $Ca^{2+}$ release corresponding to both Phase 1 and Phase 2 are shown both with and without CPA. Values are means ±SE; n = 9. CC, control control, C, control, I, ischemic. * Significantly different from No CPA. ** Significantly different from CC and C.*

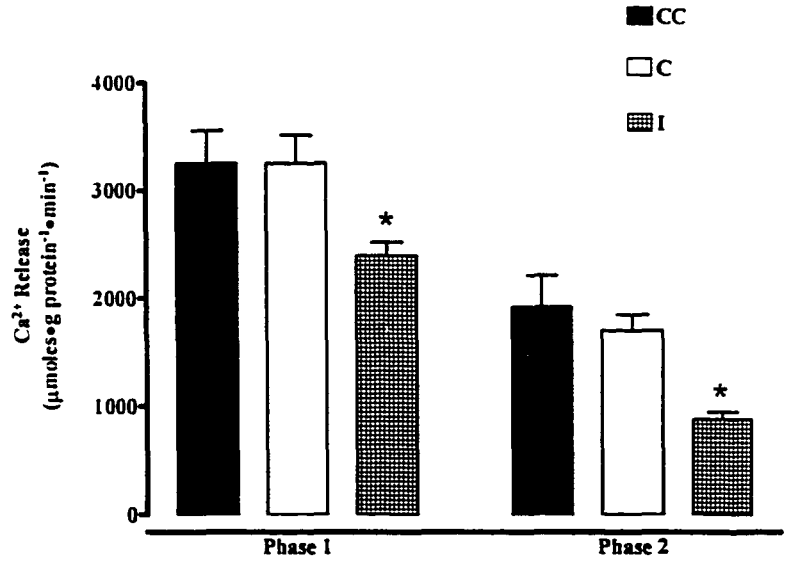



**Figure 2.6.** *Calcium release of CC, C and I SR vesicles prepared from mixed gastrocnemius and tibialis anterior muscles. $Ca^{2+}$ release was induced using $AgNO_3$ and was measured using Indo-1. The peak rates of $Ca^{2+}$ release corresponding to both Phase 1 and Phase 2 are shown. Values are means ±SE; n = 9. CC, control control, C, control, I, ischemic. * Significantly different from CC and C.*

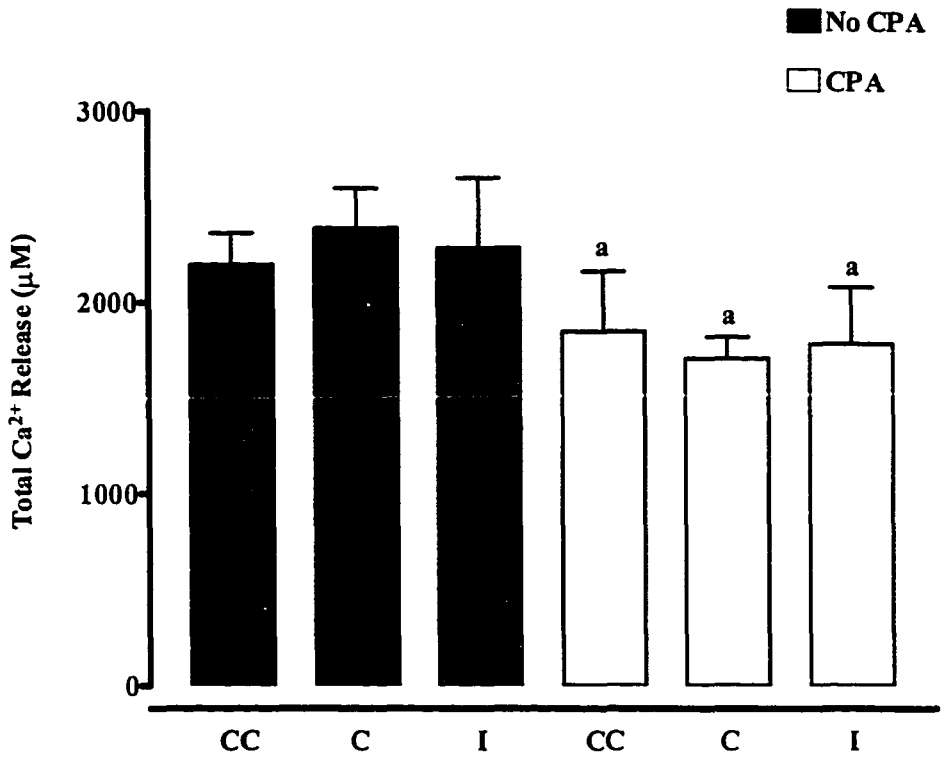



**Figure 2.7.** *Total calcium release of CC, C and I homogenates prepared from mixed gastrocnemius and tibialis anterior muscles. $Ca^{2+}$ release was induced using $AgNO_3$ and was measured using Indo-1. $Ca^{2+}$ release was measured with and without CPA to block $Ca^{2+}$ uptake. Total $Ca^{2+}$ release represents the total amount of $Ca^{2+}$ that was released from the SR until a plateau was reached and release of $Ca^{2+}$ stopped. Total $Ca^{2+}$ release corresponding to both Phase 1 and Phase 2 are shown both with and without CPA. Values are means ± SE; n = 5. CC, control control, C, control, I, ischemic. a Significant main effect ($p<0.05$) for CPA, with lower total $Ca^{2+}$ release with CPA than without CPA. There were no differences between groups within condition (CPA vs. No CPA).*

**$Ca^{2+}$-ATPase Activity.** Maximal $Ca^{2+}$-ATPase activity (μmoles•g protein$^{-1}$•min$^{-1}$) measured in homogenates between I (970 ± 54) and C (907 ± 70) was not different. Similarly, there were no differences between these muscles and CC (826 ± 46). In enriched SR vesicles, maximal $Ca^{2+}$-ATPase activity, which occurred at a $[Ca^{2+}]_f$ of approximately 6 – 10 μM in all groups (data not shown), was 34 and 27% lower ($p<0.05$) in I compared with CC and C, respectively (Fig. 2.8). There was no difference between CC and C. However, while maximal activity was depressed following I, there was no effect on enzyme kinetics

(Table 2.2). Kinetic analysis of the $Ca^{2+}$-ATPase activity-pCa curves (Fig. 2.9) showed that both the Hill coefficient and the $Ca_{50}$ were not different between groups.

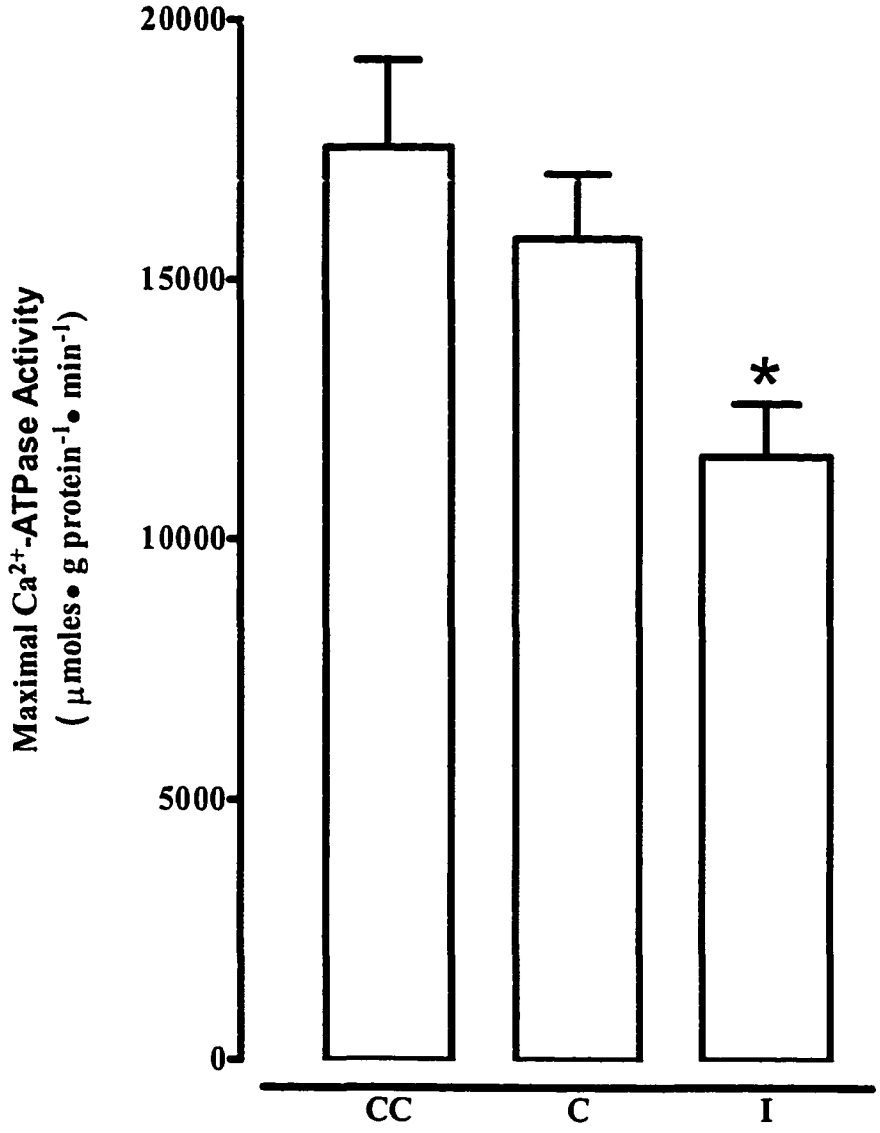



**Figure 2.8.** *Maximal SR $Ca^{2+}$-ATPase activity of CC, C and I SR vesicles prepared from mixed gastrocnemius and tibialis anterior muscles. Values are means ± SE; n = 9. CC, control control, C, control, I, ischemic. Maximal SR $Ca^{2+}$-ATPase activity occurred at a $[Ca^{2+}]_f$ of approximately 6 – 10 μM in all groups. * Significantly different ($p<0.05$) from CC and C.*

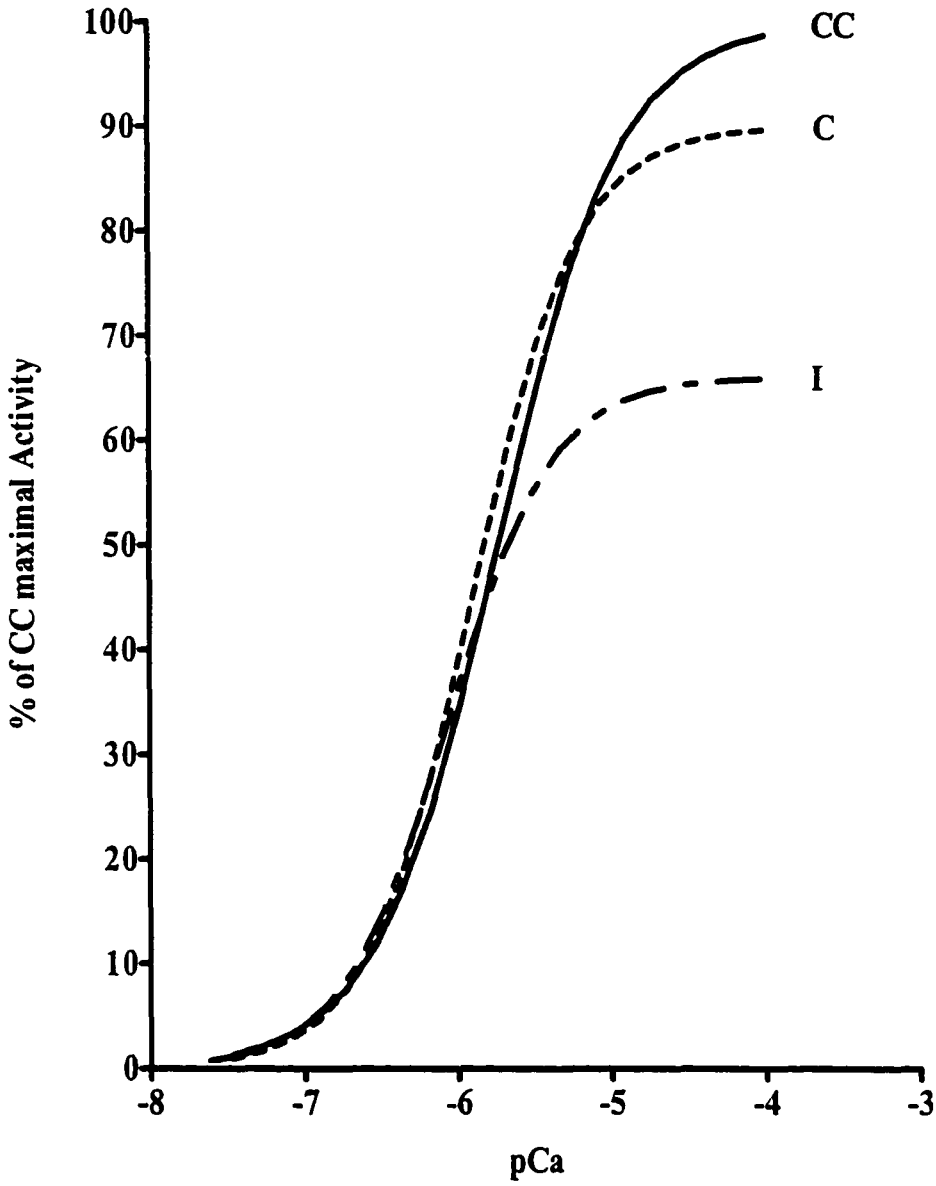



**Figure 2.9.** *Kinetic analysis showing the [$Ca^{2+}$] dependence of $Ca^{2+}$-ATPase activity of CC, C and I SR vesicles prepared from mixed gastrocnemius and tibialis anterior muscles. CC, control control, C, control, I, ischemic. Curves were fit by nonlinear regression using the average values of the Hill coefficient and $Ca_{50}$ from Table 2 for each group. Maximal activity was expressed relative to CC. Compared with CC, maximal activity was lower ($p<0.05$) in I, but there were no differences in the Hill coefficient or $Ca_{50}$ between groups.*

**Table 2.2.** *Kinetic analysis parameters of the $Ca^{2+}$-ATPase activity-pCa curves.*

| | CC | C | I |
|---|---|---|---|
| Hill coefficient | 1.1 ± 0.1 | 1.3 ± 0.1 | 1.3 ± 0.1 |
| $Ca_{50}$ | 6.1 ± 0.1 | 6.1 ± 0.1 | 6.2 ± 0.1 |

*Values are means ± SE (N=9 per group). CC, control control, C, control, I, ischemic. Kinetic characteristics were determined from plots obtained between $Ca^{2+}$-ATPase activity and pCa. Hill coefficient was determined over a range of $Ca^{2+}$-ATPase activities representing 20 to 80% of the maximal response. $Ca_{50}$ is the $Ca^{2+}$ concentration (pCa) needed to elicit 50% of the maximal $Ca^{2+}$-ATPase activity.*

**Electrophoresis and Western blot analysis.** Representative Western blots done under both non-reducing (no DTT) and reducing (with DTT) conditions, for a typical rat from each group, are provided in Fig. 2.10A and Fig. 2.10B, respectively. Total SR $Ca^{2+}$-ATPase protein contents were compared between groups using the Western blot analysis. Total protein was only analyzed on SR samples subjected to reducing conditions SDS-PAGE, since all of the $Ca^{2+}$-ATPase protein should be in the monomer form under these conditions and easily detected by the monoclonal antibody IIH11 (Molnar *et al.*, 1990). Six discrete bands were present under these conditions, corresponding to the monomer form of SERCA1 and smaller molecular weight SERCA1 products. No discrete bands corresponding to dimers or higher molecular weight aggregates were observed. Relative total protein content was taken as the sum intensity of all six bands and expressed relative to CC. There was no difference between groups in total SERCA1 protein content (Fig. 2.11).

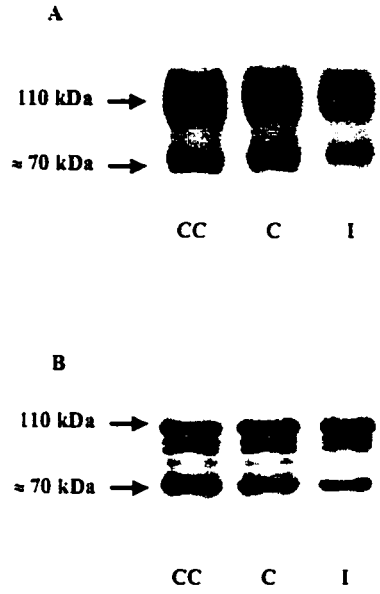



**Fig. 2.10.** *Western blot analysis of SERCA1 protein with the use of the monoclonal antibody IIH11. Blots represent the measurements from 1 CC and 1 E rat (both C and I limbs), under A) non-reducing conditions (no DTT) and B) reducing conditions (with DTT). Under non-reducing conditions, only two main bands were detected, corresponding to the monomer form of SERCA1 (110 kDa) and a smaller molecular weight (≈ 70 kDa) SERCA1 product. Six discrete bands were present under these conditions, corresponding to the monomer form of SERCA1 and smaller molecular weight SERCA1 products. CC, control control, C, control, I, ischemic.*

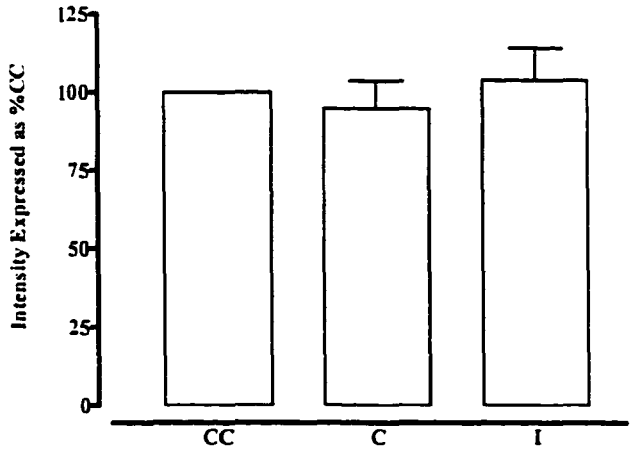



**Figure 2.11.** *Relative SERCA1 protein content in CC, C and I SR vesicles prepared from mixed gastrocnemius and tibialis anterior muslces. SERCA1 protein content was determined by reducing SDS-PAGE and Western blot analysis. Values are means ± SE; n= 9. CC, control control, C, control, I, ischemic.*

To determine if prolonged I results in SERCA1 protein oxidation and protein aggregation, SR samples from all groups were subjected to non-reducing (no DTT) and reducing (DTT) SDS-PAGE. Under non-reducing conditions, only two main bands were detected, corresponding to the monomer form of SERCA1 (110 kDa) and a smaller molecular weight (≈ 70 kDa) SERCA1 product. Under these conditions, the intensity of the band corresponding to the monomer form of SERCA1, was lower ($p<0.05$) in I, relative to CC (Fig. 2.12A). There was no difference in relative intensity of the 70 kDa bands between groups (Fig. 2.12B). When the same two bands were analyzed for relative intensity under reducing conditions, there were no differences for either band, between groups (Fig. 2.12). This finding suggests that higher molecular weight aggregates were present in ischemic SR, leading to a lower intensity of the monomer form of SERCA1 under non-reducing conditions, but not under reducing conditions.

**Fluorescence measurements.** FITC and NCD-4 binding were used as a method to assess the structure of the nucleotide binding domain and the $Ca^{2+}$ binding sites of the $Ca^{2+}$-ATPase following prolonged I, respectively. FITC covalently labels $lys_{515}$, which is close to the ATP binding site and competitively inhibits ATP binding (Pick and Karlish, 1980). Similarly, the label NCD-4 binds near the two $Ca^{2+}$ binding sites, in addition to some non-specific labeling, inhibiting binding of $Ca^{2+}$ without inhibiting the binding of ATP (Chadwick and Thomas, 1983).

A sample emission spectrum of FITC from each group is shown in Fig. 2.13A. The average maximum intensity, which occurs at 520 nm, was 26% lower in I ($p<0.05$) compared with both CC and C (Fig. 2.13B). There was no difference between CC and C. As

well, the maximum emission intensity of NCD-4 was not different between any of the groups (Fig. 2.14).

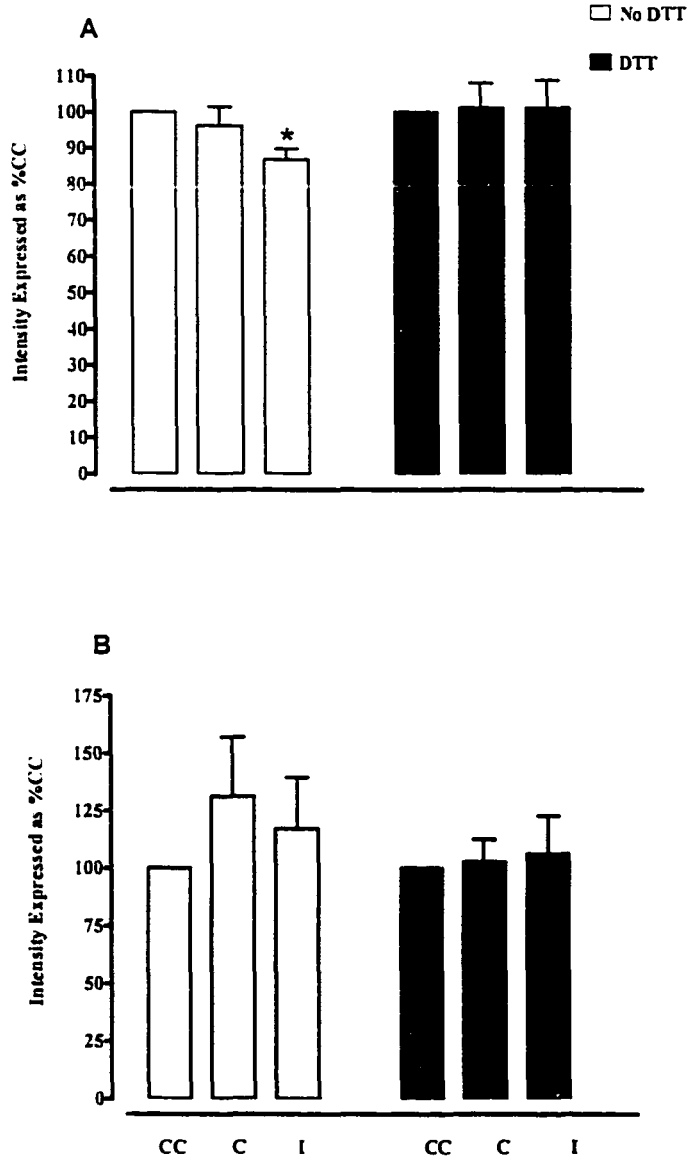



**Figure 2.12.** *Relative SERCA1 protein content in CC, C and I SR vesicles corresponding to (A) the monomer form (110 kDa), and (B) a smaller SERCA1 product (≈ 70 kDa). Protein contents were determined using non-reducing (No DTT) and reducing (DTT) SDS-PAGE and Western blot analysis. Values are means ± SE; n = 9. CC, control control, C, control, I, ischemic. *Significantly different (p<0.05) from CC.*

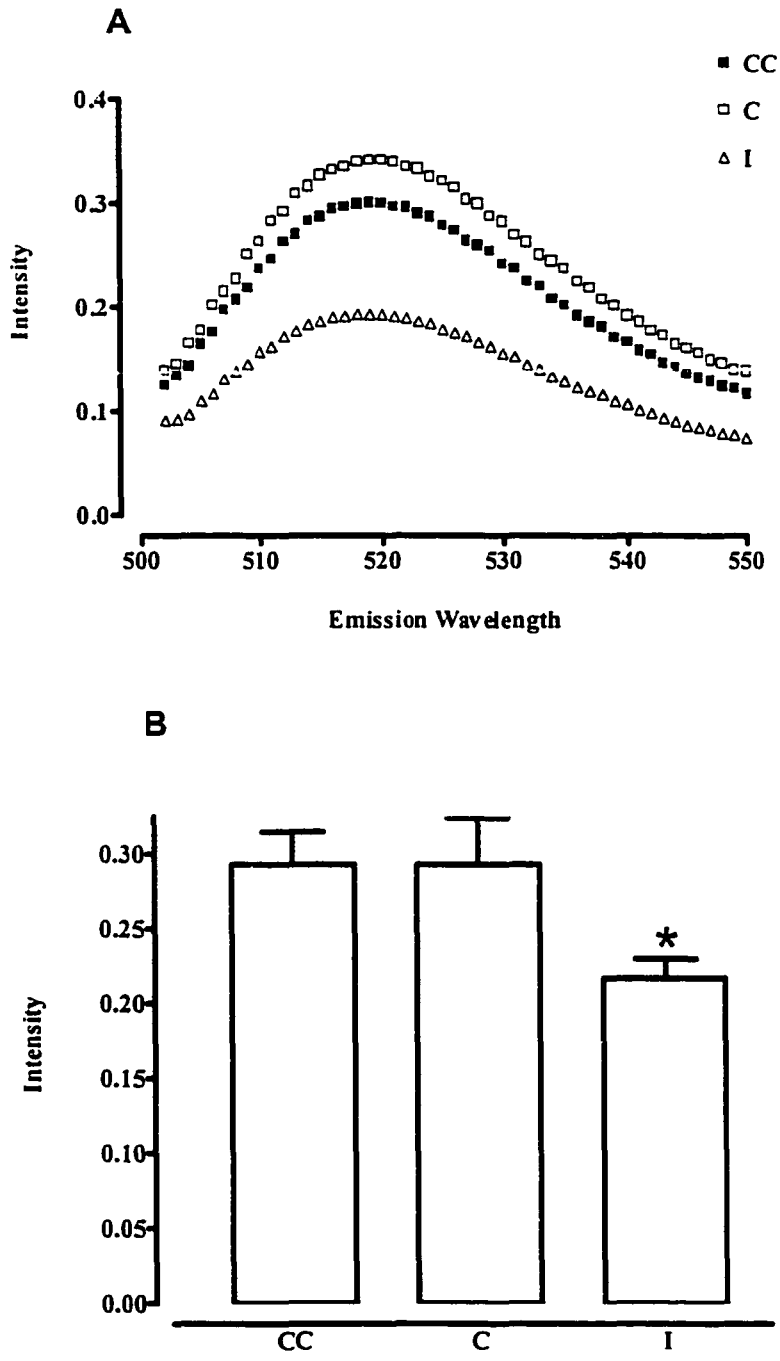



**Figure 2.13.** *Sample FITC emission spectra, one each from CC, C and I SR vesicles (A) and maximum FITC fluorescence for each group (B). Values are means ± SE; n=9. CC, control control, C, control, I, ischemic. Maximum FITC fluorescence occurs at 520 nm. *Significantly different ($p<0.05$) from CC and C.*

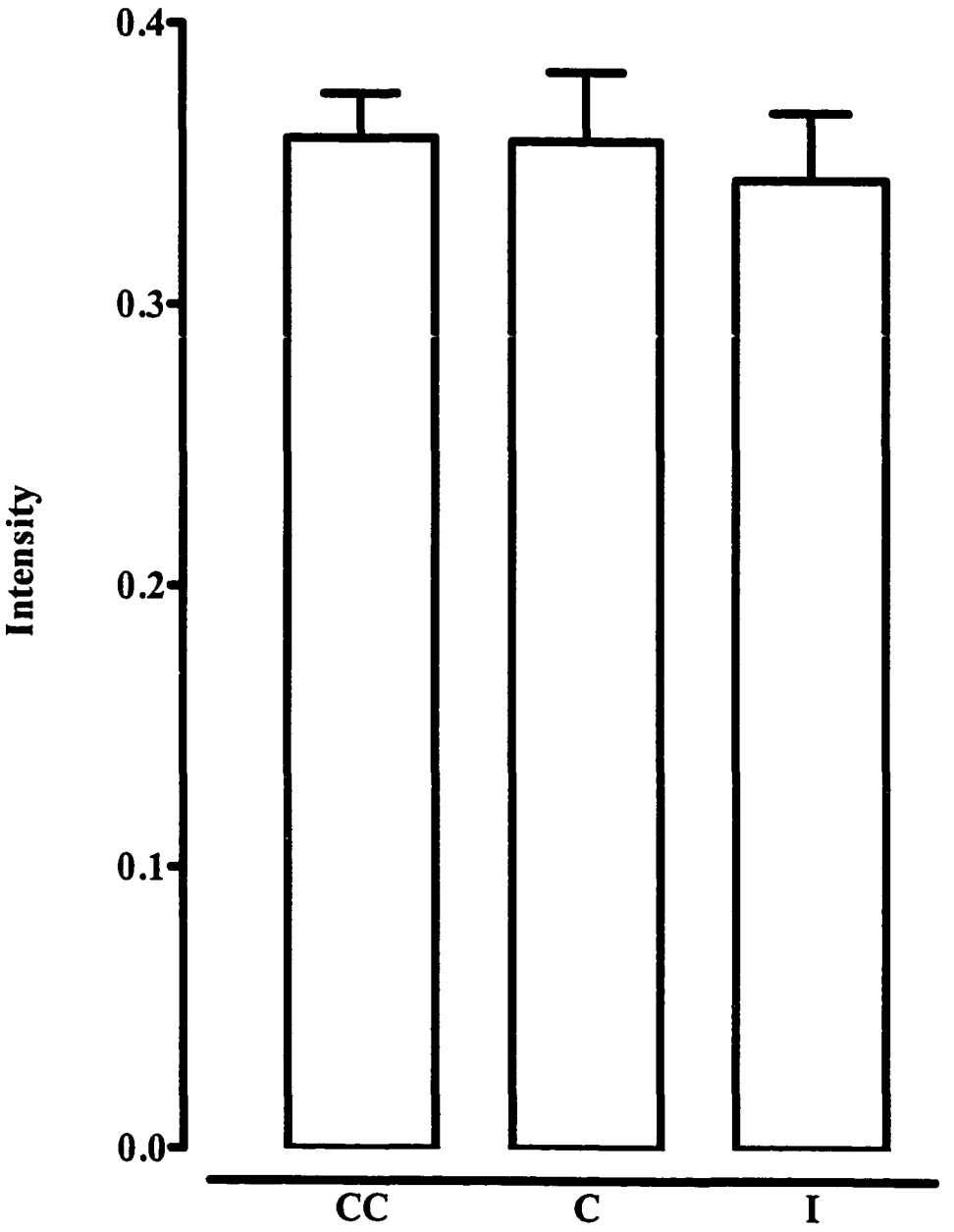



**Figure 2.14.** *Maximum NCD-4 fluorescence in CC, C and I SR vesicles prepared from mixed gastrocnemius and tibialis anterior muscles. Values are means ± SE; n = 9. CC, control control, C, control, I, ischemic. There were no significant differences between groups.*

## Discussion

In both skeletal (Rubin *et al.*, 1996) and cardiac (Opie, 1989; Kaplan *et al.*, 1997) muscle, loss of calcium homeostasis leading to cellular calcium overload appears to be a primary mechanism responsible for postischemic injury and contractile dysfunction. Generally, the same is true in most biological systems, where the loss of calcium homeostasis is central to the irreversible, and sometimes reversible, loss of normal cellular function, under numerous conditions (Belcastro *et al.*, 1998; Squier and Bigelow, 2000). The SR is the primary organelle responsible for maintaining cellular calcium homeostasis in both cardiac and skeletal muscle. Not surprisingly, alterations in SR function have been implicated in the loss of calcium homeostasis such as occurs with aging (Viner *et al.*, 1999), exercise (Williams and Klug, 1995) and I (Meno *et al.*, 1984; Kaplan *et al.*, 1992, Green *et al.*, 1997; Osada *et al.*, 1998). However, very few studies have characterized the effects of prolonged I in skeletal muslce, on SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase function.

In the present study, <u>in vitro</u> measures of SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase activity were made on both SR vesicles and muscle homogenates, prepared from rat skeletal muscle tissue that was made ischemic <u>in vivo</u>, for 4 hours. As expected, we found evidence to support our hypothesis, namely, that SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase activity are impaired with prolonged skeletal muscle I. This suggests that SR dysfunction plays a role in the observed calcium overload that occurs with skeletal muscle I (Rubin *et al.*, 1996).

The rate of $Ca^{2+}$ uptake in this study, was assessed across a range of sub-maximal $[Ca^{2+}]_f$. The reduction in $Ca^{2+}$ uptake with I was consistent across all $[Ca^{2+}]_f$, regardless of whether it was measured in muscle homogenates or SR vesicles. Unfortunately, we were

unable to determine maximal $Ca^{2+}$ uptake rates in either homogenates or SR vesicles with any confidence, therefore calculation of the Hill coefficient and the $Ca^{2+}{}_f$ at half-maximal activity was not possible. Maximal $Ca^{2+}$-ATPase activity in these samples occurs at a $[Ca^{2+}]_f$ of approximately 6-10 μM (unpublished). Since the highest $[Ca^{2+}]_f$ used to measure the rate of $Ca^{2+}$ uptake in this study was 2.0 μM, it is unlikely maximal $Ca^{2+}$ uptake rates occurred at this level.

The net rate of $Ca^{2+}$-uptake depends on two processes; namely $Ca^{2+}$ accumulation by the SR and $Ca^{2+}$ efflux from the SR. $Ca^{2+}$ accumulation is controlled by the activity of the SR $Ca^{2+}$-ATPase pump. Therefore, reductions in $Ca^{2+}$-uptake with I are likely due to reductions in maximal $Ca^{2+}$-ATPase activity. Alternatively, reductions in $Ca^{2+}$-uptake with I could be due to an increased efflux of $Ca^{2+}$ from the SR. An increased efflux of $Ca^{2+}$ may either be due to an increased permeability of the SR membrane to $Ca^{2+}$ or an increase in the passive release of $Ca^{2+}$ through CRC (Rapundalo *et al.*, 1986). There is some evidence in the heart, that I-induced reductions in SR $Ca^{2+}$ uptake are due to increases in the passive permeability of the CRC to calcium (Davis *et al.*, 1992; O'Brien *et al.*, 1997). In these studies, $Ca^{2+}$ uptake was stimulated in the presence of ryanodine, to a much greater extent in ischemic SR compared to control. However, in agreement with most other evidence in the heart (Mubagwa, 1995), we have found that ryanodine had no effect on $Ca^{2+}$ uptake measured in skeletal muscle homogenates, which was reduced following partial I in rats (Tupling *et al.*, 1997). Similarly, in the current study, the reduction in $Ca^{2+}$ uptake with prolonged I does not appear to be due to excessive $Ca^{2+}$ leakage through the CRC, as $Ca^{2+}$ uptake was reduced to the same extent in ischemic homogenate samples, measured both with and without ryanodine.

Surprisingly, most of the related studies in the heart, did not directly measure the activity of the SR $Ca^{2+}$-ATPase, but only assumed that reductions in $Ca^{2+}$ uptake, in the absence of a ryanodine effect, were due to reductions in SR calcium pumping (Rehr *et al.*, 1991; Kaplan *et al.*, 1992). It can be assumed that most of the reduction in $Ca^{2+}$ uptake with I in this study can be explained by impaired SR $Ca^{2+}$ pump function, since maximal activity of the $Ca^{2+}$ pump is reduced following 4 hours of I in rat skeletal muscle.

Clearly, elevations in cytosolic $[Ca^{2+}]_f$ with I, could be due to an impaired SR $Ca^{2+}$ pump function and $Ca^{2+}$ uptake. However, unlike the heart, in this model of skeletal muscle I, the muscle was completely quiescent and without regular fluctuation of $[Ca^{2+}]_f$. As a result, for a reduction in SR $Ca^{2+}$ pump function to contribute to the increase in $[Ca^{2+}]_f$ with I, the pump would have to be challenged by elevations in $[Ca^{2+}]_f$ from sources other than excitation-induced $Ca^{2+}$ release.

One possible source would be the basal level of SR $Ca^{2+}$ release that occurs at rest. We did not assess the rate of $Ca^{2+}$ uptake at a $[Ca^{2+}]_f$ corresponding to typical resting levels (below 100 nM), however, since $Ca^{2+}$ uptake was reduced with I at all four sub-maximal $[Ca^{2+}]_f$ measured in this study, we would assume that $Ca^{2+}$ uptake would also be reduced at resting $[Ca^{2+}]_f$ with I. Over time, this would lead to elevations in $[Ca^{2+}]_f$ and $Ca^{2+}$ overload. Moreover, basal SR $Ca^{2+}$ release may be increased at some point during the ischemic period, despite our observation that maximal $AgNO_3$-induced $Ca^{2+}$ release was reduced with I and that ryanodine had no effect on $Ca^{2+}$ uptake (discussed later). There is also good evidence that greater amounts of extracellular calcium enter the cell through the L-type $Ca^{2+}$ channel and via the $Na^+/Ca^{2+}$ exchanger with I and R in skeletal muscle (Welsh and Lindinger, 1996).

This study appears to be the first study to assess the effects of I on maximal SR $Ca^{2+}$ release in vitro, in skeletal muscle. One study in the heart has reported that EGTA-induced $Ca^{2+}$ release, measured in SR vesicles prepared from the left ventricle, was reduced following 30 min global I (Osada *et al.*, 1998). Similarly, in this study, we also found reductions in maximal $Ca^{2+}$ release with prolonged I, measured in SR vesicles. The $Ca^{2+}$ release technique we employed in this study, $AgNO_3$-induced $Ca^{2+}$ release, resulted in a $Ca^{2+}$ release response that was biphasic in nature. In SR vesicles, the peak rate of $Ca^{2+}$ release corresponding to each phase, was lower in ischemic SR vesicles compared with control.

This $Ca^{2+}$ release technique has been used to assess exercise-induced changes in SR $Ca^{2+}$ release function in SR vesicles prepared from both rat (Favero *et al.*, 1993) and frog (Williams *et al.*, 1998) skeletal muscle. The peak rate of $Ca^{2+}$ release was also reduced in these studies, following exercise. However, in these studies, and others (Ruell *et al.*, 1995; Williams and Ward, 1998), only one $Ca^{2+}$ release value was reported, corresponding to the peak positive derivative of the $Ca^{2+}$ release curve. It is uncertain from these studies, whether $AgNO_3$-induced $Ca^{2+}$ release was biphasic in nature and simply not reported, or if we are the first to observe such a response using this technique. The mechanism for this biphasic repsonse is unknown, however the clear distinction between the two phases as can be seen in Fig. 1, and the differential effects of CPA on the two phases, indicates the value of reporting data on each phase.

Unlike in SR vesicles, the peak rate of $Ca^{2+}$ release measured in homogenates was only lower in Phase 2 and not in Phase 1. The different results between SR vesicles and homogenates may be due to differential selection of a population of SR vesicles during the

isolation procedure, between ischemic and control muscle, as has been reported in the heart following I and R (Rapundalo *et al.*, 1986; Wu and Feher, 1997). On the other hand, the rapid release rate characteristic of Phase 1, as measured by Indo-1, may be affected to a greater extent by the various cytosolic $Ca^{2+}$ binding proteins than Phase 2, and this would certainly be different between SR vesicles and homogenates. Since, the effects of I on SR $Ca^{2+}$ uptake were similar between SR vesicles and homogenates, the reduction in the peak rate of $Ca^{2+}$ release corresponding to Phase 1 in SR vesicles, was likely due to I and not the isolation procedure itself.

In this study, $AgNO_3$-induced $Ca^{2+}$ release was also measured in the presence of CPA, to block $Ca^{2+}$ uptake during net $Ca^{2+}$ release in homogenates only. Given that the net $Ca^{2+}$ release by the SR is a result of the opposite activities of the CRC and the $Ca^{2+}$-ATPase, an abnormal $Ca^{2+}$ release may be caused by a dysfunction of either or both structures. Therefore, CPA was used to assess the effects of I on SR $Ca^{2+}$ release function independent of changes in $Ca^{2+}$ uptake.

For this technique to be effective, timing of CPA addition is critical. It has been shown in both actively loaded SR vesicles (Dettbarn and Palade, 1998) and skinned skeletal muscle fibres (Duke and Steele, 1998), that CPA inhibition of the SR $Ca^{2+}$ pump induces a slow release of $Ca^{2+}$ from the SR. Given the influence of both external and luminal $Ca^{2+}$ on SR $Ca^{2+}$ release (for review see Hidalgo and Donoso, 1995; Melzer *et al.*, 1995), interpretation of the effects of CPA on $Ca^{2+}$ release depends on the length of the incubation period of SR vesicles with CPA. In this study, $Ca^{2+}$ release was initiated by the addition of $AgNO_3$, immediately following addition of CPA, thus minimizing possible CPA-induced $Ca^{2+}$ leakage from the SR. Consequently, it would appear that, the effects of CPA on $Ca^{2+}$

release were directly related to inhibition of $Ca^{2+}$ pump activity and $Ca^{2+}$ uptake, and not due to altered $Ca^{2+}$ levels on either side of the SR membrane prior to release.

The results indicate that $Ca^{2+}$ release rates for Phase 1 were higher in the presence of CPA for all groups, with no differences between groups. However, for Phase 2, $Ca^{2+}$ release rates were lower with CPA for all groups. If the rate of $Ca^{2+}$ release simply reflects a balance between the rate of release and reuptake of $Ca^{2+}$ during release, then this finding is opposite to what would have been expected. This finding suggests that some $Ca^{2+}$ pump activity and $Ca^{2+}$ uptake during net $Ca^{2+}$ release, is necessary for a normal release response. In accordance with this interpretation, the reduction in $Ca^{2+}$ uptake with I may contribute to the reduction in $Ca^{2+}$ release that occurs with I, at least for Phase 2.

While most studies have reported an increase in $Ca^{2+}$ release with CPA (Makabe *et al.*, 1996; Duke and Steele, 1998; Même *et al.*, 1998; Burdyga and Wray, 1999), two studies have reported a negative effect of CPA on $Ca^{2+}$ release (Goeger and Riley, 1989; Huchet and Leoty, 1994). One possible explanation for a negative effect of CPA on $Ca^{2+}$ release has to do with a possible role for the $Ca^{2+}$ pump in counter-ion exchange during periods of net $Ca^{2+}$ release. Relatively little is known about the role of counter-ion exchange and membrane potential in the control of SR $Ca^{2+}$ transport.

Although the number of functional CRCs, as assessed by a decrease in [$^3$H]ryanodine binding capacity in SR vesicles, was not measured in this study, it would be expected that a decrease would occur with prolonged I. This finding has been reported in several studies (Holmberg and Williams, 1992; Zucchi *et al.*, 1994; Osada *et al.*, 1998) with myocardial I and R. It is possible that most of the reduction in peak $Ca^{2+}$ release for Phase 1 and Phase 2 in SR vesicles, likely can be explained by a reduced number of functioning CRCs with I.

Damage to the $Ca^{2+}$ release channel and reductions in $Ca^{2+}$ release function with I may in fact be secondary to reductions in $Ca^{2+}$ uptake and elevations in $[Ca^{2+}]_f$. This is one mechanism proposed to explain exercise-induced reductions in SR $Ca^{2+}$ release and skeletal muscle fatigue (Favero, 1999). Calcium may indirectly lead to protein damage due to its activation of the neutral protease calpain (Belcastro *et al.*, 1996). $Ca^{2+}$-activated calpain-induced proteolysis of the CRC has been demonstrated in heavy SR membranes, however, proteolysis did not alter the $Ca^{2+}$ handling or ryanodine binding properties of the heavy SR membranes (Gilchrist *et al.*, 1992). This would suggest that other mechanisms may be involved with I, to explain the reduction in $Ca^{2+}$ release that occurs.

One possibility is $O_2$ free radicals. It is well established that free radical formation plays a primary role in the etiology of I-induced damage in skeletal muscle (Rubin *et al*, 1996). We have observed large elevations in hypoxanthine and xanthine following 4 hours I (unpublished), which are substrates for the enzyme xanthine oxidase. The enzyme catalyzes the reduction of $O_2$, leading to the formation of superoxide and $H_2O_2$, and it has been proposed as a central mechanism of oxidative injury (McCord, 1985). It appears reasonable to assume that there was significant free radical production in the ischemic muscle compared with control in this study.

Very few studies have reported the effects of free radicals on SR CRCs (Kourie, 1998). It is apparent however, that free radicals can trigger rapid $Ca^{2+}$ release from the SR by modifying critical sulfhydryls in the CRC and increasing the open probability of the channel (for review see, Abramson and Salama, 1989; Favero, 1999). If this occurs with I, elevations in $Ca^{2+}$ release through the CRC at rest, may contribute to the rise in $[Ca^{2+}]_f$ and $Ca^{2+}$ overload that occurs with I. However, we found that $Ca^{2+}$ release was reduced with I.

In agreement with this, Brotto and Nosek (1996) found that hydrogen peroxide inhibited $Ca^{2+}$ release in skinned skeletal muscle fibres from the rat. It is also possible that an initial increase in the probability of the channel being in the open state is followed by irreversible loss of channel function as a result of free radical damage (Holmberg *et al.*, 1991).

The reduction in $Ca^{2+}$ uptake with I is probably related to free radical-induced damage to the $Ca^{2+}$-ATPase. The $Ca^{2+}$-ATPase contains 24 cysteine residues (Brandl *et al.*, 1986). Consequently, the SR $Ca^{2+}$-ATPase may be a principal target for modulation of muscle function by reactive oxygen species (Kukreja *et al.*, 1988). Inactivation of the SR $Ca^{2+}$-ATPase as a result of free radical formation, has been demonstrated in numerous in vitro studies (Morris and Sulakhe, 1997; Senisterra *et al.*, 1997; Viner *et al.*, 1997; Xu *et al.*, 1997). As well, there is evidence that pretreatment with oxygen free radical scavengers, superoxide dismutase and catalase, can maintain higher $Ca^{2+}$ uptake by the SR of skeletal muscle, following 3 hours of I and 19 hours of R in rat hindlimb (Lee *et al.*, 1987).

The effects of free radicals on SR $Ca^{2+}$-ATPase structure and function in vitro, have been well documented (Morris and Sulakhe, 1997; Viner *et al.*, 1999; Xu *et al.*, 1997). In this study, elucidation of the structural alterations that occur to the SR $Ca^{2+}$-ATPase in vivo, with prolonged I in rat skeletal muscle, which could explain the predicted reduction in $Ca^{2+}$-ATPase activity that occurs with I, was attempted. The results for the SR vesicles, are consistent with the hypothesis, namely that the reduction in $Ca^{2+}$-ATPase activity are accompanied by alterations in the nucleotide binding site on the enzyme.

Although the mechanisms remain unknown, these metabolic data indicate a strong possibility that free radical production, mediated during the 4 hours of I, are involved. As a result of massive reductions in the high-energy phosphates, PCr and ATP, large elevations in

hypoxanthine and xanthine, which are substrates for the enzyme xanthine oxidase, would be expected (Rubin *et al.*, 1992). The enzyme catalyzes the reduction of $O_2$, leading to the formation of superoxide and $H_2O_2$, and it has been proposed as a central mechanism of oxidative injury (McCord, 1985). Therefore, although free radical production in this study was not measured directly, it can be assumed that there was significant free radical production in the ischemic muscle compared with control.

The results from this study indicate that the structural changes in $Ca^{2+}$-ATPase with I are similar to those reported following exposure of the $Ca^{2+}$-ATPase to oxidizing conditions in vitro. In the in vitro study, it was found that exposure of the $Ca^{2+}$-ATPase to levels of hydroxyl radicals similar to that measured during postischemic R, denatures the $Ca^{2+}$-ATPase and inhibits ATPase activity by directly attacking the ATP binding site without damaging the primary structure of the enzyme (Xu *et al.*, 1997). This would suggest that prolonged I leads to structural reorganization of the nucleotide binding domain which would likely impair ATP binding and ATPase activity.

In the in vitro study, it was also found that presaturation of the active site with ATP completely protected both cardiac and skeletal muscle SR $Ca^{2+}$-ATPase function from hydroxyl radical-induced inhibition. This suggests that depletion of cellular ATP, in the region of the enzyme, induces the structural alteration to the nucleotide binding domain of the $Ca^{2+}$-ATPase. In the present study, depletion of muscle ATP stores, as observed with prolonged periods of skeletal muscle I, not only suggests formation of free radicals in skeletal muscle, but may also influence the susceptibility of the SR $Ca^{2+}$-ATPase to free radical-induced damage, specifically to the nucleotide binding domain.

There is evidence derived from exercise, of reduced SR $Ca^{2+}$-ATPase activity being related to structural alterations of the nucleotide binding domain, where ATP levels may also be reduced. Following prolonged exercise in rats (Luckin *et al.*, 1991), and chronic stimulation in rabbits (Leberer *et al.*, 1987), reductions in both maximal SR $Ca^{2+}$-ATPase activity and FITC binding capacity in SR vesicles have been reported. At least in the chronic stimulation model, pronounced reductions in ATP levels occur (Green *et al.*, 1992). Not unlike muscle I, free radicals are produced in exercise and may contribute to skeletal muscle fatigue and tissue damage (Davies *et al.*, 1982; Barclay and Hansel, 1991). In fact, recent evidence suggests that inactivation of the SR $Ca^{2+}$-ATPase following 4 days of chronic activity in rabbit fast-twitch skeletal muscle, results from protein oxidation (Klebl *et al.*, 1998).

The reductions in maximal $Ca^{2+}$-ATPase activity that were observed in this study in ischemic SR compared with CC and C, were highly correlated with the reductions in FITC binding that occurred with I. On the other hand, kinetic analysis of $Ca^{2+}$-ATPase activity shows that both the Hill coefficient and sensitivity of the enzyme to calcium ($Ca_{50}$), were unaffected. These results are consistent with a problem in binding ATP, but only in a selected population of $Ca^{2+}$ pumps. A similar effect has been postulated to occur in rabbit muscle following chronic muscle activity (Leberer *et al.*, 1987). The reduction in FITC binding is not due to reductions in $Ca^{2+}$-ATPase protein as determined by Western blot analysis. Rather, it appears that a reduction in the number of FITC binding sites / $Ca^{2+}$-ATPase protein occurred in response to the prolonged I.

The precise nature of the structural modification to the nucleotide binding domain in ischemic SR, which resulted in a reduced FITC binding capacity, cannot be directly

ascertained from this study. However, several in vitro studies have helped to characterize the free radical-induced molecular modification of the SR $Ca^{2+}$-ATPase that is correlated with its functional properties (Viner *et al.*, 1997, 1999; Senisterra *et al.*, 1997). Viner *et al.* (1997), employed a peroxyl radical generating system using the free radical initiator 2,2'-azobis(2-amidinopropane) dihydrocholride, to examine and identify oxidation-sensitive peptides within the SR $Ca^{2+}$-ATPase of fast twitch rabbit skeletal muscle. They identified six oxidatively sensitive peptides on the cytoplasmic side of the SR membrane. One of these peptide segments ($Glu_{551}$-$Arg_{604}$) is located in the nucleotide binding domain and was found to participate in the formation of intermolecular bityrosine cross-links with the identical $Glu_{551}$-$Arg_{604}$ peptide from a neighboring $Ca^{2+}$-ATPase polypeptide chain. Although this peptide does not contain the FITC binding site $Lys_{515}$ (Champeil *et al.*, 1988) or the actual ATP binding site around $Arg_{505}$ (Taylor and Green, 1989), cross-linking and aggregation in this region may interfere with both FITC and ATP binding.

In the present study, there was also evidence for aggregation of $Ca^{2+}$-ATPase in ischemic SR compared with control, as indicated by the lower relative monomer form of the protein in I. However, this difference in monomer levels was corrected in reducing gels, suggesting that aggregation in this study was due to disulfide cross-links. A similar finding was reported by Senisterra *et al.* (1997), with exposure of the SR $Ca^{2+}$-ATPase to the thiol-specific reagents, diamide and arsenite.

The difference between the present study and the study by Viner *et al.* (1997) is likely the free radical species involved in the oxidation of the $Ca^{2+}$-ATPase. The peroxyl and alkoxyl radicals generated by the system employed by Viner *et al.*, do not occur physiologically. Interestingly, Viner *et al.* (1997) found that exposure of the $Ca^{2+}$-ATPase to

$H_2O_2$ resulted in different kinetics of oxidation, particularly of the peptide sequence $Glu_{551}$-$Arg_{604}$. Therefore, the results from Viner *et al.* (1997) are not representative of an in vivo model of skeletal muscle I. In the present study, since muscle was sampled prior to R, xanthine oxidase-derived free radicals, superoxide and $H_2O_2$, were likely the primary free radicals involved.

If the reduction in maximal $Ca^{2+}$-ATPase activity and FITC binding capacity is related to the higher levels of aggregation with I in this study, likely one or more of the cysteine residues located within the nucleotide binding domain is involved. The number of free SH groups was not assessed in this study, but since aggregation was decreased in reducing gels, it is likely that cysteine oxidation and disulfide cross-linking were involved. It is also possible that intra-molecular cross-links were formed in single polypeptide chains, which would not lead to increased aggregation, but could affect ATP binding and ATPase activity.

Determining which cysteine residues are affected by prolonged I is not possible with the techniques employed in our laboratory. In a recent study, Viner *et al.* (1999) reported that modification of only $Cys_{349}$ is responsible for the modulation of the SR $Ca^{2+}$-ATPase upon exposure to peroxynitirite. This residue is located very close to the phosphorylation site at $Asp_{351}$ (Maruyama and MacLennan, 1988) but is not within the nucleotide binding domain. Peroxynitrite is formed through the interaction between nitric oxide and superoxide radical (Wang and Zweier, 1996). However, it has been shown that R after I in rat skeletal muscle is necessary for nitric oxide formation (Lepore *et al.*, 1999). Therefore, it is unlikely that peroxynitrite was involved in modulating SR $Ca^{2+}$-ATPase function and structure following prolonged I in this study.

It has been shown that labeling of $Cys_{344}$ with Br-DMC (4-(bromomethyl)-6,7-dimethoxicoumarin) or $Cys_{670}$ and $Cys_{674}$ with IAEDANS (5-((((2-iodoacetyl)amino)-ethyl)amino)naphthalene-1-sulfonic acid) does not affect $Ca^{2+}$-ATPase activity or conformational stability (Senisterra *et al.*, 1997). It is possible that other cysteine residues within the nucleotide binding domain, such as $Cys_{525}$, $Cys_{561}$ or $Cys_{614}$, were oxidized to some level during I, which could explain the reduction in both FITC binding capacity and increase in protein aggregation. However, the possibility that other functionally important amino acid residues within the nucleotide binding domain or elsewhere throughout the sequence of the $Ca^{2+}$-ATPase were altered with I, can not be ruled out.

In this study, it was determined if structural alterations in the calcium binding domain of the $Ca^{2+}$-ATPase occurs with I, using the fluorescent probe NCD-4. It was found that I had no effect on the maximum binding capacity of NCD-4 suggesting that the structure of the $Ca^{2+}$ binding domain was unaltered following prolonged I. This is in close agreement with kinetic analysis of ATPase activity in this study, as the Hill coefficient, which theoretically represents the number of calcium binding sites, was not different between groups.

As reported above, smaller molecular weight SERCA1 fragments were detected using reducing SDS-PAGE and Western blot analysis. However, the relative amount of each fragment was not different between groups (data not shown). Likely, the appearance of these products was due to proteolytic activity and / or oxidative fragmentation that took place during homogenization and preparation of SR vesicles and this should be the same for all groups. The homogenization buffer used for this study, only included one proteolytic inhibitor (PMSF) and did not include the SH reducing agent, DTT. The decision not to use

DTT was purposeful as we have reported that even 5 mM DTT in the homogenization buffer, can alter the effects of 4 hours I on SR $Ca^{2+}$-ATPase activity (Tupling *et al.*, 1999).

The effect of I on the $Ca^{2+}$-ATPase was only observed in the SR vesicles. There was no change in maximal $Ca^{2+}$-ATPase activity in the homogenates. Interestingly, we have previously observed increases in maximal activity in homogenates with shorter periods of I (Green *et al.*, 1996). It is possible that I is without effect on homogenates and that the reductions in $Ca^{2+}$-ATPase activity in SR vesicles simply reflects a selective loss of undamaged SR during the isolation procedure. This has been observed previously in ischemic hearts (Rapundalo *et al.*, 1986). On the other hand, the failure to find reductions in $Ca^{2+}$-ATPase activity in homogenates could be due to activation of other cellular ATPases, masking the effects of the inhibiting agents, or recruitment of additional enzyme by co-valent modification leading to phosphorylation or production of an activator substance during I, which binds to the $Ca^{2+}$-ATPase and activates it (Green *et al.*, 1996). It is possible that these changes become lost during the vesicle isolation process. Regardless of the mechanism, the overall effect would be to minimize the inactivation of the pool of enzymes that occurs during I.

In the present study, an I model in rat skeletal muscle known to lead to elevations in oxygen free radical production was employed. It appears that the loss of $Ca^{2+}$ homeostasis with I may be secondary to free radical induced damage of the SR $Ca^{2+}$-ATPase and/or CRC. However, it is important to remember that an <u>in vivo</u> model of skeletal muscle I was employed in this study and that SR transport function was assessed <u>in vitro</u>, under ideal conditions. Any I-induced changes in $Ca^{2+}$ uptake or $Ca^{2+}$ release measured <u>in vitro</u> must be due to intrinsic changes such as structural alterations in either one or more of the SR

proteins, the phospholipid membrane of the SR, or both. It was shown that prolonged I leads to reductions in both FITC binding capacity and protein aggregation which is associated with reductions in maximal $Ca^{2+}$-ATPase activity. These results suggest that the molecular mechanism is likely oxidation of one or more of the cysteine residues within the nucleotide binding domain of the $Ca^{2+}$-ATPase by xanthine oxidase-produced superoxide and / or $H_2O_2$. While our in vitro observations of a reduced $Ca^{2+}$ uptake and $Ca^{2+}$ release are likely important, reductions in both would be even greater in vivo due to the additional local metabolic effects (Lamb *et al.*, 1992; Zhu and Nosek, 1991; Xu *et al.*, 1996; Duke and Steele, 1999).

# CHAPTER III

## THE EFFECTS OF ISCHEMIA-REPERFUSION ON SKELETAL MUSCLE SARCOPLASMIC RETICULUM STRUCTURE AND FUNCTION WITH AND WITHOUT SUPEROXIDE DISMUTASE PLUS CATALASE

## Abstract

To investigate the hypothesis that I and R would impair SR $Ca^{2+}$-regulation in skeletal muscle, Sprague-Dawley rats (n=40), weighing 290 ± 3.5 g (mean ± SE) were randomly assigned to either a control group (CC) in which the effects of anesthetization only were studied or to an experimental group (E) in which the muscles in one hindlimb were made ischemic for 4 hours and subsequently reperfused for 1 hour (I). The non-ischemic, contralateral muscles served as control (C). In half of the E animals, a single dose of SOD + CAT was administered just prior to R. Measurements of $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase properties were made in both homgenates and SR vesicles, prepared from mixed G and TA muscles sampled from CC, C and I hindlimbs. $Ca^{2+}$ uptake was lower ($p<0.05$) with IR compared with CC, by 35% in purified SR vesicles. There were no differences between CC and C. In muscle homogenates, $Ca^{2+}$ uptake although 20-25% lower ($p<0.05$) in C, compared with CC, was not different in the IR muscle. $Ca^{2+}$ release, in muscle homogenates, using $AgNO_3$-induced $Ca^{2+}$ release, were not different between any of the groups for either Phase 1 or Phase 2. However, in purified SR vesicles, maximal $Ca^{2+}$ release was 31% higher ($p<0.05$) with IR, compared with CC for Phase 1, with no difference between CC and C. No differences existed between any of the groups for Phase 2 in purified SR vesicles. Measurements of $Ca^{2+}$-ATPase properties in homogenates and SR vesicles, indicated no effects of IR on maximal acitivity, the Hill coefficient or $Ca_{50}$. SOD + CAT treatment protected against reductions in homogenate $Ca^{2+}$ uptake in C, compared with CC ($p<0.05$) but had no effect on any other measure. These results indicate that the alterations in SR $Ca^{2+}$-regulation observed after prolonged I, are reversed within 1 hour of R.

## INTRODUCTION

The injury associated with the restoration of blood flow and tissue oxygenation following acute periods of ischemia (I), known as the reperfusion (R) syndrome, has been extensively documented in skeletal muscle (Sabido *et al.*, 1994; Rubin *et al.*, 1996). Similarly, in the heart, ischemia-reperfusion (IR) injury or myocardial stunning is a widely studied phenomenon (Hansen, 1995). It is clear that the extent of injury that occurs depends on the length of the ischemic period, however the contribution of events associated with the time-dependent effects of I or with R per se, resulting in injury, remains in question.

The central events resulting in cellular injury from periods of IR appear to occur primarily during R (McCord, 1985). In fact, skeletal muscle injury is normally greatest in areas with the highest degree of blood flow during R (Sabido *et al.*, 1994). It is thought a major mechanism leading to IR injury in both skeletal and cardiac muscle, is an increased production of $O_2$ free radicals (Peterson *et al.*, 1985; Rubin *et al.*, 1996). Since the potential sources of $O_2$ free radical production during R are greater as compared with I alone, this may explain the unexpected deleterious effects associated with R following acute I.

With the loss of ATP and total adenine nucleotides during I (DeBoer *et al.*, 1980; Jennings *et al.*, 1981; Neely and Grotyohann, 1984; Idström *et al.*, 1990; Soussi *et al.*, 1993; Rubin *et al.*, 1992; Carvalho *et al.*, 1997), the primary source of $O_2$ free radical production during the ischemic period, is the enzyme xanthine oxidase. During R, potential sources of $O_2$ free radical production include, but are not limited to, the muscle cell (xanthine oxidase, mitochondria, nitric oxide synthase [NOS] pathway), the capillary endothelial cell (xanthine oxidase, NOS pathway) and the neutrophil (NADPH oxidase, myeloperoxidase) (Sabido *et al.*, 1994; Rubin *et al.*, 1996; Lepore *et al.*, 1999). Consequently, there is a greater variety of

molecular oxygen species generated during R than during I alone and therefore, more potential target sites of attack.

One potential site of attack by $O_2$ free radicals that are produced during IR is the SR $Ca^{2+}$-ATPase (Kukreja *et al.*, 1988). Reductions in SR $Ca^{2+}$ uptake with myocardial IR are well documented (Mubagwa, 1995). Both short and more prolonged periods of myocardial I result in a decreased SR $Ca^{2+}$ uptake, whereas R following only prolonged periods of I (> 30 min) leads to further reductions in SR $Ca^{2+}$ uptake (Mubagwa, 1995). R following short periods of I (< 30min) normalizes SR $Ca^{2+}$ uptake to control levels despite sustained contractile dysfunction (Mubagwa, 1995; Kaplan *et al.*, 1997). For this reason, it is thought that impaired SR function is not related to IR induced contractile dysfunction or myocardial stunning (Kaplan *et al.*, 1997).

Surprisingly little research has been completed addressing the role of IR on SR function, in skeletal muscle. We have shown that I alone for up to 3 hours, results in a time-dependent increase in maximal SR $Ca^{2+}$-ATPase activity measured in homogenates prepared from rat skeletal muscle (Green *et al.*, 1996). Moreover, we have shown that R of skeletal muscle following I leads to a reduction in SR $Ca^{2+}$-ATPase activity, the magnitude of which dependent on the length of the ischemic period (1 to 3 hours) (Green *et al.*, 1997). It has also been shown that pretreatment with oxygen free radical scavengers, SOD and CAT, maintained higher $Ca^{2+}$ uptake by the SR of skeletal muscle following 3 hours of I and 19 hours of R in rat hindlimb (Lee *et al.*, 1987). Thus, free radical formation is likely a mechanism for impaired SR function with IR in skeletal muscle. In a recent study, a reduction in maximal SR $Ca^{2+}$-ATPase activity following 4 hours I in rat skeletal muscle was observed (Chapter II). The reduction in $Ca^{2+}$-ATPase, which was associated with a

reduction in FITC binding and increased aggregation of $Ca^{2+}$-ATPase monomers, was likely due to oxidation of one or more cysteine residues within the nucleotide binding domain (Chapter II).

In the present study, the added effects of R for 1 hour following 4 hours I, on SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase function in rat skeletal muscle were assessed. Based on previous work, it was expected that SR function would be impaired even further with R due to more potential sources of $O_2$ free radical production generated during R. It was also expected that treatment with oxygen free radical scavengers (SOD + CAT), just prior to R, would improve SR function compared with IR alone. It was hypothesized that any effects of IR and treatment with SOD and CAT on SR $Ca^{2+}$-ATPase activity would be associated with similar effects on FITC binding. The efficacy of SOD + CAT treatment for improving force recovery during R was also tested in a separate group of animals by monitoring peak twitch force output of the gastrocnemius-plantaris-soleus complex during I and R.

## Methods

### Animal description and care

Adult female Sprague-Dawley rats weighing 290 ± 3.5 g (mean ± SE) were housed in an environmentally controlled room (temperature 22-24°C, 40-60% relative humidity) with reversed light/dark cycles. Animals were fed ad libitum on laboratory chow and water until the time of the experiment. All experiments were initiated at approximately the same time each day to avoid large diurnal variations in muscle glycogen (Conlee *et al.*, 1976).

Experimental protocols were approved by the Animal Care Committee of the University of Waterloo.

**Experimental Groups**

To investigate the effects of complete I, followed by R, on SR function, animals were randomly assigned to CC (n = 10) and two E (n = 20) groups. E groups were divided based on the type of treatment they received. In one group (ET), a single dose of SOD at 60 000 units/kg plus CAT at 500 000 units/kg, dissolved in 2 ml sterile saline, was administered intravenously through a tail vein, 10 – 20 min prior to R. The other E group was just given 2 ml sterile saline prior to R. The CC group did not receive any treatment. For each E and ET animal, the experimental condition, 4 hours of total I and 1 hour of R, was randomly assigned to one hindlimb (I or IT) while the contralateral limb served as a control limb (C or CT). Due to tissue requirements for the isolation procedure used to obtain SR vesicles, experiments were conducted on one CC, 2 E and 2 ET animals each day.

**Experimental Protocol**

Before the induction of I, the rats were weighed and anesthetized. Anesthesia was initially accomplished using an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body wt) and then was maintained using supplementary intraperitoneal injections, as required. Throughout the ischemic periods, body temperature was maintained between 37 and 39 °C, by having the rats lay in a prone position on a warm heating pad. I was induced by placing a tourniquet around the upper hindlimb and proximal to the knee joint. To ensure total occlusion of blood flow to the hindlimb, a 350-mmHg pressure was employed (Walker *et al.*, 1989; Fish *et al.*, 1993). Total I was confirmed based on almost total depletion of muscle phosphocreatine and ATP following 4 hours of I (Chapter II). R consisted of simply

deflating and removing the tourniquet. After removing the tourniquet, the area directly under the tourniquet was also massaged for approximately 2 min to help support blood flow (Lee *et al.*, 1987).

At the end of the 1 hour R period, a small piece of white G muscle was rapidly sampled from each of the E and ET limbs, and frozen in liquid nitrogen for later analysis of muscle metabolites. The remainder of the G muscle (both red and white portions) along with the entire TA muscle were excised and placed in ice cold buffer, to be used for SR isolation by differential centrifugation. The G and TA muscles from each limb of the CC animal were sampled in the same manner, immediately following anesthetization.

**Sample preparation for SR assessment in vitro.**

All muscles were prepared according to Heilmann *et al.* (1977). Mixed G and TA muscles were diluted approximately 1:5 (w/v) in homogenizing buffer containing (in mM) 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.5), 250 sucrose, 0.2% sodium azide and 0.2 phenylmethylsulfonyl fluoride (no DTT) and mechanically homogenized with a polytron homogenizer (PT 3100) at 16500 rpm, for 2x30sec bursts. Aliquots of muscle homogenate were quick frozen in liquid nitrogen and stored at –70 to -80°C for later analysis of SR function. To obtain an enriched SR membrane fraction, a combination of two SR isolation protocols were used (Eletr and Inesi, 1972; Heilmann *et al.*, 1977). The homogenate was centrifuged at 5500 g for 10 min to remove cellular debris, and the supernatant was filtered through 4 layers of gauze to remove as much fat as possible. The supernatant was then transferred to new tubes and centrifuged at 12500 g for 18 min. These pellets were discarded and the spin was repeated. Again, the supernatant was transferred to new tubes and centrifuged at 50000 g for 52 min. These pellets were resuspended in 10 ml

homogenizing buffer plus 600 mM KCl, and allowed to incubate at 4°C for 30 min. This suspension was then centrifuged at 15000 g for 10 min to pellet nearly all the mitochondria. The supernatant was centrifuged at 50000 g for 52 min. The final pellet, enriched in SR membranes (no sucrose cushion), was resuspended in homogenizing buffer at a protein concentration of 2-6 mg/ml. SR isolation was carried out by differential centrifugation using a Beckmann Ultracentrifuge with a 70.1 Ti fixed-angle rotor.

**Analytic Procedures**

**Muscle metabolite analysis.** To determine the effects of I on muscle metabolic behaviour, metabolites of the high-energy phosphate system [PCr, Cr, Pi, glycogen and lactate] were assessed. Metabolites were measured in freeze-dried tissue after PCA extraction by fluorometric procedures as previously reported (Green *et al.*, 1987). All concentrations were expressed as micromoles per gram of dry weight.

**SR $Ca^{2+}$ uptake measurements.** Oxalate supported $Ca^{2+}$ uptake was measured in muscle homogenates and purified SR using the $Ca^{2+}$ flourescent dye Indo-1 according to methods of O'Brien (O'Brien, 1990; O'Brien *et al.*, 1991) with minor modifications. Fluorescence measurements were made on a spectrofluorometer (RatioMaster™ system, Photon Technology International) equipped with dual emission monochromators. The measurement of $[Ca^{2+}]_f$ using the Indo-1 procedure is based on the difference in the maximal emission wavelengths between the $Ca^{2+}$-bound form of Indo-1 and the $Ca^{2+}$-free form. The excitation wavelength was 355 nm and the emission maxima were 485 and 405 nm for $Ca^{2+}$-free (G) and $Ca^{2+}$-bound (F) Indo-1, respectively. Photon counts per second were recorded simultaneously for both emission wavelengths. The $Ca^{2+}$-independent (background) fluorescence was measured in the reaction medium (without Indo-1) at each emission

wavelength before starting the experiment. Background fluorescence was automatically corrected prior to starting each assay using the Felix software (Photon Technology International).

The reaction buffer for muscle homogenates (SR in parentheses) contained 200 (100) mM KCl, 20 mM HEPES, 10 mM $NaN_3$, 5 μM TPEN, 5 mM oxalate, 15 (10) mM $MgCl_2$ and 10 mM PEP, pH 7.0. Prior to collecting emission spectra, 18 U/ml each of LDH and PK and 1.5 μM Indo-1 were added to a cuvette containing 2 ml of reaction buffer. Also, 3 μl of $CaCl_2$ (10 mM) was added each trial to achieve an initial $[Ca^{2+}]_f$, prior to starting the reaction, of approximately 2.5 μM with isolated SR and 2.0 μM with homogenates. Immediately after data collection was initiated, 40 μl of homogenate (20 μl SR) was added to the cuvette. Shortly after the addition of homogenate or SR, 5 mM ATP was added to initiate $Ca^{2+}$-uptake.

As $Ca^{2+}$ decreases because of active SR $Ca^{2+}$ uptake, F decreases, G increases, and the ratio of F to G decreases. The ratio (R) is used to calculate $[Ca^{2+}]_f$. With the use of Felix software, the ionized $Ca^{2+}$ concentration was calculated by the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_f = K_d * (G_{max}/G_{min}) (R - R_{min}) / (R_{max} - R) \qquad (1)$$

where $K_d$ is the equilibrium constant for the interaction between $Ca^{2+}$ and Indo-1, $R_{min}$ is the minimum value of R at addition of 250 μM EGTA, $G_{max}$ is the maximum value of G at addition of 250 μM EGTA, $G_{min}$ is the minimum value of G at addition of 1 mM $CaCl_2$ and $R_{max}$ is the maximum value of R at addition of 1 mM $CaCl_2$. The $K_d$ value for the $Ca^{2+}$-dye complex is 250 and 135 nM for muscle homogenates and purified SR vesicles, respectively

(Grynkiewicz *et al.*, 1985). For all $Ca^{2+}$ uptake trials, $R_{min}$ and $R_{max}$ were not determined until $Ca^{2+}$ uptake had plateaued, which occurs at approximately 100 nM $[Ca^{2+}]_f$.

Before the rate of $Ca^{2+}$ uptake was calculated, the generated curve from **equation 1**, $[Ca^{2+}]_f$ versus time, was smoothed over 21 points using the Savitsky-Golay algorithm. The rate of $Ca^{2+}$ uptake was then analyzed at 4 separate free calcium concentrations (0.5, 1.0, 1.5 and 2.0 μM) for isolated SR and only the lowest 3 free calcium concentrations for muscle homogenates. First, linear regression was done on a range of values 100 nM above and below the desired $[Ca^{2+}]_f$. The rate of $Ca^{2+}$ uptake was then determined by differentiating the linear-fit curve and expressed in μmoles·g $protein^{-1}$· $min^{-1}$.

**SR $Ca^{2+}$ release measurements.** $Ca^{2+}$ release was measured on both muscle homogenates and SR vesicles according to the methods of Ruell (Ruell *et al.*, 1995), with minor modifications. $Ca^{2+}$ release assays were conducted as for $Ca^{2+}$ uptake, except that when the $[Ca^{2+}]_f$ declined to a plateau, 3 μl $AgNO_3$ was added to give a final concentration of 141 μM. The reaction was then allowed to proceed for approximately 3 min. With the addition of $AgNO_3$, $Ca^{2+}$ release consistently proceeded in 2 distinct phases. There was an initial rapid rate of release (Phase 1) followed by a slower, more prolonged rate of release (phase 2) (see Study 1). As for $Ca^{2+}$ uptake, the generated curve from **equation 1** was smoothed over 21 points and differentiated. The maximal rate of $Ca^{2+}$ release was calculated by taking the maximum positive derivative for each phase and expressed in μmoles·g $protein^{-1}$· $min^{-1}$.

**SR $Ca^{2+}$-ATPase activity measurements.** Spectrophotometric (Schimadzu UV 160U) measurement of SR $Ca^{2+}$-ATPase activity was performed on homogenates using procedures developed by Simonides and van Hardeveld (1990) and SR samples according to

Leberer *et al.* (1987) with minor modifications. Total ($Mg^{2+}$-activated)-ATPase activity was measured in the presence of the $Ca^{2+}$ ionophore A-23187, across a range of $CaCl_2$ concentrations. Basal activity was measured in the presence of 40 μM CPA, which completely inhibits SR $Ca^{2+}$-ATPase activity (Seidler *et al.*, 1989). The difference between total and basal activities represents the $Ca^{2+}$-activated ATPase activity. Maximal activity and the $Ca^{2+}$ dependency of $Ca^{2+}$-ATPase activity were assessed by adding 1-11 μl of 100 mM $CaCl_2$ in 0.5 μl additions. $Ca^{2+}$-ATPase activity increases with $[Ca^{2+}]_f$ until a plateau occurs, once maximal activity is reached. The $[Ca^{2+}]_f$ corresponding to each $CaCl_2$ addition was assessed separately, on a different SR aliquot, using dual-wavelength spectrofluorometry and the $Ca^{2+}$ fluorescent dye, Indo-1. $Ca^{2+}$-ATPase activity was then plotted against pCa and the Hill coefficient along with the $Ca_{50}$. These properties were determined through non-linear regression with computer software (GraphPad Software Inc.) using the following sigmoidal dose-response equation

$$Y = Y_{bot} + (Y_{top} - Y_{bot})/(1+10^{(LogCa_{50} - X)} \bullet n_H)$$

where $Y_{bot}$ is the value at the bottom of the plateau, $Y_{top}$ is the value at the top of the plateau, $LogCa_{50}$ is the logarithm of $Ca_{50}$, the concentration that gives a response halfway between $Y_{bot}$ and $Y_{top}$ and $n_H$ is the Hill coefficient. For calculation of these properties, only a portion of the curve, which corresponded with between 20 and 80% of maximal activity was used. For SR $Ca^{2+}$ uptake, release and ATPase assays, protein was determined by the method of Lowry as modified by Schacterle and Pollock (Schacterle and Pollock, 1973). On a given day, an equal number of samples from each condition were analyzed in duplicate.

**Fluorescence measurements.** Fluorescence measurements were made on an SLM-4800S spectrofluorometer (SLM Instruments, Urbana, IL). FITC (Sigma) and NCD-4

(Molecular Probe) were stored at a concentration of 5 mM in ethanol at –20 °C. FITC emission spectra (490 to 550 nm) were collected by exciting samples at 490 nm. FITC labeling was done by washing the SR samples once in wash buffer with no DTT, then resuspending the samples in FITC labeling buffer (wash buffer plus 2.5 μM FITC, pH 8.8), and vortexing gently in darkness for 20 min at 25 °C. The SR samples were then washed again in ordinary wash buffer to remove unbound label. NCD-4 emission spectra were collected by exciting samples at 340 nm and scanning the emission intensity from 400 to 430 nM at 1 nm increments. NCD-4 labeling was done by washing the SR samples once in wash buffer with no DTT, then resuspending in NCD-4 labeling buffer (wash buffer plus 150 μM NCD-4, pH 6.2) and incubating in darkness for 3 hours at 25 °C. As before, the sample was washed to remove unbound label.

**Muscle Force Measurements.** In a separate group of E and ET animals (n = 5), the effects of IR on electrically evoked muscle twitch force was assessed for the gastrocnemius-plantaris-soleus complex from each hindlimb. Surgical preparation and the animal stimulation apparatus has been described in detail elsewhere (Chin *et al.*, 1995; Carvalho *et al.*, 1997). For all animals, $P_t$ was measured at rest, 15, 30, 45, 60, 120, 180 and 240 min of I and 5, 10, 15, 30, 45 and 60 min of R. Twitch force was obtained via direct muscle stimulation using stainless steel electrodes, with a single 0.2 ms pulse, at 70 volts. Muscle stimulation was performed using a Grass S48 Stimulator while force data was collected on line and analyzed using the Watscope Data Acquisition Unit and Software (Northern Digital Inc. © 1985-1987). An independent shunt calibration was performed for each force transducer daily. A shunt calibration was employed to avoid problems associated with mechanical calibration. Prior to induction of I, optimal length for maximal $P_t$ ($L_o$) and

maximum voltage for optimum muscle twitch force were established. In determining $L_o$, muscle twitches were separated by 30 s to avoid a fatigue or potentiation effect. After $L_o$ and supramaximal voltage were established, the muscle was allowed to equilibrate for 5 min before inducing I. Twitch force was expressed relative to the initial resting value and calculated as a percentage of initial force.

**Statistical Analysis.** For all measurements, a one-way ANOVA was used to test for differences between means. Where significant differences were found, Tukey's post hoc tests were used to compare specific means. For all comparisons, statistical significance was accepted at $p < 0.05$. All data are expressed as means ± SE.

## RESULTS

**Muscle metabolites.** Muscle metabolites for each group are shown in Table 3.1. With the exception of muscle glycogen, there were no differences between CC, C and CT for any of the metabolites measured. Although muscle glycogen was approximately 10% lower ($p<0.05$) in both C and CT compared with CC, this difference was considerably less than the 86% reduction ($p<0.05$) in glycogen measured in I and IT, compared with CC. As expected, muscle ATP and PCr were nearly fully depleted in I and IT. Muscle Cr was approximately 1.5 fold-higher ($p<0.05$) in both I and IT compared with CC, C and CT. Similarly, Pi was approximately 2.8-fold higher ($p<0.05$) and lactate was approximately 5.7-fold higher ($p < 0.05$) in I and IT, compared with CC, C and CT.

**Table 3.1.** *Effects of IR on muscle metabolite concentrations.*

| | CC | C | I | CT | IT |
|---|---|---|---|---|---|
| ATP | 28.2 ± 0.6 | 28.2 ± 0.3 | 2.1 ± 0.6 * | 27.6 ± 0.5 | 2.4 ± 1.1 * |
| PCr | 64.3 ± 8.2 | 57.9 ± 9.3 | 5.7 ± 1.3 * | 64.7 ± 3.2 | 5.5 ± 2.7 * |
| Cr | 103 ± 8.5 | 107 ± 9.5 | 158 ± 8.4 * | 102 ± 4.5 | 157 ± 8.4 * |
| Pi | 63.5 ± 8.6 | 62.6 ± 9.9 | 175 ± 5.9 * | 50.1 ± 1.9 | 176 ± 8.1 * |
| Lactate | 33.6 ± 7.8 | 39.4 ± 9.3 | 189 ± 15.7 * | 30.6 ± 3.1 | 206 ± 17.2 * |
| Glycogen | 132 ± 6.2 | 119 ± 5.4 ⁺ | 19.1 ± 3.1 * | 116 ± 4.5 ⁺ | 17.5 ± 2.7 * |

*Values are means ± SE in μmol/g dry wt with the exception of Glycogen, which is in μmol glucosyl units/g dry wt (N=10 per group). CC, control control, C, control, I, Ischemic, CT, control treatment, IT, ischemic treatment, PCr, phosphocreatine, Cr, creatine, Pi, inorganic phosphate. *Significantly different (p<0.05) from CC, C and CT. ⁺Significantly different (p<0.05) from CC.*

**$Ca^{2+}$-ATPase Activity.** Surprisingly, there were no differences (p>0.05) in maximal SR $Ca^{2+}$-ATPase activity measured in both muscle homogenates and isolated SR vesicles between groups (Fig. 3.1). Moreover, kinetic analysis of the $Ca^{2+}$-ATPase activity-pCa curves showed that both the Hill coefficient and the $Ca_{50}$ were not different (P>0.05) between groups in either muscle homogenates or isolated SR vesicles (Table 3.2).

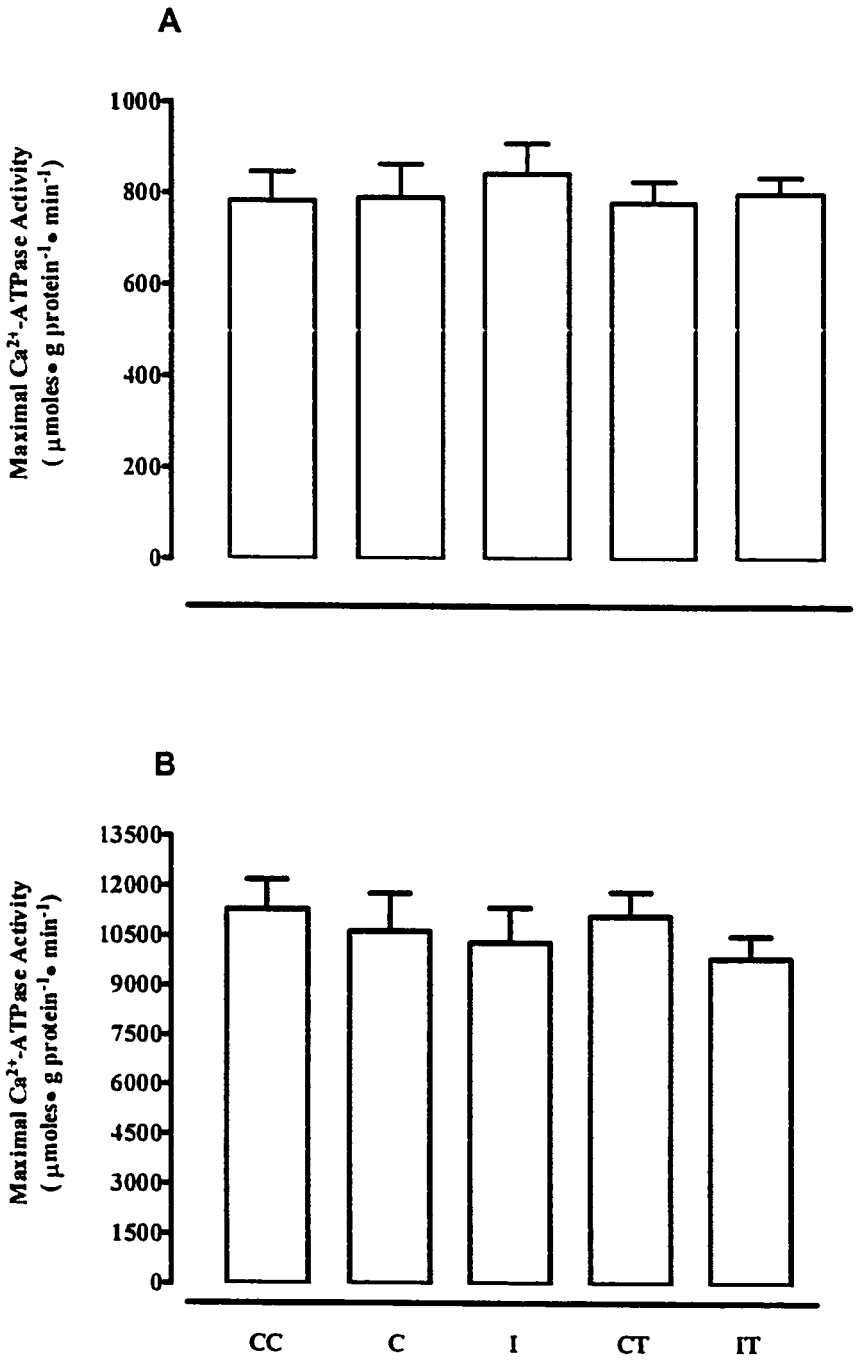



**Figure 3.1.** *Maximal SR $Ca^{2+}$-ATPase activity of CC, C, I, CT and IT, measured in A) muscle homogenates and B) isolated SR vesicles. CC, control control, C, control, I, ischemic, CT, control treatment, IT, ischemic treatment. Values are means ± SE; n = 10. Maximal SR $Ca^{2+}$-ATPase activity occurred at a $[Ca^{2+}]_f$ of approximately 6 – 10 µM in all groups.*

**Table 3.2.** *Kinetic properties of the $Ca^{2+}$-ATPase activity –pCa curves in homogenates and isolated SR vesicles.*

| | CC | C | I | CT | IT |
|---|---|---|---|---|---|
| **Homogenates** | | | | | |
| Hill coefficent | 1.4 ± 0.3 | 1.3 ± 0.3 | 1.3 ± 0.1 | 1.5 ± 0.5 | 1.4 ± 0.1 |
| $Ca_{50}$ | 6.3 ± 0.1 | 6.2 ± 0.1 | 6.2 ± 0.1 | 6.4 ± 0.1 | 6.3 ± 0.1 |
| **SR vesicles** | | | | | |
| Hill coefficient | 1.1 ± 0.1 | 1.2 ± 0.2 | 1.3 ± 0.2 | 1.1 ± 0.1 | 1.2 ± 0.2 |
| $Ca_{50}$ | 6.0 ± 0.1 | 6.1 ± 0.1 | 6.0 ± 0.1 | 6.1 ± 0.1 | 6.1 ± 0.1 |

*Values are means ± SE; n=10 per group. CC, control control, C, control, I, Ischemic, CT, control treatment, IT, ischemic treatment. Hill coefficient was obtained from Hill plots of the section of the $Ca^{2+}$-activity versus calcium concentration, corresponding from 20 to 80% of normal activity. $Ca_{50}$ is defined as the calcium concentration of half-normal activity.*

**Fluorescence measurements.** FITC and NCD-4 binding were used as a method to assess the structure of the nucleotide binding domain and the $Ca^{2+}$ binding sites of the $Ca^{2+}$-ATPase following prolonged IR, respectively. There were no differences between any of the groups in either FITC (Fig. 3.2A) or NCD-4 (Fig. 3.2B) binding.

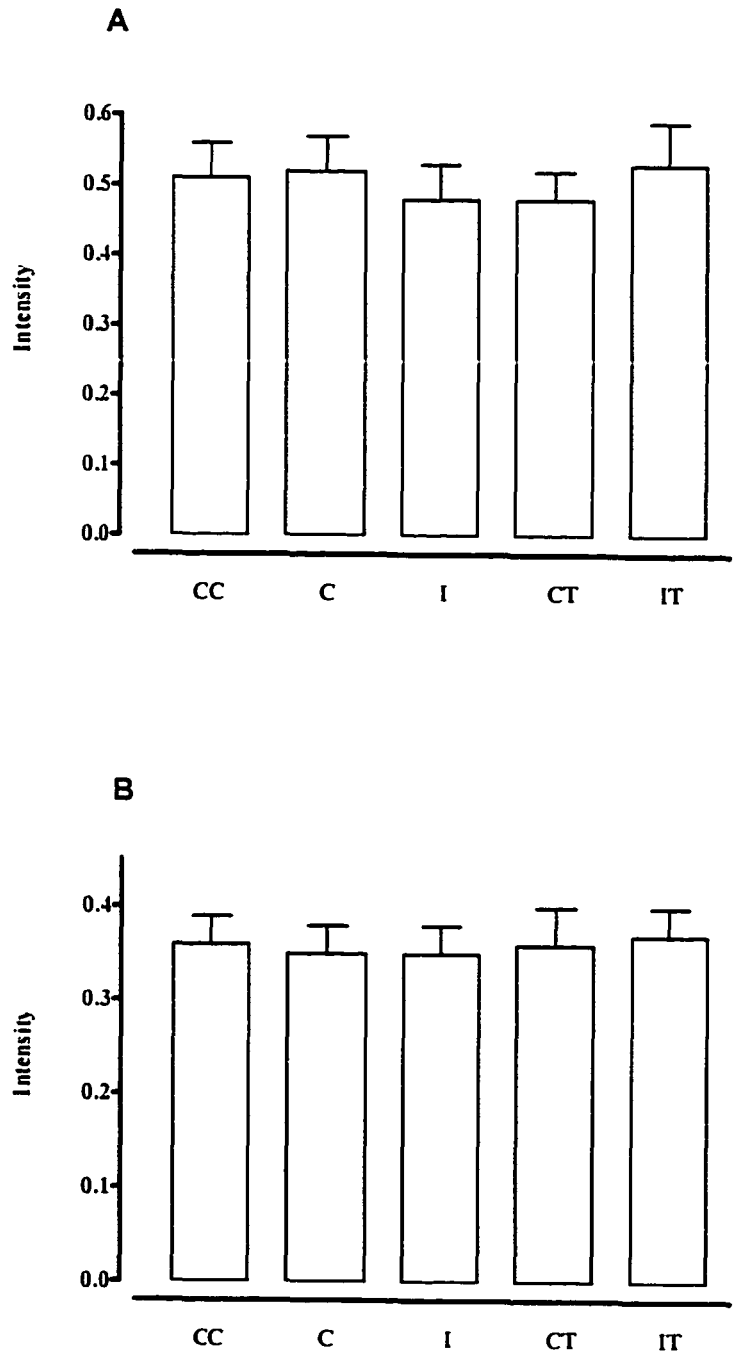



**Figure 3.2.** *Maximum FITC fluorescence (A) and maximum NCD-4 fluorescence (B) in CC, C, I, CT and IT SR vesicles prepared from mixed gastrocnemius and tibialis anterior muscles. Values are means ± SE; n = 10 per group. CC, control control, C, control, I, ischemic, CT, control treatment, IT, ischemic treatment. There were no significant differences between groups.*

**$Ca^{2+}$ uptake.** $Ca^{2+}$ uptake was assessed at 3 and 4 different $[Ca^{2+}]_f$ in muscle homogenates and purified SR vesicles, respectively. In muscle homogenates, $Ca^{2+}$ uptake was 20-25% lower ($p<0.05$) in C, compared with CC, measured at both 0.5 and 1.0 μM $[Ca^{2+}]_f$ (Fig. 3.3). $Ca^{2+}$ uptake was also 15-17% lower ($p<0.05$) in C compared with CT at 0.5 and 1.5 μM $[Ca^{2+}]_f$, with a strong trend ($p=0.08$) for lower $Ca^{2+}$ uptake in C compared with CT at 1.0 μM $[Ca^{2+}]_f$. There was also a strong trend ($p=0.08$) for lower $Ca^{2+}$ uptake in IT compared with CC, measured at both 0.5 and 1.0 μM $[Ca^{2+}]_f$. There were no differences between CC, CT and I at any $[Ca^{2+}]_f$. In purified SR vesicles, $Ca^{2+}$ uptake was lower ($p<0.05$) in both I and IT across all $[Ca^{2+}]_f$, compared with CC, by 30-38% (Fig. 3.4). $Ca^{2+}$ uptake was also approximately 25% lower ($p<0.05$) in IT compared with CT across all $[Ca^{2+}]_f$. There were no differences between CC, C and CT in $Ca^{2+}$ uptake, however at 1.0 μM $[Ca^{2+}]_f$, there was a trend ($p=0.09$) for lower $Ca^{2+}$ uptake in C compared with CC.

**$Ca^{2+}$ release.** Due to the biphasic response of $Ca^{2+}$ release (see Fig. 2.1), we obtained two measures of maximal $Ca^{2+}$ release corresponding to each phase, for each trial. In muscle homogenates, there were no differences in $AgNO_3$-induced $Ca^{2+}$ release between any of the groups for either Phase 1 or Phase 2 (Fig. 3.5). However, in purified SR vesicles, maximal $Ca^{2+}$ release was 31% higher ($p<0.05$) in I, compared with CC for Phase 1, with no differences between any of the other groups (Fig. 3.6). No differences existed between any of the groups for Phase 2 in purified SR vesicles.

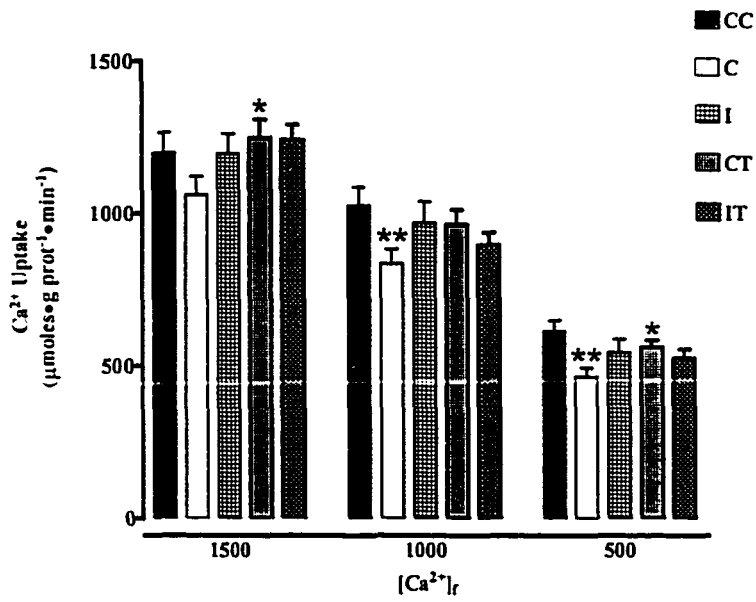



**Figure 3.3.** *Calcium uptake of CC, C, I, CT and IT homogenates prepared from mixed gastrocnemius and tibialis anterior muscles. CC, control control, C, control, I, ischemic, CT, control treatment, IT, ischemic treatment. $Ca^{2+}$ uptake was measured using Indo-1 and at 3 sub-maximal $[Ca^{2+}]_f$ (1500, 1000 and 500 nM). Values are means ± SE; n = 10. * Significantly different (p<0.05) than C. ** Significantly different (p<0.05) than CC.*

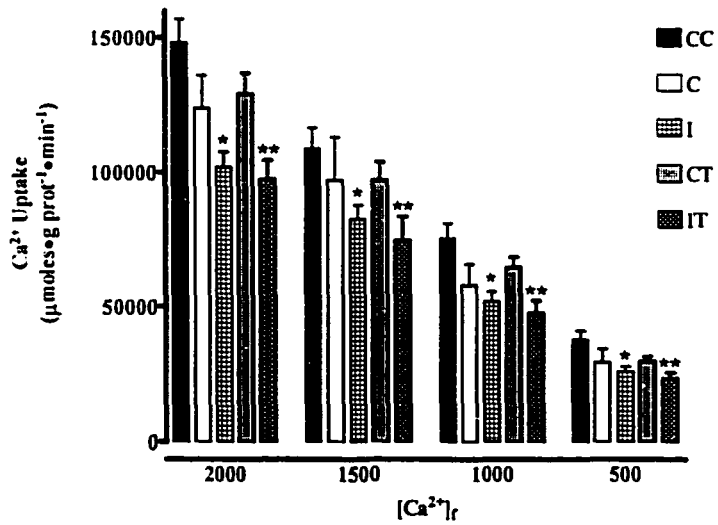



**Figure 3.4** *Calcium uptake of CC, C, I, CT and IT SR vesicles prepared from mixed gastrocnemius and tibialis anterior muscles. CC, control control, C, control, I, ischemic, CT, control treatment, IT, ischemic treatment. $Ca^{2+}$ uptake was measured using Indo-1 and at 4 sub-maximal $[Ca^{2+}]_f$ (2000, 1500, 1000 and 500 nM). Values are means ± SE; n = 10. * Significantly different (p<0.05) than CC. ** Significantly different (p<0.05) than CC and CT.*

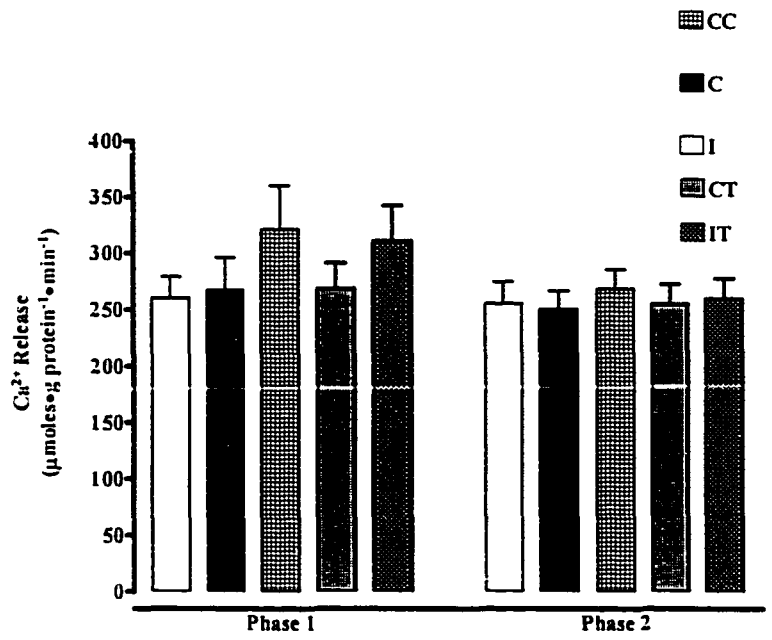



**Figure 3.5.** *Calcium release of CC, C, I, CT and IT homogenates prepared from mixed gastrocnemius and tibialis anterior muscles. CC, control control, C, control, I, ischemic, CT, control treatement, IT, ischemic treatment. $Ca^{2+}$ release was induced using $AgNO_3$ and was measured using Indo-1. The peak rates of $Ca^{2+}$ release corresponding to both Phase 1 and Phase 2 are shown. Values are means ± SE; n = 10.*

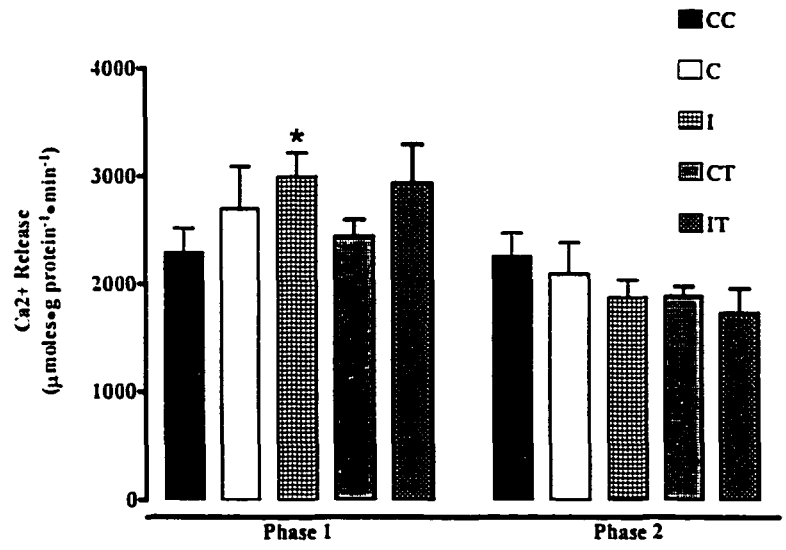



**Figure 3.6.** *Calcium release of CC, C, I, CT and IT SR vesicles prepared from mixed gastrocnemius and tibialis anterior muscles. CC, control control, C, control, I, ischemic, CT, control treatment, IT, ischemic treatment. $Ca^{2+}$ release was induced using $AgNO_3$ and was measured using Indo-1. The peak rates of $Ca^{2+}$ release corresponding to both Phase 1 and Phase 2 are shown. Values are means ± SE; n = 10. * Significantly higher ($p<0.05$) than CC.*

**Force Measurements.** The changes in $P_t$ with IR are presented in Fig. 3.7. Just after 1 hour of I, the muscle was incapable of producing force and remained in this state for the duration of the ischemic protocol. R for 1 hour did not result in force recovery. Treatment with SOD + CAT, just prior to R, had no effect on force recovery in the gastrocnemius-plantaris-soleus complex following I. Importantly, $P_t$ force output was maintained in C and CT and even increased slightly above resting values throughout the IR protocol. There were no differences in $P_t$ between C and CT at any time point.

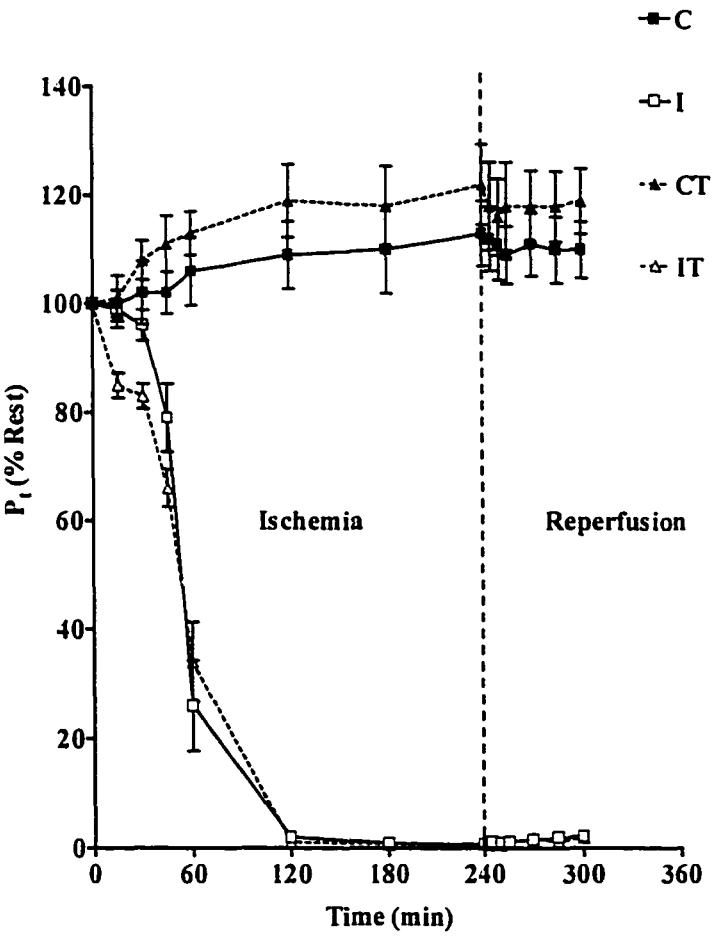



**Figure 3.7.** *Changes in peak twitch tension ($P_t$) during I-R of C, I, CT and IT gastrocnemius-plantaris-soleus complex. Values are means ± SE; n = 5. C, control, I, ischemic, CT, control treatment, IT, ischemic treatment.*

## DISCUSSION

In this study, the effects of R following prolonged I on skeletal muscle SR function in vitro, were assessed. Previously, it was shown that prolonged I alone for 4 hours resulted in reduced SR $Ca^{2+}$ release, $Ca^{2+}$ uptake and maximal $Ca^{2+}$-ATPase activity, presumably due to increased $O_2$ free radical production (Chapter II). Since $O_2$ free radical production is believed to be even greater with R following acute I, it was hypothesized that SR function would be impaired even further with 4 hours I and 1 hour R compared with 4 hours I alone. Moreover, it was postulated that treatment with free radical scavengers would attenuate the damage to the $Ca^{2+}$-ATPase during IR, reducing the depression in activity.

Both maximal $Ca^{2+}$-ATPase activity and the kinetic properties, Hill coefficient and $Ca_{50}$, were not different between IR and control groups in both muscle homogenates and isolated SR vesicles. Moreover, homogenate $AgNO_3$-induced $Ca^{2+}$ release was not different between groups for both Phase 1 and Phase 2, while in isolated SR vesicles, Phase 1 $Ca^{2+}$ release was actually higher in I compared with CC. $Ca^{2+}$ uptake was approximately 30% lower in I compared with CC, however only in isolated SR vesicles and not in homogenates. Somewhat unexpectedly, IR in one hindlimb lead to reductions in homogenate $Ca^{2+}$ uptake in the contralateral control limb compared with CC, with a similar trend observed in SR vesicles.

Comparing these results in isolated SR fractions with the effects of I alone, it appears that SR function, including $Ca^{2+}$-ATPase activity, $Ca^{2+}$ release and $Ca^{2+}$ uptake, recovers substantially with just 1 hour of R in skeletal muscle. In the heart, SR function recovers following short periods of IR, but is depressed even further with R following prolonged periods of I (Mubagwa, 1995). The absolute duration of the ischemic period is not

comparable between skeletal muscle and cardiac muscle in terms of the time-course and severity of injury that occurs. However, 4 hours of I is comparable to prolonged I in the heart in terms of the metabolic response (Jennings *et al.*, 1981; Harris *et al.*, 1986; Idström *et al.*, 1990; Koretsune and Marban, 1990) and the extent of necrosis that takes place (Reimer *et al.*, 1981; Harris *et al.*, 1986). Therefore, it is unclear why R following prolonged I in skeletal muscle allows recovery of SR function, as occurred in this study, but not in the heart. One conspicuous difference between the two preparations is the level of contractile activity. In the current study, skeletal muscles were generally inactive. However, the heart remains active.

Numerous reports have shown that exposure of SR vesicles to $O_2$ free radicals in vitro, leads to protein oxidation and a reduction in $Ca^{2+}$-ATPase activity (Morris and Sulakhe, 1997; Viner *et al.*, 1999; Xu *et al.*, 1997). It is also well established that both $O_2$ free radicals and altered SR function play an important role in the etiology of IR injury in both skeletal (Rubin *et al*, 1996) and cardiac (Mubagwa, 1995) muscle. Thus, clearly reductions in SR $Ca^{2+}$-ATPase activity should have been expected in this study, as are observed with I alone (Chapter II). It would appear that mechanism(s) which cause a restoration in SR $Ca^{2+}$-ATPase activity in SR fractions must have also been present during R, to allow recovery of $Ca^{2+}$-ATPase activity to occur.

It is possible that the reduction in $Ca^{2+}$-ATPase activity that occurs in vitro with I alone is reversible upon R or that recovery of $Ca^{2+}$-ATPase activity with R reflects recruitment of a latent pool of enzyme that is not activated even under optimal conditions and is unaffected by I. If the later is true, the latent pool of $Ca^{2+}$ pumps would normally be

oriented such that FITC would be unable to bind to $Lys_{515}$, otherwise FITC binding should have still been reduced from I (see Chapter II).

Activation of the SR $Ca^{2+}$-ATPase above normal has been reported to occur during recovery from low intensity exercise in rats (Ferrington *et al.*, 1996). In that study, the increase in $Ca^{2+}$-ATPase activity was accompanied by increases in active $Ca^{2+}$-ATPase pump units as determined by the concentration of phosphorylated enzyme intermediate formed from ATP. It is unknown whether FITC binding would have increased in relation to increases in active $Ca^{2+}$ pump units since FITC binding was not determined. The authors postulated that increases in the number of active $Ca^{2+}$-ATPase pump units was in response to increased cytosolic calcium levels in an attempt to restore $Ca^{2+}$ homeostasis following exercise.

Our results indicate that the effects of I on SR $Ca^{2+}$-ATPase function in SR fractions are reversible with R. However, this would have to involve a mechanism independent of muscle metabolism. When measured <u>in vitro</u>, maximal SR $Ca^{2+}$-ATPase activity is reduced following 4h I in rat skeletal muscle, despite being measured under supposedly optimal conditions (see Chapter II). Considering the severe metabolic alterations that occur with 4 hours I in rat skeletal muscle (see Chapter II), it is possible that sustained incubation of the SR $Ca^{2+}$-ATPase <u>in vivo</u>, in the presence of one or more metabolic byproducts, plays a role in reducing SR $Ca^{2+}$-ATPase activity <u>in vitro</u>. However, since R restored $Ca^{2+}$-ATPase activity but resulted in minimal metabolic recovery in this study, it is unlikely that the metabolic response to I alone or to IR has an effect on SR $Ca^{2+}$-ATPase activity measured <u>in vitro</u>. It should be noted however, that <u>in vivo</u>, under the extreme metabolic perturbations

that occur with IR, large depressions in SR $Ca^{2+}$-ATPase activity would result (Zhu and Nosek, 1991; Korge and Campbell, 1995).

It has been shown that heat shock and expression of heat-shock protein 72 (HSP 72) prior to IR in the heart, is associated with an increase in maximal SR $Ca^{2+}$-ATPase activity (O'Brien *et al.*, 1997). Unfortunately, few studies have examined the expression and localization of HSP 72 with IR in skeletal muscle. However, it is well known that oxidative stress, leading to protein destabilization and exposure of hydrophobic residues, induces the synthesis of heat shock proteins (for review see Freeman *et al.*, 1999). One study has shown that I alone and IR in the heart increases the expression of HSP 72, with much higher levels found with IR compared with I alone (Yu *et al.*, 1999). Collectively, these results imply that heat-shock protein elevations with R may normalize SR $Ca^{2+}$-ATPase activity following I, measured in vitro.

It's puzzling that $Ca^{2+}$-ATPase activity was unaffected by IR in this study, measured in both muscle homogenates and isolated SR vesicles, whereas in a previous study, we found that homogenate $Ca^{2+}$-ATPase activity was reduced with 1, 2 and 3 hours I followed by 2 hours R (Green *et al.*, 1997). The differences can't be explained by differences in muscle fibre type since reductions in $Ca^{2+}$-ATPase activity with IR in the earlier study were noted in both the soleus and EDL muscles. However, considering that increases in homogenate $Ca^{2+}$-ATPase activity were noted in those same muscles following 1, 2 and 3 hours I alone (Green *et al.*, 1996) much of the reduction in $Ca^{2+}$-ATPase activity with R, simply reflected the reversal of the I-induced increase in activity. Nonetheless, $Ca^{2+}$-ATPase activity was reduced below normal with R in the earlier study but not in the present study. An obvious explanation may simply be that the lengths of the R period were different.

Perhaps, with a longer duration of R in this study (i.e. > 1 hour), these results would be different.

Despite normalization of maximal $Ca^{2+}$-ATPase activity in both muscle homogenates and isolated SR vesicles with R following I, $Ca^{2+}$ uptake remained depressed, at least in isolated SR vesicles. Thus, the efficiency of $Ca^{2+}$ transport is reduced with prolonged IR in skeletal muscle. This is not surprising since we have shown that this also occurs with partial I in rat skeletal muscle (Tupling *et al.*, 1997). A number of possibilities exist to explain a reduction in efficiency of SR $Ca^{2+}$ transport including 1) a change in $Ca^{2+}$ binding properties of the $Ca^{2+}$-ATPase, 2) CRC activation and increased passive $Ca^{2+}$ release and 3) an increase in membrane permeability to $Ca^{2+}$ and excessive $Ca^{2+}$ leakage.

These results show that the $Ca^{2+}$ binding properties of the $Ca^{2+}$-ATPase were not affected by IR since there were no changes in either NCD-4 binding or the Hill coefficient for $Ca^{2+}$-ATPase activity. Due to limited sample, $Ca^{2+}$ uptake in the presence of ryanodine in order to assess the activation state of the CRC at rest, was not measured in this study. However, we have shown that where a reduction in efficiency of $Ca^{2+}$ transport occurs with partial I in rat skeletal muscle, ryanodine has no effect on $Ca^{2+}$ uptake (Tupling *et al.*, 1997). The reduction in $Ca^{2+}$ uptake that occurs following 4 hours I alone is also unaffected by ryanodine and thus not due to activation of the CRC (see Chapter II). Therefore, it is believed that activation of the $Ca^{2+}$ release channel and increased passive $Ca^{2+}$ release does not contribute to the reduced efficiency of $Ca^{2+}$ transport observed in this study.

The remaining possibility is that SR membrane permeability to $Ca^{2+}$ was increased as a result of oxidative damage. An impressive number of studies have reported various levels of oxidative membrane damage following IR in both skeletal muscle (Fantini and

Yoshioka, 1993; Seyama, 1993; Grisotto *et al.*, 2000) and the heart (Mubagwa, 1995; Morris and Sulakhe, 1997). Moreover, the effects of $O_2$ free radicals on membrane permeability and ion transport in vitro are well documented (Kourie, 1998). Consequently, SR membrane damage involving lipid peroxidation may explain the reduction in $Ca^{2+}$ uptake following IR in this study, since there were no changes in $Ca^{2+}$-ATPase activity.

Membrane damage may also explain the finding that the early rapid rate of $AgNO_3$-induced $Ca^{2+}$ release was slightly higher with IR in isolated SR vesicles. Alternatively, it is well known that $O_2$ free radicals can trigger rapid $Ca^{2+}$ release from the SR by modifying critical SH groups in the CRC and increasing the open probability of the channel (for review see, Abramson and Salama, 1989; Favero, 1999). However, it was found that $Ca^{2+}$ release was reduced with 4 hours I alone (see Chapter II) and based on pilot data, it is assumed that the concentration of $AgNO_3$ employed in this study (141 μM) maximally activates all CRCs in each sample. It appears that the increase in $Ca^{2+}$ release observed in isolated SR vesicles with IR was not a result of modifications to the open probability of the CRCs. Instead, flux through the CRCs could be increased with IR due to changes in some aspect of the complex control of $Ca^{2+}$ release (i.e. phosphorylation- dephosphorylation of SR proteins), or increased flux through a leaky SR membrane could explain the increase in $Ca^{2+}$ release with IR.

Another possibility exists to explain the increase in $Ca^{2+}$ release that occurred only in SR vesicles and not in muscle homogenates. The different results between SR vesicles and homogenates may be due to differential selection of a population of SR vesicles during the isolation procedure, between IR and control muscle, as has been reported in the heart following I and R (Rapundalo *et al.*, 1986; Wu and Feher, 1997). In other words, it's

possible that vesicles more enriched in CRCs were obtained after SR isolation from IR muscle compared with control muscle. Furthermore, damaged SR membranes from IR muscle may have preferentially been selected during isolation, which would also explain the different $Ca^{2+}$ uptake results observed between homogenates and isolated SR vesicles.

In this study, the efficacy of SOD + CAT treatment on improving SR function with IR was also tested. Numerous studies have shown that SOD + CAT treatment attenuates IR injury in both skeletal muscle (Lee *et al.*, 1987; Yokota *et al.*, 1989; Seyama, 1993) and cardiac muscle (Netticadan *et al.*, 1999; Temsah *et al.*, 1999). With respect to the SR, Lee *et al.* (1987) found that pretreatment with SOD + CAT maintained higher rates of SR $Ca^{2+}$ uptake following 3 hours I and 19 hours R in rat skeletal muscle. Furthermore, hearts treated with SOD + CAT prior to 30 min I and 60 min R had preserved SR function in relation to protected SR protein phosphorylation (Netticadan *et al.*, 1999) and protected SR gene expression (Temsah *et al.*, 1999). However, in another study, it was shown that SOD + CAT treatment had no effect on myocardial SR $Ca^{2+}$ uptake following 15 min I and 15 min R since SR $Ca^{2+}$ uptake recovered in hearts reperfused both with and without SOD + CAT (Rehr *et al.*, 1991).

Given the general absence of an effect of IR on skeletal muscle SR function in this study, it was not surprising to find that SOD + CAT treatment was without effect in I, on all SR parameters measured. However, interestingly, homogenate $Ca^{2+}$ uptake was higher in contralateral control muscle with SOD + CAT than with saline treatment alone. Also, in SR vesicles, $Ca^{2+}$ uptake was higher in C compared with I after SOD + CAT treatment (i.e. CT > IT) with no difference between C and I, in saline treated animals. Collectively, these results suggest that SOD + CAT treatment protected against IR-induced reductions in $Ca^{2+}$

uptake that normally occur in skeletal muscle from the contralateral limb, with prolonged IR.

In discussing possible mechanisms for the effects of IR in one hindlimb on skeletal muscle SR function in the contralateral limb, it is important to note that no change in SR function occurs in the contralateral limb following 4 hours I alone (see Chapter II). Therefore, though it has been reported that myocardial I increases sympathetic nervous system activity and release of norepinephrine 1000-fold (Schömig, 1990) and that β-blockers protect against IR-induced changes in cardiac SR function (Rana *et al.*, 2000), the reduction in $Ca^{2+}$ uptake in the contralateral control limb observed in this study is not likely mediated via any IR-induced hormonal response.

Given that SOD + CAT treatment prevented the IR-induced reduction in $Ca^{2+}$ uptake in the contralateral limb, the mechanism must have involved an $O_2$ free radical mediated process. Experimental evidence suggests that hindlimb IR may initiate a systemic inflammatory response leading to injury of remote organs such as the liver (Brock *et al.*, 1999). It's possible that this also occurs in skeletal muscle tissue. Depending on the degree of inflammation, infiltrating white blood cells may adhere to the endothelium and cause narrowing of the microvasculature which, in combination with SNS mediated vasocontriction, may result in hypoxic tissue damage. Moreover, white blood cells (and neutrophils in particular) can produce $O_2$ free radicals (Sabido *et al.*, 1994). The effectiveness of SOD + CAT treatment in protecting the contralateral limb from IR-induced changes in SR function corresponds with this mechanism.

The microcirculatory changes in IR parallel those seen in inflammation (Sabido *et al.*, 1994). It has been shown in skeletal muscle that less than 15% of the muscle is being

perfused with blood 24 hours post-IR (Lepore *et al.*, 1999). This phenomenon is referred to as no-reflow (Ames III *et al.*, 1968). The extent of no-reflow that occurred in this study was not determined but such a mechanism could partially explain the lack of metabolic recovery with R observed in this study and therefore lack of force recovery. Significant no-reflow may also have prevented adequate delivery of SOD + CAT to the ischemic tissue which may help explain the lack of improvement in muscle metabolism, SR $Ca^{2+}$ uptake and contractile function in ischemic tissue with SOD + CAT treatment. Finally, it should be noted that interpreting the effects of IR on SR function is problematic where significant no-reflow occurs since reperfused tissue can not be sampled independently from non-perfused tissue with ease.

In summary, 4 hours I and 1 hour R in rat skeletal muscle leads to severe metabolic and contractile function perturbations but only minimal changes in SR function. There were no changes in SR $Ca^{2+}$-ATPase activity with IR and both the increase in SR $Ca^{2+}$ release and reduction in SR $Ca^{2+}$ uptake with IR were only observed in isolated SR vesicles and not in muscle homogenates. However, SR $Ca^{2+}$ uptake in the contralateral limb is reduced following IR and this is prevented with SOD + CAT treatment. It is concluded that SOD + CAT treatment protects against remote tissue injury following prolonged skeletal muscle IR in rats.

# CHAPTER IV

## GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

## DISCUSSION

The primary objective for this thesis was to investigate the changes that occur in the regulation of $[Ca^{2+}]_f$ in skeletal muscle by the SR during I and IR. Specifically, the goal was to determine the effects of I and IR on SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and $Ca^{2+}$-ATPase activity, measured in vitro in both crude muscle homogenates and isolated SR vesicles. Secondly, the fluorescent probes, FITC and NCD-4, were used to determine if structural alterations to the $Ca^{2+}$-ATPase enzyme occur with I and IR, in association with the expected reduction in $Ca^{2+}$-ATPase activity with I and IR. To determine the involvement of $O_2$ free radicals in the IR-induced changes in muscle mechanical function and SR function in vitro, SOD + CAT treatment was administered in one group of rats just prior to R.

It was hypothesized that SR $Ca^{2+}$ uptake, $Ca^{2+}$ release and maximal $Ca^{2+}$-ATPase activity, measured in both muscle homogenates and SR vesicles, would be reduced with I and IR. Reductions in FITC binding were also expected with both I and IR. Furthermore, it was hypothesized that SOD + CAT treatment during IR, would protect against reductions in all of the above, and improve recovery of $P_t$ during R. The major findings are summarized in Table 4.1.

**Table 4.1.** *Summary table of the major results in muscle homogenates (SR in parentheses) from both the I and IR studies, compared against CC.*

| | ISCHEMIA | | ISCHEMIA-REPERFUSION | | | |
|---|---|---|---|---|---|---|
| | C | I | C | I | CT | IT |
| $Ca^{2+}$ uptake | → (→) | ↓* (↓*) | ↓$^{\tau}$ (→) | → (↓) | → (→) | → (↓$^{\tau}$) |
| $Ca^{2+}$ release | | | | | | |
| - Phase 1 | → (→) | → (↓*) | → (→) | → (↑) | → (→) | → (→) |
| - Phase 2 | → (→) | ↓* (↓*) | → (→) | → (→) | → (→) | → (→) |
| $Ca^{2+}$-ATPase | | | | | | |
| - max activity | → (→) | → (↓*) | → (→) | → (→) | → (→) | → (→) |
| - Hill coefficient | → (→) | → (→) | → (→) | → (→) | → (→) | → (→) |
| - $Ca_{50}$ | → (→) | → (→) | → (→) | → (→) | → (→) | → (→) |
| FITC binding | (→) | (↓*) | → (→) | → (→) | → (→) | → (→) |
| NCD-4 binding | (→) | (→) | → (→) | → (→) | → (→) | → (→) |

*C, control, I, ischemic, CT, control treatment, IT, ischemic treatment. $Ca_{50}$ is the $Ca^{2+}$ concentration (pCa) needed to elicit 50% of the maximal $Ca^{2+}$-ATPase activity. →, not different from CC, ↓, lower ($p<0.05$) compared with CC, ↑, higher ($p<0.05$) compared with CC. * Significantly different ($p<0.05$) from C. $^{\tau}$ Significantly different ($p<0.05$) from CT.*

Using a 4 hour I model, it was found $Ca^{2+}$-uptake and $Ca^{2+}$ release, measured in both muscle homogenates and isolated SR vesicles, and maximal $Ca^{2+}$-ATPase activity, measured in isolated SR vesicels, were all reduced with prolonged I and recovered with 1 hour of R. The reductions in maximal $Ca^{2+}$-ATPase activity with I, were accompanied by similar reductions in FITC binding in SR vesicles, with no changes in NCD-4 binding. There were no changes in either the Hill coefficient or the $Ca_{50}$, with I and IR. With IR, SOD + CAT

treatment had no effect on either mechanical function or SR function in ischemic muscle, but improved SR $Ca^{2+}$ uptake in contralateral control muscle.

These appear to be the first studies to assess the effects of I and IR on SR $Ca^{2+}$ release in vitro, in skeletal muscle. The technique used to measure $Ca^{2+}$ release in these studies, employed $AgNO_3$ as the releasing stimulus. $AgNO_3$ is a SH oxidizing agent which triggers the opening of the CRC by oxidizing critical SH groups on the CRC (Abramson and Salama, 1989). Given that SH oxidation may have occurred during both I and IR, it is possible that the CRC SH groups which are sensitive to $AgNO_3$-induced oxidation, may have been modified in the I and IR muscle, compared with control. If this were true, the effectiveness of $AgNO_3$ as a releasing agent could potentially be different between ischemic and control muscle. Nevertheless, the results suggest that the CRC is modified with I, leading to a reduction in $Ca^{2+}$ release which subsequently recovers with R. The biphasic $Ca^{2+}$ release response consistently observed in all assay trials, using $AgNO_3$ as the releasing agent, is a novel finding. The mechanism for this biphasic response is unclear, however the clear distinction between the two phases, and the differential effects of CPA on the two phases, indicates the value of reporting data on each phase.

One of the most interesting findings from the I study, was that complete inhibition of SR $Ca^{2+}$-ATPase activity with CPA, increased the early rapid rate of release (Phase 1) in all groups and decreased the slower more prolonged rate of release (Phase 2) in all groups. Moreover, the percent reduction in Phase 2 release with CPA was lower in I compared with CC and C. These results suggest that reductions in $Ca^{2+}$ uptake with I may be mechanistically linked to reductions in $Ca^{2+}$ release that occur with I. This notion is supported by the finding that both $Ca^{2+}$ uptake and $Ca^{2+}$ release were recovered with IR, at

least in homogenates. The coupling mechanism between SR $Ca^{2+}$ uptake and $Ca^{2+}$ release remains unclear, however one possible role for $Ca^{2+}$ uptake during net $Ca^{2+}$ release, is to provide a pathway for counter-ion exchange.

It has been emphasized that measurement of SR function in vitro is performed under supposedly optimal conditions, and that alterations in SR function in vitro with I and IR must reflect structural alterations to the SR that occurred during I and IR in vivo, that persist throughout sample preparation and measurement of SR function in vitro. Furthermore, given the severe metabolic changes that occurred with I and IR in this study, clearly, reductions in SR $Ca^{2+}$-ATPase activity, $Ca^{2+}$ uptake and $Ca^{2+}$ release would be expected in vivo (Inesi and Hill, 1983; Zhu and Nosek, 1991; Lamb *et al.*, 1992; Favero *et al.*, 1995; Xu *et al.*, 1996), with I and IR.

These studies also appear to be the first to attempt to identify the precise structural modifications that occur to the SR $Ca^{2+}$-ATPase with I and IR, that may account for changes in SR $Ca^{2+}$-ATPase activity, measured in vitro. Reductions in FITC binding in I, compared with both CC and C, accompanied the I-induced reduction in maximal $Ca^{2+}$-ATPase activity, measured in isolated SR vesicles, compared with both CC and C. There was also an increased aggregation of $Ca^{2+}$-ATPase protein in I, compared with CC. Collectively, these results suggest that the reduction in maximal SR $Ca^{2+}$-ATPase activity with I is related to structural modification of the nucleotide binding domain, probably as a result of free-radical induced oxidation of one or more cysteines. There were no changes in NCD-4 binding with I, suggesting that the $Ca^{2+}$-binding sites of the $Ca^{2+}$-ATPase were unaffected by I.

Despite complete recovery in maximal $Ca^{2+}$-ATPase activity with just 1 hour of R, following 4 hours I, it remained important to determine if structural alterations to the

nucleotide binding domain or the $Ca^{2+}$ binding domain occurred with IR. Dissociation of changes in $Ca^{2+}$-ATPase activity and FITC binding with IR, would indicate that alterations in structure of the nucleotide binding domain do not induce reductions in maximal $Ca^{2+}$-ATPase activity, with I. However, since recovery of maximal $Ca^{2+}$-ATPase activity with R, was matched by FITC binding capacity, this provides further support for the argument that inactivation of the SR $Ca^{2+}$-ATPase with I, measured in vitro, is due to structural modification of the nucleotide binding domain. NCD-4 binding was still not different between groups, following IR.

One aim of this thesis, was to determine if the effects of I and IR on SR function measured in vitro, were the same in both muscle homogenate and isolated SR vesicle preparations. It was found, that I-induced alterations in SR $Ca^{2+}$ uptake and $Ca^{2+}$ release were similar in both homogenates and isolated SR vesicles. One exception was maximal $Ca^{2+}$-ATPase activity measured in homogenates, which was not changed with I. In fact, there was a trend, albeit insignificant, for an increase in homogenate maximal $Ca^{2+}$-ATPase activity with I. This finding is inconsistent with the results that were found for isolated SR vesicles in this study, but supports an earlier finding from our laboratory, namely that there was a time-dependent increase in homogenate maximal $Ca^{2+}$-ATPase activity with skeletal muscle I in rats (Green *et al.*, 1996). In that study, $Ca^{2+}$-ATPase activity was only assessed in homogenates and not in isolated SR vesicles.

The lack of significance for an increase in homogenate $Ca^{2+}$-ATPase activity in the present study, may simply be related to the length of the ischemic period, which was longer (4 hours) in this study, compared with the earlier study (up to 3 hours). However, the mechanism underlying the differences between homogenates and SR vesicles in the present

study, remains uncertain. One possibility is that I is without effect on homogenates and that the reductions in $Ca^{2+}$-ATPase activity in SR vesicles simply reflects a selective loss of undamaged SR during the isolation procedure. Similar results of the effects of I on $Ca^{2+}$ uptake in both preparations, does not support this possibility. On the other hand, the failure to find reductions in $Ca^{2+}$-ATPase activity in homogenates could be due to activation of other cellular ATPases, masking the effects of the inhibiting agents, or recruitment of additional enzyme by co-valent modification, leading to phosphorylation or production of an activator substance during I, which binds to the $Ca^{2+}$-ATPase and activates it (Green *et al.*, 1996). It is possible that these changes become lost during the vesicle isolation process.

The method used to measure SR $Ca^{2+}$-ATPase activity in homogenates is based on the assay developed by Simonides and van Hardeveld (1990). These authors were able to demonstrate the specificity of their procedure for measuring SR $Ca^{2+}$-ATPase activity, by systematically inhibiting the other major ATPases that are present in the homogenate. Furthermore, in our modifications, basal ATPase activity is determined by selectively inhibiting the SR $Ca^{2+}$-ATPase with CPA, and SR $Ca^{2+}$-ATPase activity is subsequently determined by subtracting basal ATPase activity from total ATPase activity. However, the assay was developed using only muscle homogenates prepared from control muscle tissue. It is unclear whether the other ATPases, namely acto-myosin ATPase, $Na^{+}$-$K^{+}$-ATPase and mitochondrial ATPase, are inhibited to the same extent, using this assay, on homogenates prepared from ischemic tissue.

On the other hand, only the effects of I on maximal $Ca^{2+}$-ATPase activity were different, whereas the effects of I on the Hill coefficient and $Ca_{50}$ were identical, between homogenate and enriched SR vesicle preparations. One limitation of this study, was that

only sub-maximal and not maximal $Ca^{2+}$ uptake was measured in both muscle homogenates and isolated SR vesicles. It is possible that the discrepent findings for maximal $Ca^{2+}$-ATPase activity, between homogenate and isolated SR preparations, may have also reflected differences in maximal $Ca^{2+}$ uptake between the two preparations. If that was the case, then a differential yield of SR vesicles during isolation, from ischemic muscle compared with control, could explain the different results obtained on homogenates versus isolated SR vesicles.

Surprisingly maximal $Ca^{2+}$-ATPase activity with IR was not different between groups, measured in both homogenate and isolated SR vesicle preparations. As discussed earlier, it is possible that either the reductions in maximal $Ca^{2+}$-ATPase activity that occurred with I are reversed with R, or that there is recuitment of additional pumps which are able to compensate for those $Ca^{2+}$ pumps affected by I. However, which strategy dominates, can not be determined from the results obtained in this thesis.

Unlike $Ca^{2+}$-ATPase activity, different results for both $Ca^{2+}$ uptake and $Ca^{2+}$ release were found between muscle homogenates and isolated SR vesicles, with IR. Compared with CC, $Ca^{2+}$ uptake was lower with IR, only measured in isolated SR vesicles, and Phase 1 $Ca^{2+}$ release was higher following IR, also only measured in isolated SR vesicles and not in muscle homogenates. This suggests that there could also be a differential yield of SR vesicles isolated from IR skeletal muscle compared with control, as has been found for the heart with myocardial IR (Rapundalo et al., 1986).

Another major goal of this thesis, was to determine the role of $O_2$ free radicals in IR-induced changes in SR function and mechanical function, since $O_2$ free radicals are thought to be involved in the etiology of IR injury (Rubin *et al.*, 1986). It was hypothesized that

treatment with the free radical scavengers, SOD + CAT, just prior to R, would protect SR function and improve mechanical function during R. It was found that SOD + CAT treatment did not improve mechanical function and had no effect on SR function from ischemic muscle, but did protect against SR damage that normally occurs in contralateral control muscle with IR.

Based on previous findings (Carvalho *et al.*, 1997), contractile dysfunction following prolonged periods of skeletal muscle I would be expected and easily explained by the metabolic changes that occur. The muscle's inability to produce force would persist as long as muscle ATP levels remained low during R. Moreover, despite recovery of SR function measured in vitro with R, the I-induced metabolic changes, which are unaltered by R, would directly impair SR function in vivo, including both $Ca^{2+}$ uptake and $Ca^{2+}$ release (Inesi and Hill, 1983; Zhu and Nosek, 1991; Lamb *et al.*, 1992; Favero *et al.*, 1995; Xu *et al.*, 1996). On that basis, together with reduced ATP levels, impaired SR $Ca^{2+}$ cycling likely contributes to the persistent fatigue observed in this study with with R, following 4 hours I (Allen *et al.*, 1995).

It has been shown that capillary red blood cell velocity is essentially zero, 90 minutes following 4 hours normothermic I in rat EDL muscle (Potter *et al.*, 1993). There is a strong possibility that severe no-reflow also occurred in the present study, with 4 hours I and just 1 hour R. This no-reflow phenomenon likely explains the lack of recovery in both muscle metabolism and contractile function that was observed in this study. On the other hand, recovery of SR function in vitro did occur with R. It is possible that I-induced intrinsic alterations in SR function recover early during R as a result of reactive hyperemia, which has been shown to occur following 2 and 3 hours of I in rat skeletal muscle, immediately

upon release of the tourniquet (Skjeldal *et al.*, 1993; Potter *et al.*, 1994). Previously, we have reported that recovery of SR $Ca^{2+}$ uptake measured in vitro following I, occurs following only 7 minutes of R in rat skeletal muscle (Fan *et al.*, 1998).

Given that SR function from ischemic muscle recovered with R in animals that did not receive SOD + CAT treatment, any additional effect of SOD + CAT treatment on SR function in ischemic muscle, would not be expected. However, this does not exclude the possibility that SOD + CAT treatment may help prevent alterations in SR function that occur with IR in vivo. Perhaps some oxidation of the SR $Ca^{2+}$-ATPase, CRC or membrane, occurs in vivo, but does not persist throughout preparation of SR vesicles and measurement of SR funciton in vitro. There is evidence that this does occur if DTT is included in the homogenization buffer, (Tupling *et al.*, 1999). Furthermore, SOD + CAT treatment may be benficial for minimizing damage to cellular structures, other than the SR, and preventing other changes associated with IR injury in skeletal muscle (Rubin *et al.*, 1996).

In hindsight, it would have proved more informative to include a treatment group in the I study, since $O_2$ free radicals were likely involved in both the reduction in maximal $Ca^{2+}$-ATPase activity and FITC binding, and the increased aggregation of $Ca^{2+}$-ATPase protein. Since the source of $O_2$ free radicals with I alone, is likely xanthine oxidase, perhaps pre-treatment with allopurinol, an inhibitor of xanthine oxidase and effective oxidant scavenger (Rubin et al., 1996), or xanthine oxidase depletion with a tungsten diet, may have prevented the I-induced changes in SR structure and function. This would be worth testing in the future.

From a clinical perspective, other pharmacological agents, such as $Ca^{2+}$ antagonists, nitric oxide synthase inhibitors, iron chelators or other $O_2$ free radical scavengers other than

SOD + CAT, may have been more effective in improving mechanical function and preventing alterations in SR function with I and IR. However, the primary objective of this thesis, from a biochemical perspective, was to determine the effects of I and IR on skeletal muscle SR structure and function in vitro, and to assess the role of $O_2$ free radicals in any changes that occur. Nevertheless, further work is warranted to assess the roles of I- and IR-induced increases in $O_2$ free radicals, $[Ca^{2+}]_f$ and decreased adenine nucleotides on changes in SR structure and function with I and IR.

## CONCLUSIONS

The results obtained from the two major studies completed for this thesis, suggest that SR $Ca^{2+}$ uptake and $Ca^{2+}$ release measured in vitro in both muscle homogenates and SR vesicles, and maximal $Ca^{2+}$-ATPase activity, only in SR vesicles, are impaired with I alone, but recover with R. The reduction in maximal SR $Ca^{2+}$-ATPase activity in SR vesicles with I is related to structural modification of the nucleotide binding domain, probably as a result of free-radical induced oxidation of one or more cysteines. Unlike I alone, SR $Ca^{2+}$ uptake in the contralateral limb is impaired with IR, probably due to increased free radical production, since SOD + CAT treatment prevented the reduction in $Ca^{2+}$ uptake in the contralateral limb. It is clear that the effects of I and IR on SR function measured in vitro, depends on the IR schedule and the type of preparation, homogenates or isolated SR vesicles.

## FUTURE DIRECTIONS

This work raises many issues for future investigation. From a clinical perspective, it appears that impaired SR $Ca^{2+}$ cycling is associated with I- and IR-induced skeletal muscle injury. However, whether alterations in SR function is an adaptive response to I and IR, perhaps to reduce energy utilization, or is actually a mechanism to cause I and IR injury, by contributing to increases in $[Ca^{2+}]_f$, is uncertain. Clearly, measurements of cellular $[Ca^{2+}]_f$ during I and IR in vivo, would help to elucidate the role that SR alterations have in I and IR injury.

Comparing our results from earlier studies (Green *et al.*, 1996; Green *et al.*, 1997) and the results presented in this thesis, it appears that the length of both the I and R period, influences the alterations in SR function that occur with I and IR. Further work to elucidate the time-course dependent changes in SR function with I and IR, relative to the extent of injury (reversible vs. irreversible) that takes place, would also be important from a clinical perspective.

From a biochemical perspective, it remains unclear whether SR isolation results in a differential yield of SR vesicles, depending on whether vesicles are prepared from ischemic or control muscle. This is a significant issue, since FITC and NCD-4 binding must be measured on isolated SR vesicle preparations. Studying the I-induced structural alterations that occur to the $Ca^{2+}$-ATPase in vivo, would not be appropriate if SR isolation confounded interpretation of the results. By measuring SR function in the supernatant and pellet fractions following each spin with SR isolation, this would provide information on any differences in the yield of SR, between ischemic and control muscle (Rapundalo *et al.*, 1986).

The mechanism(s) that allows protection of SR $Ca^{2+}$-ATPase activity in homogenates but not in SR vesicles, with I, needs to be determined. Further work concerning the validity of the assay for measuring SR $Ca^{2+}$-ATPase activity in muscle homogenates, prepared from ischemic muscle, needs to be done. Furthermore, developmental work should be performed to determine if measurement of FITC binding and NCD-4 binding can be done on muscle homogenates or crude homogenates (i.e., one low-speed spin). This would allow assessment of structural changes to the $Ca^{2+}$-ATPase that occur with I and IR, without concern for potential loss of protective mechanisms with SR isolation.

One factor not investigated in this thesis, was the nature of the structural modification to the CRC that occurs with I and IR. In the future, any change in the number of functional CRCs with I and IR should also be determined using [$^3$H]ryanodine binding. Likewise, the effects of I and IR on the SR membrane were not determined in this study. In future studies, it would be important to determine the effects of I and IR on the SR membrane, since membrane damage appears to be involved in the alterations in SR $Ca^{2+}$ cycling that occur with I and IR.

The mechanisms responsible for the biphasic $Ca^{2+}$ release response observed in these studies with $AgNO_3$, also warrants further investigation. It is unclear if a similar response would occur with other releasing agents, such as caffeine, or if such a response is important physiologically. Moreover, the effects of CPA on $Ca^{2+}$ release should prove an exciting future research direction. It would appear that control of SR $Ca^{2+}$ uptake and $Ca^{2+}$ release are tightly coupled, such that inhibition of $Ca^{2+}$ uptake impairs $Ca^{2+}$ release. However, the

mechanisms involved and the nature of coupling between $Ca^{2+}$ uptake and $Ca^{2+}$ release, remains in question.

The role of increased $[Ca^{2+}]_f$ with I and IR, on alterations in SR structure and function, remains unclear. Increases in $[Ca^{2+}]_f$ are generally believed to be harmful to the cell, due to its activation of proteolytic enzymes (Jackson *et al.*, 1984; Klenerman *et al.*, 1995; Belcastro *et al.*, 1996), however, there is also speculation that an increase in the number of active $Ca^{2+}$ pumps occurs in response to increases in $[Ca^{2+}]_f$ (Ferrington *et al.*, 1996). This may be a potential mechanism to explain the recovery in SR $Ca^{2+}$-ATPase activity that occurs with R, in the face of increased $O_2$ free radicals that are known to reduce $Ca^{2+}$-ATPase activity. Similarly, the effects of changes in the aggregation state of the $Ca^{2+}$-ATPase and the role of HSPs in the normalization of $Ca^{2+}$-ATPase activity that occurs with R, should be an exciting area for future research.

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# APPENDIX I

## DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) is a general method for detecting protein denaturation or unfolding (Lepock *et al.*, 1990). With this method, temperature transitions are detected by measuring the heat absorbed during a thermotropic transition as the temperature is scanned upward at a defined rate. Reductions in the transition temperature for thermal denaturation ($T_m$) measured by DSC, is a sensitive method for detecting protein denaturation to the $Ca^{2+}$-ATPase (Lepock et al., 1990).) To determine if inactivation of the SR $Ca^{2+}$-ATPase with I and IR, is due to denaturation of the $Ca^{2+}$-ATPase, thermal denaturation of isolated SR vesicle preparations from all groups, was assessed by DSC. It was hypothesized that both I and IR would induce significant denaturation of the SR $Ca^{2+}$-ATPase, which would be reflected by a lower $T_m$ in SR vesicles prepared from I and IR muscle, compared with control.

## METHODS

**Differential Scanning Calorimetry**. A Microcal-2 differential scanning calorimeter interfaced to a DEC Pro 380 computer was used to obtain all scans. The protein content of the SR samples was 2-5 mg/ml, and a scan rate of 1 °C/min was used. For the DSC measurements, the SR was washed once in wash buffer with no DTT and then resuspended in a solution of 100 mM KCl, 5 mM DTT and 1 mM EGTA, pH 7.0. The noisier scans were smoothed by fast Fourier transform filtering. In all cases, the samples were rescanned and this scan, which showed no transitions, was subtracted from the first scan to correct for intrinsic base line curvature. The change in specific heat ($\Delta C_p$) upon denaturation was corrected as previously described (Lepock et al., 1990). All curves were then fitted for irreversible denaturation assuming two-state, irreversible

denaturation of the form $N \xrightarrow{K_i} D$ with the data points of the exothermic transition removed before fitting (Lepock et al., 1990). The temperature for thermal denaturation $T_m$ was obtained from the best fit curves.

## RESULTS

**Table A.1.** *Thermal denaturation temperatures (°C) for $Ca^{2+}$-ATPase from all muscles from both the ischemia and ischemia-reperfusion studies.*

| | CC | C | I | CT | IT |
|---|---|---|---|---|---|
| I | 46.1 | 49.8 | 50.0 | | |
| IR | 47.6 | 46.4 | 48.5 | 45.0 | 47.6 |

*All samples for each group (n=9) were combined and values represent one scan for each group. I, ischemia, IR, ischemia-reperfusion, CC, control control, C, control, I, ischemic, CT, control treatment, IT, ischemic treatment.*

## DISCUSSION

These were the first studies attempting to assess the extent of SR $Ca^{2+}$-ATPase protein denaturation in SR samples prepared from skeletal muscle, following I and IR in vivo, using DSC. The results obtained are somewhat confusing. Unexpectedly, with I, it appears as though SR $Ca^{2+}$-ATPase from both I and C, is more tolerant to thermal denaturation, compared with CC. With IR, the results suggest that SR $Ca^{2+}$-ATPase from the contralateral control muscle may be slightly less tolerant to thermal denaturation, compared

with both I and CC. These results differ from the changes in $Ca^{2+}$-ATPase activity that occurs, at least with I.

The $T_m$ values in CC, are somewhat low, relative to what has been reported previously for rabbit skeletal muscle when heated in 1 mM EGTA (49 °C) (Lepock *et al.*, 1990). The reason for these low values are uncertain, however, the results suggest that some protein denaturation to the SR $Ca^{2+}$-ATPase may have occurred during isolation of SR vesicles. Since DTT was not included in the homogenization buffer, or at any step during SR isolation, likely oxidation of the $Ca^{2+}$-ATPase occurred during SR isolation leading to protein denaturation. Nevertheless, it appears that both ischemic and control $Ca^{2+}$-ATPase with I, and ischemic ATPase, with and without SOD + CAT treatment, with IR, are protected against protein oxidation and denaturation that potentially occurs during isolation of SR vesicles. Likewise, protein denaturation of the $Ca^{2+}$-ATPase, that is expected to occur with both I and IR, is not observed using DSC.

With I, there was evidence for increased aggregation of the $Ca^{2+}$-ATPase (see Chapter II). Protein aggregation is a general outcome when proteins are denatured, either by oxidation (Senisterra et al., 1997), or heat (Kampinga, 1993; Lepock et al., 1995; Senisterra *et al.*, 2000). On that basis, denaturation of the $Ca^{2+}$-ATPase likely occurred with I. However, SR vesicles prepared from I muscle, were more tolerant to thermal denaturation, compared with CC. The $T_m$ measured in I, was actually slightly higher than previously reported values for SR $Ca^{2+}$-ATPase isolated from rabbit skeletal muscle ($\approx$ 49 °C) (Lepock et al., 1990).

Several possibilities exist to explain the protection afforded ischemic SR $Ca^{2+}$-ATPase to a subsequent heat stress. The most obvious explanation is that 4 hours I may

result in increased HSP synthesis. It is well known that oxidative stress, leading to protein destabilization and exposure of hydrophobic residues, induces the synthesis of heat shock proteins (for review see Freeman *et al.*, 1999). One study has shown that I alone and IR in the heart increases the expression of HSP 72, with much higher levels found with IR compared with I alone (Yu *et al.*, 1999). Unfortunately, few studies have examined the expression and localization of HSP 72 with I or IR in skeletal muscle. However, if HSP synthesis was elevated with 4 hours I in the present study, this may be the mechanism for the protection of SR $Ca^{2+}$-ATPase to thermal denaturation, that was observed for ischemic muscle.

This interpretation doesn't adequately explain the finding that the $T_m$ in C was also higher, compared with CC, and not different from I. It appears a higher $T_m$ with I, was specific to the E animal, idependent of limb, compared with the CC animal. This suggests that another variable, associated with the experimental procedure, but not associated with I per se, was responsible for the different $T_m$ response between experimental and control animals. During the ischemic protocol, E rats layed in a prone position on a warm heating pad to maintain body temperature (between the heating pad and the rat) at 37 – 39 °C. Unfortunately, intramuscular temperature was not monitored. It is possible that muscle temperatures were elevated in both limbs, which may have elicited a heat shock response.

On the other hand, with IR, $T_m$ was only higher in I and IT compared with CC. This does not lend support to the notion that elevated muscle temperatures, specific to the E animal, induced thermotolerance in both I and C, since muscle temperature should have been the same in both the I and IR studies. Perhaps localalized skeletal muscle I leads to a systemic response that allows thermotolerance in both ischemic and non-ischemic muscle,

whereas IR, induces thermotolerance in ischemic muscle but leads to a systemic response unfavourable for thermotolerance in non-ischemic muscle.

The fact that both maximal $Ca^{2+}$-ATPase activity and FITC binding were lower with I, but $T_m$ was not lower with I, is also difficult to explain. One possibility is that I-induced inactivation of the enzyme, due to structural modification of the nucleotide binding domain, does not necessarily reflect significant protein denaturation, which would result in thermal instability. On the other hand, partial inactivation of the $Ca^{2+}$-ATPase and reduced FITC binding may reflect a population of enzyme that is irreversibly denatured. Proteins that are irreversibly denatured show no transitions during DSC (Lepock et al., 1990).

Interpretation of the DSC results obtained in these studies is highly speculative. Nonetheless, further DSC studies are warranted, to determine the extent of SR $Ca^{2+}$-ATPase denatruation that occurs during I and IR, in vivo. Furthermore, the role of HSPs in skeletal muscle I and IR needs to be examined. It remains to be demonstrated that newly synthesized HSPs with I and IR are localized to the SR $Ca^{2+}$-ATPase and increase thermotolerance of the $Ca^{2+}$-ATPase in vitro.

# APPENDIX II

## SR VESICLE CHARACTERIZATION

## CONTAMINATION PROFILE AND TOTAL SERCA PROTEIN

**Mitochondrial Contamination**

In order to characterize the SR vesicles obtained with the SR isolation protocol employed in these studies (Eletr and Inesi, 1972; Heilmann *et al.*, 1977), pilot studies were performed to determine the %mitochondrial contamination in the SR fractions, by measuring citrate synthase, a marker enzyme for muscle mitochondria. Citrate synthase activity was determined in both homogenate and SR vesicle preparations using methods previously established (Green *et al.*, 1984). Percent mitochondrial contamination was calculated as the ratio of SR vesicle and homogenate activity expressed per gram tissue. Based on 17 separate isolations, only using control G and TA muscles, the % mitochondrial contamination was calculated to be 5.8 ± 0.8%. This level of mitochondrial contamination is similar to results published previously (Chin *et al.*, 1994) and is considered to be an acceptable level. Percent mitochondrial contamination was not compared between groups in the above studies (i.e. CC, C, I, CT and IT).

**Contamination by Other Proteins**

Non-reducing and reducing SDS-PAGE gels were analyzed for total SERCA protein and visually inspected for contamination by other proteins. Total SERCA protein levels were determined by scanning densitometry and values were expressed as a % of the CC value. Comparisons were made between groups for total SERCA protein for the I study only (Table A.2). A sample gel from each condition (non-reducing vs. reducing) is shown in Figure A.1.

**Table A.2.** *Total SR $Ca^{2+}$-ATPase protein in SR vesicles for CC, C and I determined from non-reducing and reducing SDS-PAGE gels by scanning densitometry.*

| | CC | C | I |
|---|---|---|---|
| Non-Reducing | 100 | 99.2 ± 3.0 | 93.7 ± 3.2 |
| Reducing | 100 | 113 ± 12 | 111 ± 8.8 |

*Relative SR $Ca^{2+}$-ATPase protein content in CC, C and I SR vesicels prepared from mixed gastrocnemius and tibialis anterior muscles. SR $Ca^{2+}$-ATPase protein content was determined by non-reducing and reducing (DTT) SDS-PAGE. Values are means ± SE. CC, control control, C, control, I, ischemic.*

Under non-reducing conditions, there was a strong trend (P=0.06) towards a lower total SERCA protein in I, compared with CC. This is consistent with the western blot data, where the monomer form of SERCA1 potein levels were lower in I, compared with CC, under non-reducing conditions. However, under reducing conditions, there were no differences between groups for both SDS-PAGE gels (SERCA) and Western blots (SERCA1). This finding suggests that total SERCA protein was not altered with ischemia but there was significant protein aggregation between adjacent SERCA molecules that occurred with ischemia.

The level of contamination by other proteins, in the SR vesicles, was also compared between groups (CC vs. C vs. I) by visual inspection of SDS-PAGE gels, run under both non-reducing (Fig. A.1a) and reducing (Fig. A.1b) conditions. As can be seen from the non-reducing gel, the amount of contamination from other proteins was minimal for all groups and there were no real differences between groups. Under reducing conditions, approximately 10 new protein bands were detected for all the groups, with no differences between groups. Comparing the number of new bands between these gels and the western

the western blots for SERCA1 that were run under reducing conditions, it appears that protein aggregation between the SR $Ca^{2+}$-ATPase and other proteins within the cell also occurred, but differences between groups were difficult to detect.

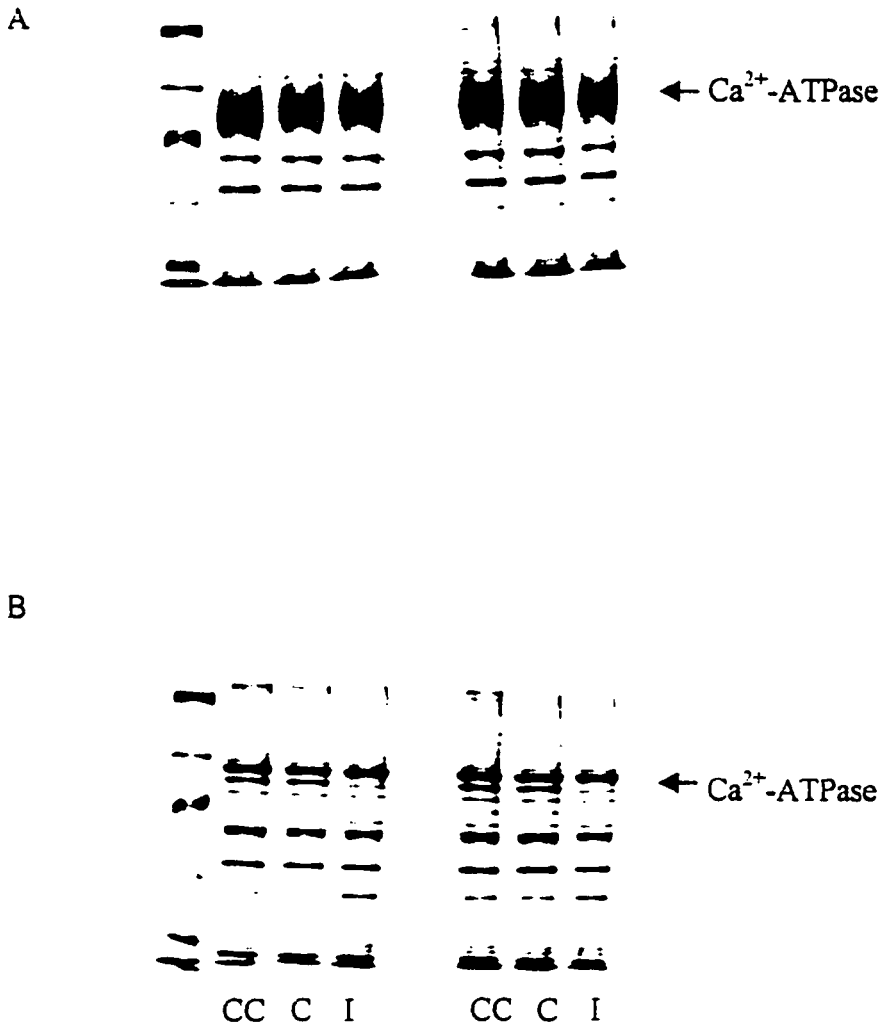



**Fig. A.1.** *SDS-PAGE gels run under non-reducing (A) and reducing (B) conditions. Total SERCA protein content was determined by scanning densitometry under both non-reducing and reducing conditions. Gels were also visually inspected for the level of contamination by other proteins which was compared between groups. There were no differences in total SERCA protein levels or the level of contamination between groups.*