EFFECTS OF ALTERED PHYSICAL ACTIVITY ON HUMAN SKELETAL MUSCLE SARCOPLASMIC RETICULUM CALCIUM REGULATION

by

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DECLARATION

This dissertation summarizes original, previously unpublished work conducted at the Victoria University of Technology, in the Department of Human Movement, Recreation and Performance, and at the Department of Life Science and Technology.

This dissertation is the result of work performed by the author. However, considerable collaboration was also involved in the studies involving exercising humans, due to the complexity of the studies and the invasive procedures undertaken. The muscle biopsies were conducted by qualified medical personnel. Mr. Steve Fraser assisted with the organisation of subjects for the experimental trials and in conducting the exercise tests. Dr. Michael M^{c}Kenna, Associate Professor Michael Carey and Dr. Steve Selig helped in conducting the exercise tests and analyses in Biochemistry Laboratory. Dr. Xiao Nan Wang also assisted with the muscle metabolite assays and fibre type analyses conducted in the Biochemistry Laboratory and in conducting the exercise tests.

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ABBREVIATIONS

Cardio-respiratory

$\dot{V}_E$	Pulmonary Ventilation
$\dot{V}_{O2_{peak}}$	Peak pulmonary oxygen consumption
$\dot{V}_{CO2}$	Pulmonary carbon dioxide output
HR	Heart rate
BP	Blood Pressure

Muscle Function

1/2 RT	Half relaxation time

Metabolism

ADP	Adenosine diphosphate
ATP	Adenosine 5' triphosphate

Time

h	Hour
min	Minute
ms	Millisecond
s	Second

Other

SLTx	Single lung transplant recipients
BLTx	bilateral lung transplant recipients
HLTx	Heart-lung transplant recipients
EDL	extensor digitorum longus
SOL	soleus
TPEN	N,N,N',N'-tetrakis(2-pyridyl-methyl)ethylenediamine
[ ]	Concentration

ABSTRACT

The sarcoplasmic reticulum (SR) regulates intracellular [Ca^{2+}] and therefore largely determines the rates of contraction and relaxation in skeletal muscle. However, little is known about SR function in human skeletal muscle. This thesis therefore investigated several factors that could potentially influence SR function in human skeletal muscle, including the effects of acute exercise, training status, chronic inactivity and the influence of muscle fibre type. A final study investigated the effects of an immunosuppressive drug on SR function in rat skeletal muscle.

Study 1. No studies have investigated the effects of fatigue on SR Ca^{2+} release and Ca^{2+} uptake in human muscle and this was investigated in eight healthy untrained men. Fatigue was induced by 50 maximal isokinetic knee extensor contractions. Two muscle biopsies were taken from vastus lateralis at rest and a single biopsy at fatigue to assess skeletal muscle SR function. The rates of SR Ca^{2+} release (Ag^{+}-induced) and Ca^{2+} uptake (oxalate supported) were analysed fluorimetrically using Indo-1 in crude muscle homogenates. SR Ca^{2+}ATPase activity in crude muscle homogenates was measured spectrophotometrically. Muscle fibre types (Type I, IIA and IIB) were measured histochemically, and the muscle metabolites, ATP, PCr, lactate, glycogen IMP and pH were also determined. With fatigue, muscle torque fell by 43.4±3.3% (mean±SEM, $P<0.05$), whilst the rates of muscle SR Ca^{2+} release, Ca^{2+} uptake and the activity of Ca^{2+}ATPase were reduced by 42.1±3.8%, 43.0±5.2% and 38.5±4.2%, respectively ($P<0.01$). Muscle ATP, PCr, glycogen and pH were all decreased, whilst IMP and lactate were elevated with fatigue ($P<0.05$). Positive relationships were found between each of muscle ATP, PCr and glycogen and the percentage depression in each of muscle SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity ($P<0.05$); negative correlations were found for muscle Cr, IMP, lactate and pH ($P<0.05$). Thus, intense fatiguing exercise induced marked declines in SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity, which most likely play an important role in the torque decline with fatigue. The mechanisms for the decline in SR function cannot be determined from this study, but structural alterations in SR proteins are indicated, since these effects were measured *in-vitro*. Possible differences in the severity of *in-vivo* more than *in-vitro* changes cannot be determined in this study. However, it is possible that the dramatic metabolic perturbations observed may lead to an even more marked *in-vivo* depression in SR function with fatigue.

Study 2. Little is known about the importance of muscle fibre composition and the effects of training on SR function in resting human muscle. Further, possible protective effects of

training against the depressed SR function with fatigue have not previously been analysed. Thus, skeletal muscle performance, fibre types, SR function and metabolites were compared between eight untrained (UT), eight resistance trained (RT) and eight endurance trained (ET) subjects. All subjects underwent a maximal incremental exercise test to determine their peak oxygen uptake ($\dot{V}O_2$ peak), and isokinetic knee extensor tests to determine their torque-velocity relationships and muscle fatiguability. Vastus lateralis muscle biopsies were taken at rest and following 50 maximal isokinetic knee extensor contractions and analysed for SR function, fibre types and metabolites. The ET group had a higher $\dot{V}O_2$ peak than the RT and UT groups (67.6±1.5, 43.8±3.6, 44.4±1.8 $ml.kg^{-1}.min^{-1}$, respectively, $P<0.005$) and a lower fatigue-induced decline in muscle torque (29.9±4.3, 47.4±5.0 and 43.4±3.3 %, respectively, $P<0.005$). Thigh muscle cross sectional area was greater in RT than in ET and UT ($P<0.05$). Both muscle fibre type and training status influenced muscle SR at rest. Positive relationships were found between the proportion of Type II muscle fibres and each of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity ($r>0.56$, $P<0.005$). ET had a higher proportion of Type I fibres than RT and UT (67.4±3.3, 43.6±4.9, 50.7±2.9%, respectively, $P<0.01$) and in resting muscle, had lower rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity than UT ($P<0.05$). In resting muscle, RT had a lower SR Ca^{2+} uptake than UT ($P<0.05$), despite no differences in fibre type proportions. SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity each declined with fatigue in all groups ($P<0.05$). The decline in SR function with fatigue was less in ET than in RT and UT ($P<0.01$) but when expressed as a percentage decline, no significant differences were apparent between groups. In conclusion, SR function was clearly related to the fibre composition in human skeletal muscle but also differed with training status. The lower SR function in resting muscle in ET is consistent with a lesser proportion of Type II fibres. Chronic endurance and resistance training did not prevent the decline in SR function with fatigue. The attenuated reduction in SR function with fatigue in ET most likely reflected their lower proportion of Type II fibres but may also be consistent with a lesser fatigue effect on SR in Type I fibres. Other training effects were suggested in RT by the lesser SR Ca^{2+} uptake at rest that might indicate additional training effects on muscle SR.

Study 3. The effects of chronic inactivity on human skeletal muscle SR function have not been investigated. Lung transplant recipients (LTx) have been chronically inactive and exhibit poor exercise performance despite organ transplantation and subsequent rehabilitation. This study investigated whether SR function was abnormal in seven transplant patients compared to seven age- and sex-matched healthy controls. All subjects

underwent an incremental exercise test, with a lower $\dot{O}_2$ peak found in LTx than in controls (18.7±1.5 vs. 36.9±2.4 ml.kg^{-1}.min^{-1}, respectively, P<0.01). Two resting biopsies were taken from vastus lateralis muscle and analysed for fibres type, SR function and metabolites. LTx (P<0.01) had a lower proportion of Type I fibres (25.0±4.4 vs. 56.5±2.1 %) and a higher proportion of Type IIB muscle fibres (40.7±8.0 vs. 3.1±1.4 %) than controls. Opposing effects were found between changes in fibre types and SR function, with lower resting muscle SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity in LTx than in controls (32.5 ±9.2, 28.6±7.5 and 22.1±5.5 %, respectively, P<0.05). Muscle metabolites were also abnormal, with lower resting muscle ATP and pH and higher IMP and lactate in LTx (P<0.05). Thus, LTx demonstrated skeletal muscle fibre type abnormalities, which are consistent with the effects of long-term disuse. SR function was abnormally low in LTx, particularly when fibre composition is considered, and this may be an important contributor to their poor muscular performance.

Study 4. The abnormal exercise performance and muscle SR characteristics found in LTx may result from chronic disuse, but may also be affected by disease and by the immunosuppressive drugs taken. No studies have investigated the effects of the immunosuppressive drug Cyclosporin A (CsA) on SR function in skeletal muscle. Therefore the acute *in-vitro* effects of CsA (0, 1, 25 and 50 μg/ml CsA) on SR function were investigated in the homogenates from extensor digitorum longus (EDL) and soleus (SOL) muscles in eight Sprague-Dawley rats. The rates of SR Ca^{2+} release, Ca^{2+} uptake and the activity of Ca^{2+}ATPase in EDL were 2-4 times higher than in SOL (3.8±0.2, 2.6±0.1, 2.0±0.2 -fold, respectively). SR Ca^{2+} release was reduced at the respective CsA concentrations in EDL (P<0.05) by 20.2±4.0%, 30.0±4.9% and 36.7±4.4% and in SOL (P<0.05) by 24.7±4.3%, 33.9±4.5% and 45.3±4.4%. The rates of SR Ca^{2+} uptake and Ca^{2+} ATPase were also similarly reduced by CsA, compared with controls (P<0.05). Thus, acute exposure to CsA depressed SR function in both EDL and SOL muscle homogenates, which suggests that CsA could also inhibit skeletal muscle SR function in lung transplant recipients.

In summary, each of the factors examined in this thesis was found to have important effects on skeletal muscle SR characteristics. Consistent with other mammalian muscle, human muscle SR function was significantly correlated with muscle fibre composition. Skeletal muscle SR function was depressed with muscular fatigue, and was lower at rest in both endurance trained and resistance trained individuals compared to controls. SR function was impaired in lung transplant recipients, which may in part be due to chronic disuse, as well as the effects of the immunosuppressive drug CsA.

CHAPTER I. INTRODUCTION

Movement is essential for survival and encompasses an enormous diversity of muscle contractile function, for walking, running, eating, crying, smiling, or even standing still. These movements result from skeletal muscle contractions and therefore all depend on excitation-contraction coupling. Calcium ions (Ca^{2+}) play a key role in skeletal muscle excitation-contraction coupling (Ruegg 1992). Following activation of the voltage sensors within t-tubular membranes, Ca^{2+} is released from the sarcoplasmic reticulum (SR), elevating cytosolic calcium concentration ($[Ca^{2+}]$), allowing cross bridge binding at the myofibrillar proteins and subsequent force production. Increased Ca^{2+}ATPase enzyme activity results in Ca^{2+} re-uptake by the SR and induces muscle relaxation due to dissociation of actin-myosin filaments. Thus, the SR regulates cytosolic $[Ca^{2+}]$, which largely determines the rates of skeletal muscle contraction and relaxation.

Disturbances in SR Ca^{2+} regulation have been linked with muscular fatigue, disuse and muscle pathology (Kim et al. 1982, Westerblad et al. 1991, Fitts 1994, Benders et al. 1997). However, few studies have investigated the effects of fatigue, training and disuse on muscle SR function in human skeletal muscle.

This thesis examines several factors affecting SR Ca^{2+} regulation in human skeletal muscle, through *in-vitro* measurements of SR Ca^{2+}uptake, Ca^{2+} release and Ca^{2+}ATPase activity in muscle homogenates. The effects of muscle fatigue, as well as those of resistance and endurance training, as well as muscle fibre type on muscle SR Ca^{2+} regulation are investigated. The role of SR Ca^{2+} regulation is also investigated in lung transplant patients, who suffer from chronic extreme muscular disuse. Since additional factors might influence muscle SR Ca^{2+} regulation in these patients, a further study investigated the effects of the immunosuppressive drug Cyclosporine on SR Ca^{2+} regulation in rat skeletal muscle.

CHAPTER II REVIEW OF LITERATURE

2.1 Skeletal Muscle Sarcoplasmic Reticulum Structure and Function

2.1.1 Overview of Excitation-Contraction Coupling

Excitation-contraction coupling (ECC) in skeletal muscle is the series of events linking muscle fibre membrane depolarisation to muscle contraction, as shown in Figure 2.1. Briefly, ECC consists of the following processes. After neuromuscular transmission, an action potential is propagated over the entire surface of the muscle membrane (sarcolemma) and spreads down into the transverse tubule network (T tubule). Propagation of the action potential in the T tubule activates a voltage sensor which triggers sarcoplasmic reticulum (SR) release of Ca^{2+} into the cytosol. The free Ca^{2+} in the cytosol is bound to the regulatory proteins (troponin) allowing cross- bridge bending attachment and responsible for the power stroke that pulls the thin actin filament inward. Finally, the SR actively transports Ca^{2+} back into its longitudinal tubules (LT), via ATP-driven calcium pump (Ca^{2+}ATPase) located in the membrane of the SR. Once Ca^{2+} has been lowered sufficiently in the cytosol, the actin and myosin filaments then dislocate and the thin filaments slide back to their original position, leading to muscle relaxation. Thus, the SR continually controls the myoplasmic [Ca^{2+}] and thereby regulates force development and relaxation.

2.1.2 Historical Perspectives

Early experimental work examining the role of SR in muscle ECC focussed on factors influencing muscle relaxation. A "factor" in muscle "juice" able to relax isolated myofibrils and demembranated muscle fibres was discovered by Marsh (1951) and Bendall (1952). A few years later, Kumagai et al. (1955) and Portzehl (1957) discovered

that a "microsomal fraction", which could be separated by high-speed differential centrifugation, was present in the relaxing activities of muscle juice. These vesicles were found to consist mainly of fragments of SR (Hasselbach 1961). Hasselbach (1964) and Portzehl et al. (1964), first demonstrated evidence for calcium sequestration by the SR in *vivo*. Fluorescent dyes were exploited for the analysis of Ca^{2+} turnover in muscle cells (Baylor and Hollingworth 1988). Recent experiments have used fluorescent dyes such as Fura-2 and Indo-1, combined with very sensitive transducers for measuring [Ca^{2+}] and force levels in single fibres (Westerblad and Allen 1991, Williams and Fay 1990).

In recent years, researchers have investigated aspects of muscle activation, examining the role of Ca^{2+} in ECC (Ashley et al. 1970, Ashley et al. 1991, Franzini-Armstrong et al. 1994, Melzer and Luttgau 1995, Bers and Fill 1998), dihydropyridine receptors (Lamb and Walsh 1987, Rios and Pizarro 1991, Lamb 1992, Meissner and Lu 1995, Lu et al. 1995, Sorrentino 1995) and ryanodine receptors (RyRs) (Franzini-Armstrong 1975, Dulhunty et al. 1992, 1996, Airey et al. 1993, Coronado et al.1994, Hosey et al. 1996, Sutko and Airey 1996). Using a variety of techniques, investigators are now gaining increasing understanding of the intracellular processes regulating ECC.

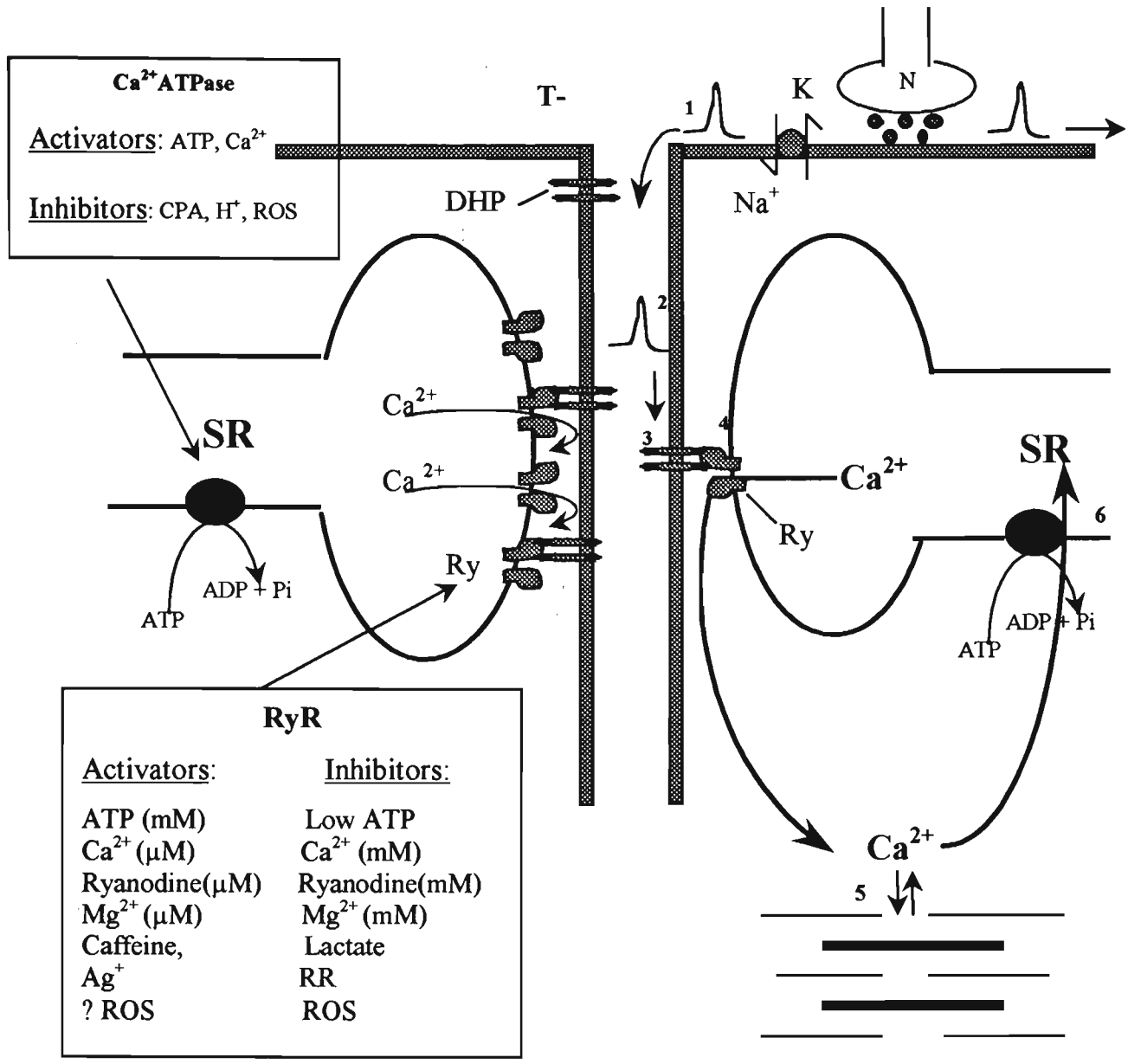


Figure 2.1 Schematic diagram of Ca^{2+} regulation during excitation -contraction coupling in skeletal muscle. Modified from Sutko and Airey (1996).

1. Sarcolemma depolarization; 2. T- tubular depolarization; 3. DHPR charge movement; 4. RyR - voltage regulated Ca^{2+} release; 5.Ca^{2+} binding to troponin, actomyosin hydrolysis of ATP and cross-bridge force development and cycling rate; 6. Ca^{2+} reuptake to SR via Ca^{2+}ATPase. N, Nerve cell terminal; RyR, ryanodine receptor; CPA, cyclopiazonic acid; RR, ruthenium red; DHPR, dihydropyridine receptor; ROS reactive oxygen species; — actin filament, ▬ myosin filament.

2.1.3 Sarcoplasmic Reticulum Structure

The SR is a fine membranous network of interconnected tubules surrounding each myofibril within the muscle (Figure 2.2). The end of each SR segment expands within these segments to form sac-like regions, referred to as the terminal cisternae. These are the Ca^{2+} storage sites and they lie in close proximity, but do not directly contact, the adjacent T-tubules. The T- tubule is an invagination of the sarcolemma, which runs from the surface of the muscle cell in a complicated network, into all interior portions of the fibre. The T-tubule and the SR terminal cistern on either side forms a triad, as observed by electron microscopy (Figure 2.3). This area is also called the T-SR junction. In frog skeletal muscle, there is a 15nm gap between the T-Tubular membrane and the junctional membrane of the terminal cisternae (Block et al. 1988). A "foot" - like structure forms a bridge in this gap, and forms a regular tetragonal lattice of about 800 "feet" μm^{-2} junctional membrane. The "feet" occupy approximately 27% of the cisternal membrane and amounts to about 1500 $cm^2\ g^{-1}$ muscle (Franzini-Armstrong 1975, Franzini-Armstrong et al. 1988). The characteristic size and shape of the feet structures are a square or quatrefoil-shaped particle of ~ 20nm on each side, with a 2 nm hole in the centre, as determined from purified J-SR protein (Inui et al. 1987, Lai et al. 1988a, Wagenknecht 1989, 1997). The feet structures represent the Ca^{2+} release channels of the SR (Rios and Pizarro 1991).

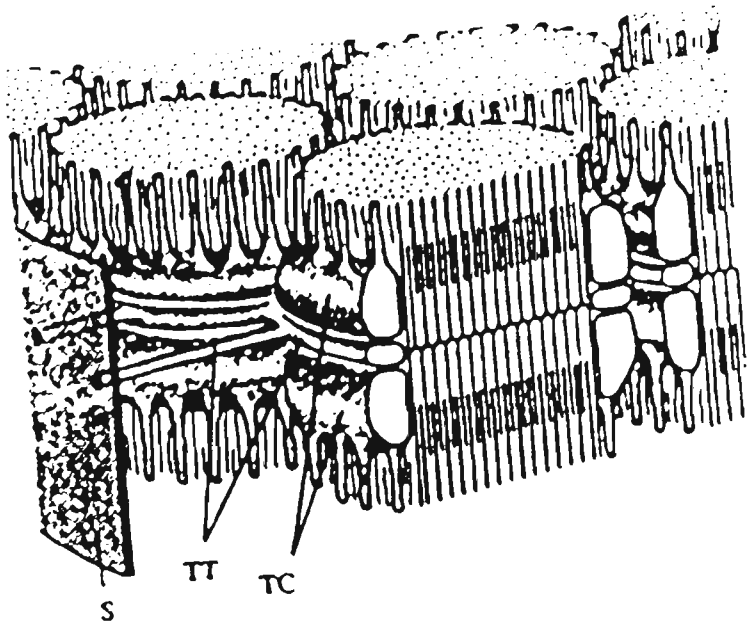


Figure.2.2 Schematic drawing of a section from an amphibian muscle fibre showing myofibrils surrounded by terminal cisternae (TC) of the SR and the transverse tubular (TT) which form the elements of the triad. The T-system opens to the extracellular space at the sarcolemma. From Melzer et al. (1995).

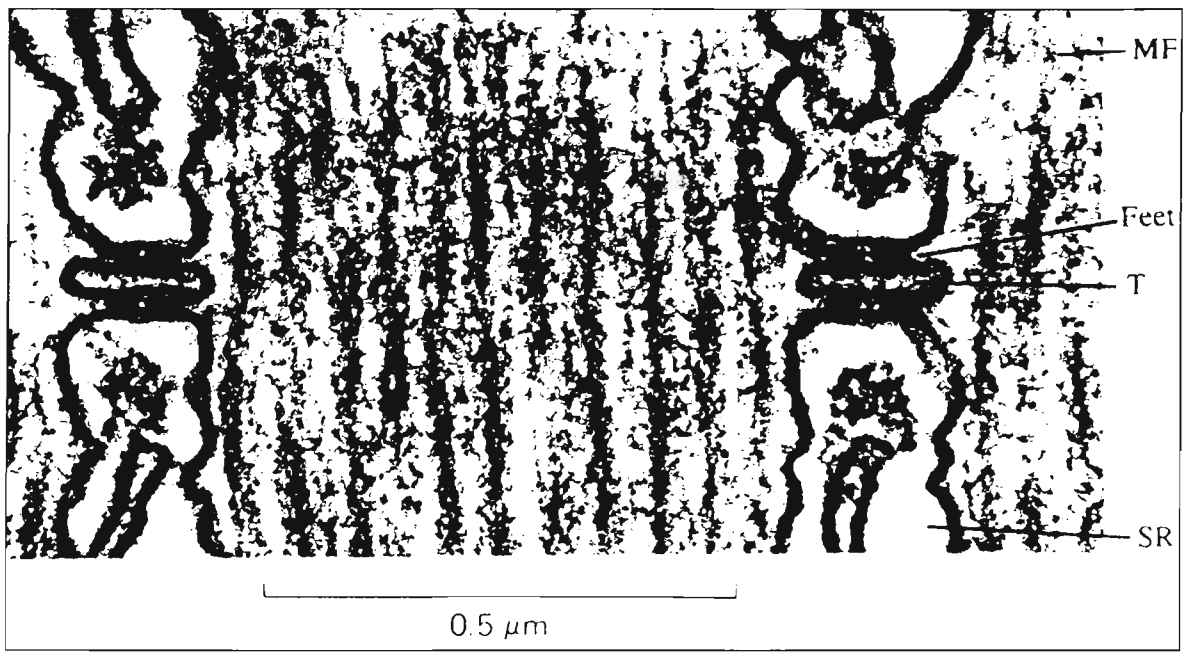


Figure.2.3 Longitudinal electron micrograph through part of a toadfish muscle fiber showing two triads. Note the flattening of the T-tubules (T), the junctional feet linking the tubules to the adjacent SR membranes (SR) and myofibril (MF). From Franzini-Armstrong (1980).

2.1.4 Sarcoplasmic Reticulum Ca^{2+}release

The main function of the T tubule is the transmission of electrical activity from the surface membrane into the central portions of the fibre. This then triggers massive release of Ca^{2+} from the terminal cisternae of the SR into the cytosol. In the latter process there are at least two key membrane proteins involved, known as the dihydropyridine receptors (DHPRs), which are located in the T tubule membrane, and the ryanodine receptors (RyRs), which are the physiological Ca^{2+} release channel, and are located in the SR terminal cisternae membrane.

2.1.4.1 Dihydropyridine Receptor

There is a type of L-channel (large or long-lasting) found in high density in the transverse tubular membranes of skeletal muscle fibres, which is the DHPR, so named because it is bound and blocked by dihydropyridine (MacLennan et al. 1997). The DHPR is voltage-dependent, and appears to act as a voltage sensor to transmit a signal from the T tubule to the RyR in the SR (Melzer et al. 1995). This is thought to be the major mechanism by which Ca^{2+} is released from the SR in skeletal muscle (Chandler et al. 1976).

Diamond-shaped arrays or "tetrads" of four particles in the junctional T tubule (jT) have been observed in toad fish swim bladder by Block et al. (1988). The authors suggested that each of the four particles making up the jT tetrad is a DHPR. Mammalian skeletal muscle contains a similar tetradic structure. The DHPR complex consists of 5 subunits: α_1 (185kDa), α_2 (143kDa), β (54kDa), γ (30kDa) and δ (26kDa). The α_1 subunit of skeletal muscle has been isolated and characterized (Tanabe et al. 1987). The α_1 subunit has the DHP binding site, the voltage-sensing region, the Ca^{2+} channel function, and the primary role of controlling Ca^{2+} release from the SR. The α_1 subunit contains four repeats, each with 6 hydrophobic segments (S1-S6) which are thought to span the T-tubular membrane. S4 of the repeats is regarded as the voltage-sensing element (Block et al. 1988, Lamb

1992, Hosey et al. 1996). Experiments with chimeras of the α_1 DHPRs subunits from skeletal and cardiac muscle have shown that the intracellular loop between the third repeat of the α_1 subunits is critical for skeletal muscle -type E-C coupling (Lamb 1998).

At least two isoforms of the DHPR α_1 subunit are expressed in skeletal muscle, known as the skeletal muscle, or cardiac muscle DHPR isoforms (Pereon et al. 1997). Recently, Pereon et al. (1998) examined the expression of DHPR α_1 subunit isoforms in rat diaphragm, soleus and EDL muscles. The DHPR skeletal muscle isoform mRNA was expressed at the highest levels in the EDL muscle, whilst both the diaphragm and soleus muscles expressed significant levels of the DHPR cardiac isoform mRNA (Pereon et al. 1998).

2.1.4.2 Ryanodine Receptor

The physiological Ca^{2+} release channel of the SR is known as the Ryanodine Receptor (RyR) as it has a high-affinity binding for the plant alkaloid, ryanodine. The RyR was first isolated by Pesah et al (1986) and Inui et al. (1987). The RyR contains ~5030 amino acids (Takeshima et al. 1989). Under the electron microscope, samples manifested a four leaf structure spanning the junction gap between T- system and SR (Ferguson et al. 1984). The cytoplasmic part of the RyR protein is 29 × 29× 12 nm, with a 14 × 14 × 7 nm baseplate which is inserted into the TC membrane (Radermacher, 1994). The RyR from the triadic junction of skeletal muscle, cardiac and brain tissue have now been cloned and sequenced (Takeshima et al. 1989, Zorzato et al. 1990, Nakai et al. 1990, Otsu et al. 1990, Hakamata et al. 1994). There are three main RyR isoforms in mammalian tissues, referred to as RyR_1 (Figure 2.4), RyR_2 and RyR_3 Each of the RyRs is a separate gene product, but the sequence identity is 66-70% (Smith et al. 1988, Ogawa 1994, Coronado et al. 1994, Otsu et al. 1990, Giannini et al. 1995). All RyRs have a similar molecular weight of

400,000 - 450,000 as estimated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) (Inui et al. 1987, Lai et al. 1988a).

In 1990, Zorzato et al. cloned cDNAs encoding human RyR. Although the rabbit and human sequences are very similar, analysis of the human sequence indicated a more complex structure of the transmembrane part of the receptor than the rabbit (Takeshima et al. 1989). The substitution of Arg for Gly2344 in the human skeletal muscle is associated with malignant hyperthermia (Phillips et al. 1994, 1996). Also, Zucchi et al. (1994) have shown reduced Ca^{2+} release in ischaemic – reperfused cardiac muscle. The involvement of RyR in malignant hyperthmia and other skeletal muscle diseases was recently reviewed by Zucchi and Ronca-Testoni (1997).

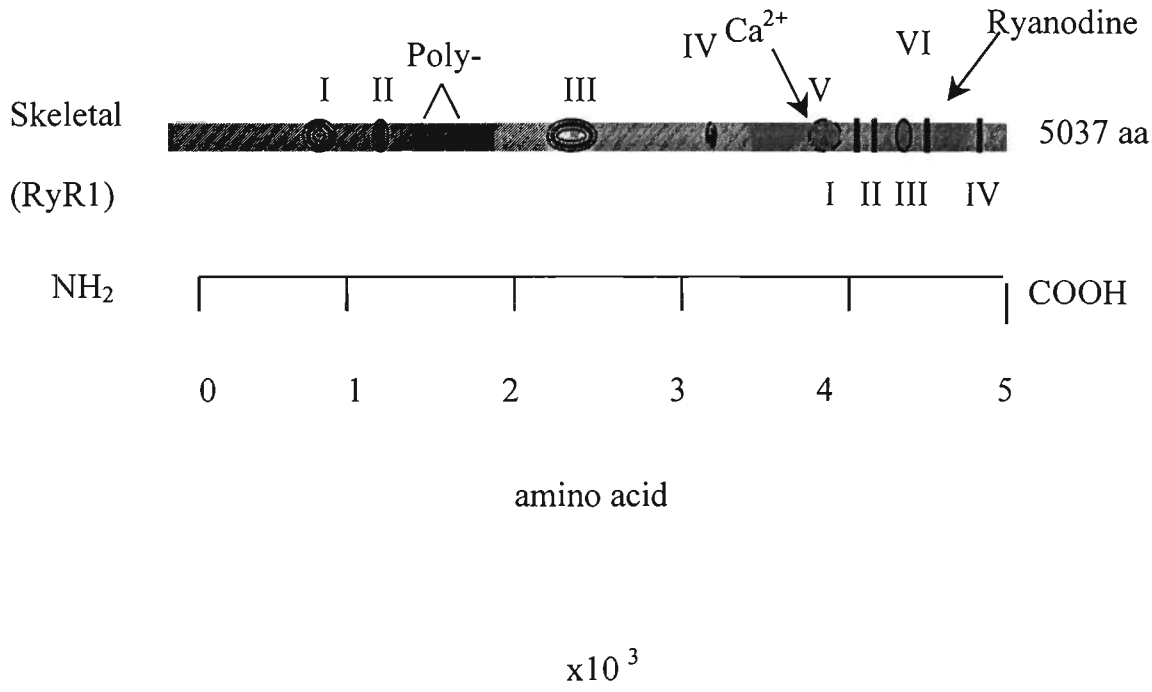


Figure 2.4 Proposed topography of ryanodine receptors (RyR1). From Marks, (1997). Analyses of deduced amino acid (aa) sequence obtained from cDNA cloning and protease sensitivity mapping of surface-exposed regions. Six regions of protease sensitivity have been identified in skeletal RyR1 (labelled I-VI). High and low -affinity ryanodine-binding

sites are located near carboxy terminus as is a Ca^{2+}- binding site (Ca^{2+} inside box). A polyglutamic acid region (Poly-Glu) is unique to RyR1.

2.1.4.3 Mechanisms of SR Ca^{2+} release

Three possible mechanisms have been proposed to explain the signal transmission at the triad, coupling the depolarized T-tubular membrane to SR Ca^{2+} release. These mechanisms are Ca^{2+}-induced Ca^{2+} release (CICR, Endo et al. 1970, Ford and Podolsky 1970), involvement of a second messenger mechanism (Vergara 1985, Volpe 1985) and a direct mechanical link between the DHPR and the RyR (Chandler et al. 1976). (Figure 2.5).

The CICR mechanism involves Ca^{2+} ions passing through the Ca^{2+} channel in the T-tubular DHPR into the cytosol, binding to a site on the RyR, and causing SR Ca^{2+} release. The second messenger mechanism proposes that T-tubular membrane depolarization activates in some way (may be via the DHP receptor) phospholipase C (PLC), which cleaves phosphatidylinositol-(4,5)-bisphosphate (PIP_2) into inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to the RyR, inducing Ca^{2+} release from the SR. The mechanical model proposed in the simplest form the control of Ca^{2+} release by the potential of the T-tubular membrane and has become the most accepted (Melzer et al. 1995). This involves the charge being rigidly connected with a channel-blocking particle, which blocks the Ca^{2+} release channel under resting conditions; the intramembrane charge movement initiates Ca^{2+} release.

Of these three mechanisms, the latter mechanism is believed to be the most important in skeletal muscle (Lamb 1992). Recently Sutko and Airey (1996) suggested that the presence of alternating DHPR and RyR might indicate that two different mechanisms. Those RyR opposed by DHPR would most likely be activated by a mechanical link, whereas those RyR not opposed by DHPR could be activated by the CICR mechanism.

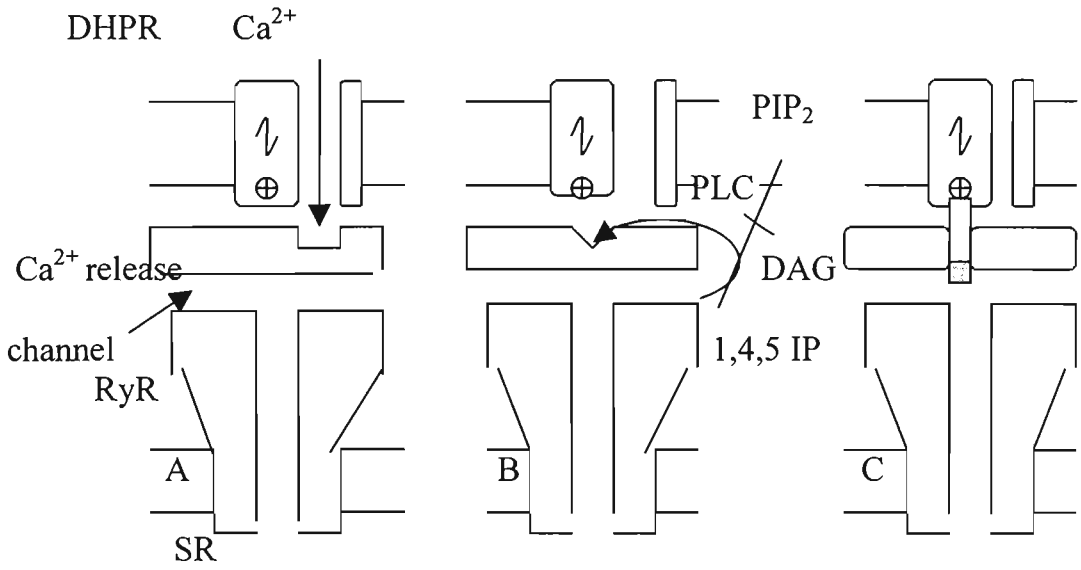


Figure 2.5 Schematic diagram of the triad with three possible mechanisms of signal transmission between the DHPR in the T-tubular membrane and the RyR in the membrane of the terminal cisterna of the SR. A. Ca^{2+}- induced Ca^{2+} release. B. Second messenger mechanism. C. Mechanical model. From Melzer et al. (1995).

The density of the DHPR in skeletal muscle was 50 to 100 times higher then in other tissues (Fosset et al. 1983). Block et al. (1988) reported a DHPR to RyR ratio of 2.0. However, the DHPR to RyR ratio is variable in skeletal muscle from different species, ranging from 0.5 to 2.0 (Franzini-Armstrong and Jorgensen 1994). The densities of DHPR and RyR are different in different species, muscles and fibre types (Sutko and Airey 1996). The DHPR content was higher in dystrophic than in normal chicken muscle (Moro et al. 1995). A recent study found a significantly reduced DHPR to RyR ratio in the EDL, soleus and pooled muscles in the old rat compared to young and middle age rats (Renganathan et al. 1997). There do not appear to be any measures of the DHPR to RyR ratio in human muscle.

2.1.5 Factors Regulating SR Ca^{2+} Release

Generally, Ca^{2+} release is modulated by endogenous ligands, but many other compounds also affect SR release channels. Endogenous modulators and regulatory compounds have become useful tools in the analysis of ECC and are shown in Table 2. 1.

Table 2.1 Modulators of the SR Ca^{2+} Release Channel

Activators	Inhibitors
Ca^{2+} (μM)	Ca^{2+} (mM)
Mg^{2+} (μM)	Mg^{2+} (mM)
ATP (mM)	Low ATP
Ryanodine (μM)	Ryanodine (mM)
Caffeine	Ruthenium Red
$InsP_3$	Calmodulin
Ag^{+}	Lactate
? ROS	ROS

2.1.5.1 SR membrane potential

Release of calcium from the SR depends on opening of other channels to allow a counter current to flow with calcium, to prevent large changes in the potential difference across the membrane, which would impede the calcium fluxes.

At very low (10^{-11} M) concentrations of ryanodine, the RyR have a low conductance state, which allows calcium to leak from the terminal cisternae of the SR and to be recycled with ATP utilization (Bianchi 1997). With concentrations of ryanodine greater than 10^{-6} M, resting muscle slowly contracts. Furthermore, when the RyR complex is formed during stimulation, μM ryanodine will inhibit the muscle twitch, and if formed before stimulation, these can accelerate the development of contraction (Bianchi 1997).

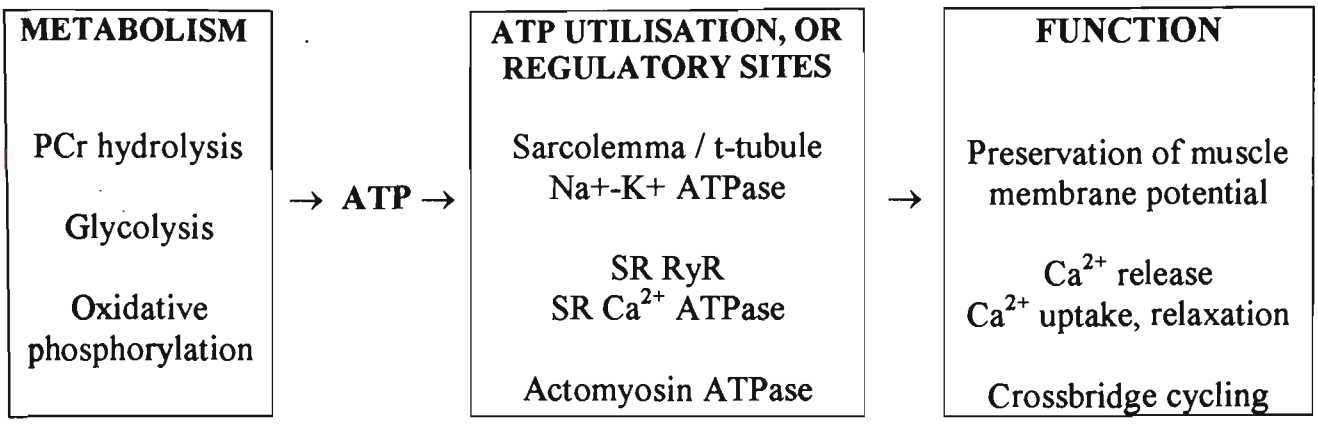
2.1.5.2 Cytosolic and intraluminal SR Ca^{2+} concentrations

The cytosolic [Ca^{2+}] and SR luminal [Ca^{2+}] are both important factors for regulation of the Ca^{2+} release channels. The open probability (P_0) of RyR channels, and thus, Ca^{2+} release from SR, has a bell-shaped dependence on cytoplasmic [Ca^{2+}]. The RyR P_0 is near zero with nanomolar cytoplasmic [Ca^{2+}], is increased with μM [Ca^{2+}], and falls between 0.2 and 1.0 mM Ca^{2+} in skeletal muscle (Rüegg, 1992). When SR lumenal Ca^{2+} concentration was increased from < 0.1 μM to approximately 250 μM, Ca^{2+} channel activities were enhanced. Those were associated with a long open time constants at negative holding potentials, but the channel activity remained low at positive holding potentials. However, when the SR luminal [Ca^{2+}] was increased to 1, 5, and 10 mM, decreased channel activities at negative holding potentials and increased activities at positive holding potentials were found (Tripathy and Meissner 1996).

2.1.5.3 Muscle energy supply and substrate depletion

Calcium regulation by SR is a series of processes requiring ATP and thus energy consumption. For example, ATP is required for Ca^{2+} transport into the SR by the Ca^{2+} pump and for Ca^{2+} release from SR through the Ca^{2+} release channel. Thus SR function is dependent upon energy provision by metabolic processes within the cell. A schematic representation linking ATP, SR function and muscle contractile function is shown in Figure 2.6. In skeletal muscle the transition from rest to the active state may involve a 100-fold increase in the demand for ATP (Lüttgau, 1992). No other cell type in the body has to cope with such massive increases in ATP utilisation.

Figure 2.6 Roles of cellular ATP in skeletal muscle function.



The cytosolic ATP concentration may effect the SR Ca^{2+} release. [ATP] can activate the Ca^{2+} release channel of the skeletal muscle in vesicle-ion studies (Meissner 1994, Smith et al. 1986). Tripathy and Meissner (1996) examined the rabbit skeletal muscle Ca^{2+} release channels (ryanodine receptors). They showed that addition of 2 mM $[ATP]_{cyto}$ whilst keeping the free cytosolic $[Ca^{2+}]$ at 45 nM, greatly increased the number of the short channel openings. The Ca^{2+} release channel is optimally activated only by the combined presence of Ca^{2+} and ATP (Meissner 1994).

2.1.5.4 Muscle pH

The mean resting intracellular pH reported in muscle fibres varies from~7.2-7.0, being dependent on the buffer medium used and temperature of measurement (Aickin and Thomas 1977, Juel, 1988). In human muscle resting pH is about 7.1 (Allsop et al. 1990). A dramatic decrease in muscle pH occurs in exhaustive exercise (Hultman and Sahlin 1980). In dynamic exercise muscle pH has been reported to fall to 6.6 (Sahlin et al. 1975), 6.5 (Hermansen and Osnes 1972, Sahlin 1978, Spriet et al. 1989) and using NMR to 6.2 (Wilson et al. 1988).

Different results on pH effects on SR function have been found in isolated RyR channel and intact fibre preparations. The effect of decreased muscle pH on SR function has recently received attention. Rios et al. (1994) hypothesized that the decline of the pH could interfere with Ca^{2+} binding to either the internal surface of the DHPR or to the

release channel itself. Following reconstitution of the purified RyR channel into a lipid bilayer, Ma et al. (1988) found the channel Po (open) was maximal at pH 7.4 but was almost null at pH 6.5. At a low pH the channel opening was decreased in frequency and duration. Godt and Nosek (1989) reported that acidification impedes cross-bridge function and is responsible for a decline in force. Recent studies have challenged the force depressing role of lower pH in mammalian muscle fatigue and indicate that the effect of pH depends on the temperature. Similar findings were obtained in skinned rabbit and intact single mouse fibres (Pate et al. 1995 and Westerblad et al. 1997a). The results showed the expected large force decline effect of lowered pH at 10 °C, but only a 10% decline in force at ~30 °C. Thus, acidosis has little direct effect on force production at physiological temperature (Westerblad et al. 1997a). Lamb et al. (1992) and Lamb and Stephenson (1994) attributed all of the decline in force at low pH to direct inhibition of the contractile apparatus, and concluded that even a pH at 6.2 did not effect Ca^{2+} release in rat and toad skinned muscle fibres. Thus, whilst isolated RyR may be inhibited by acidosis, no effect was seen when the RyR remained in a fibre with functional E-C coupling. However, studies demonstrated a reduced Ca^{2+} uptake at acidic pH in both species (Stephenson 1998). On this basis, an acidosis-mediated slow Ca^{2+} uptake was hypothesised to lead to a reduced Ca^{2+} releas (Fitts and Balog 1996).

In summary, the preparation (fibres or channels) used to determine temperatures effect in SR Ca^{2+} release. The results observed in lower pH state. At physiological temperatures a lower pH does not reduced SR Ca^{2+} release in intact muscle fibres in the rat and in the toad. However, in isolated RyR preparation, the effect of H^{+} was highly cooperative.

2.1.5.5 Lactate

Lactate ([Lac^{-}]) is a product from glycolysis, which occurs anaerobically only in the cell cytosol. Lactate accumulation has long been appreciated as an index of accelerated glycolysis (Katz and Sahlin 1988) and occurs when the rate of glycolysis exceeds that of

pyruvate oxidation, resulting in increases in both [NADH] and [H^+] and a decline in [NAD^+]. The regeneration of NAD^+ by lactate dehydrogenase (LDH, Equaation 1) also enables glycolysis to continue:

Equation 1 $\quad Pyruvate + NADH + H^+ \longleftrightarrow lactate^- + NAD^+$

Muscle [Lac^-] may rise by 10 to 27- fold after intense exercise (Boobis et al. 1983, Cheetham et al. 1986, McCartney et al. 1986). The accumulation of Lac^- begins close to the onset of exercise, consistent with the maximal activation of glycogenolysis and glycolysis (Jacobs et al. 1983, Jones et al. 1985). There is some support for the accumulation of lactate playing a major role in the development of local muscular fatigue, when fatigue occurs during high intensity, and short duration exercise, within the time frame of ~10 s to 15 min (Tesch et al. 1978, Holloszy, 1973). Recently, addition of lactate decreased force development in working dog muscle and skinned rabbit fibres (Hogan et al. 1995, Andrews et al. 1996).

Recently, Favero et al. (1997) investigated the effects of lactate on skeletal muscle SR function in SR vesicles from rabbit white skeletal muscle. They demonstrated that 10 to 30 $mmol.l^{-1}$ lactate inhibited SR Ca^{2+} release that was stimulated by either Ca^{2+} or caffeine. This decreased Ca^{2+} release was due to a reduced number of open SR Ca^{2+} release channels, as shown by reduced Ca^{2+}- and caffeine-stimulated [^3H] ryanodine-binding. They further found when the RyR was reconstituted in a lipid bilayer, that 10-30 $mmol.l^{-1}$ lactate significantly inhibited the SR Ca^{2+} release channel activity. Thus, elevated [Lac^-] may disrupt excitation-contraction coupling, causing decreased SR Ca^{2+} release and reduced Ca^{2+} transients, leading to lower cytosolic [Ca^{2+}]. Thus, elevated cytosolic [Lac^-] may play a key role in the reduction in force output that occurs with fatigue, as discussed later.

2.1.5.6 Mg^{2+}

Alterations in skeletal muscle cytosolic [Mg^{2+}] will affect SR Ca^{2+} regulation and thus, may directly influence muscle function (Westerblad and Allen 1992a, Lamb and Stephenson 1991). The total magnesium concentration per unit muscle water is ~14 mM, with ~ 6mM diffusible in the myoplasm, for example bound to ATP, creatine phosphate, or parvalbumin (Blatter 1990, Raju et al. 1989). The free ionized [Mg^{2+}] in the myoplasm in the resting state, is in the range of 0.8-1.0 mM, when estimated with Mg^{2+}- sensitive microelectrodes, or the Mg^{2+}- sensitive indicator Furaptra (Somlyo et al. 1985). During contraction Mg^{2+} moves into the SR as a counter ion to the release Ca^{2+} (Somlyo et al. 1985); on the other hand, it is displaced by Ca^{2+} from binding sites on troponin or parvalbumin.

In both intact and mechanically skinned fibres Lamb and Stephenson (1991, 1994) demonstrated that under physiological conditions for Mg^{2+} (1mM) and ATP (2-8mM), the SR Ca^{2+} release channels were closed, despite the strong stimulatory effect of ATP on channel opening. When cytosolic [Mg^{2+}] was reduced from 1mM by a factor of 10-20 fold or more, the SR Ca^{2+} release channels opened in the presence of ATP for a substantial proportion of time, causing rapid Ca^{2+} release from SR (Meissner 1986, Lamb and Stephenson 1991, 1994). T-tubule depolarisation in 1mM [Mg^{2+}] induced Ca^{2+} release from SR, but an increase in [Mg^{2+}] from 1 to 3 mM inhibited Ca^{2+} release. When [Mg^{2+}] was raised to 10mM, depolarisation and voltage sensors activated in the T-tubule could not open the SR Ca^{2+} release channels. It is possible that Mg^{2+} is bound to a low-affinity inhibitory site on the SR Ca^{2+} release channel, thus keeping the channel closed under resting conditions. Once the Mg^{2+} is removed from the inhibitory site by some factors, e.g. DHP receptor complexes, the SR Ca^{2+} release channel will open in the presence of ATP. However, high [Mg^{2+}] will remain a potent inhibitor of the SR Ca^{2+} release channels

(Lamb and Stephenson 1991, 1994). This may be an important inhibitory factor in fatigue, as discussed later.

2.1.5.7 Free radicals

A growing body of evidence indicates that free radicals also affect skeletal muscle SR function (Reid 1996). Cellular accumulation of free radicals has been associated with an impaired SR membrane integrity and indicated as a possible cause of exercise induced damage to skeletal muscle SR (Davies et al. 1982). However, the reactive oxygen species (ROS) produced by skeletal muscle also promote excitation-contraction coupling and indeed, appear to be obligatory for optimal contractile function (Reid 1996). At more than 1mM hydrogen proxide (H_2O_2) increased peak twitch stress, lengthened time-to-peak tension and half-relaxation time, in muscle fibre bundles from rat diaphragm. However, when the fibres were exposed to catalase (1 to 10^5 U/ml), which scavenges H_2O_2, the above twitch characteristics and the twitch-to-tetanus force ratio were decreased in a dose-dependent manner (Reid et al. 1993).

Exposure to 1 mM H_2O_2 for 5 minutes dramatically inhibited SR Ca^{2+} release in rat skeletal muscle, but had no effect on the isometric force-producing properties of the contractile apparatus, or on SR Ca^{2+} uptake (Brotto and Nosek 1996). In contrast, 5 mM H_2O_2 increased ryanodine binding to its receptor in SR vesicles isolated from fast-twitch skeletal muscle and caused a rapid SR Ca^{2+} release (Favero et al. 1995). Oba et al. (1996) found that 6 mM H_2O_2 initially potentiated the twitch tension in single frog skeletal muscle fibres, whereas inhibition of twitches was seen after 15 minutes. This potentiation may be induced by oxidizing the sulphydryl groups (SH) of DHP receptors, subsequently eliciting Ca^{2+} release from the SR. The major difficulty in interpreting these studies is that the H_2O_2 concentration in skeletal muscle is not known.

Systematic studies of rat diaphragm have shown that ECC is acutely altered by manipulating NO concentrations and that NO opposes the action of ROS, by inhibiting

ECC. Inhibitors of NO synthesis increase rat diaphragm twitch and submaximal tetanic force production, whereas NO depresses force production and increases fatigue (Kobzik et al. 1994). The results of Ca^{2+} flux measurements suggested that NO might inhibit the RyR, the principal Ca^{2+} release pathway in striated muscle (Mészáros et al. 1996). NO donors, such as S-nitroso-N-acetylpenicillamine (SNAP), L-arginine nitro-L-argine methylester-sensitive NO-synthase, were found to reduce the rate of Ca^{2+} release from isolated skeletal muscle SR and skeletal muscle homogenates in the rabbit (Mészáros et al. 1995).

2.1.5.8 RyR and calcium release in skeletal muscle

There is very little published literature on DHPR or RyR receptors in human skeletal muscle. Nitrendipine is an inhibitor of the DHPR channel (Smith et al. 1987). Desnuelle et al. (1986) analyzed the properties of nitrendipine receptors in skeletal muscle from normal young boys and in boys with Duchenne muscular dystrophy. Their results suggest that nitrendipine binding sites or nitrendipine-sensitive channel Ca^{2+} binding sites were not altered in Duchenne muscular dystrophy (Desnuelle et al. 1986). Lunde and Sejersted (1997) measured the concentration of [^{3}H] ryanodine binding sites in skeletal muscles in humans and in rats. The maximal binding to EDL and soleus muscles in the rat was 59.1 and 16.2 pmol.g^{-1} wet wt, whereas, in human gluteal muscles, the binding was only 12.3 pmol g^{-1} wet wt. Klitgaard et al. (1989) reported that the [^{3}H]-ryanodine binding site concentration in the vastus lateralis muscle was two-fold higher in a group of old healthy men compared to a group of young healthy men, but was reduced in the strength-trained group compared with old control group. The concentration was 0.025±0.01 and 0.050±0.05 pmol.mg^{-1} in the healthy young and old controls, respectively (Klitgaard et al. 1989) Valdivia et al. (1991) reported an abnormally high skeletal muscle [^{3}H] ryanodine receptor binding site concentration in humans with malignant hyperthermia. The concentration was 0.18±0.09 pmol.mg^{-1} in the healthy controls (Valdivia et al. 1991).

Ruell et al. (1995) reported $AgNO_3$-induced Ca^{2+} release in human skeletal muscle homogenates using Indo-1 after Ca^{2+} loading of the vesicles during the Ca^{2+}ATPase–mediated uptake process. O'Brien and Lee (1997), recently reported using indo-1 to measure muscle homogenate, Ca^{2+} release in large amounts (~g) of human muscle in the presence and absence of ryranodine and caffeine. Warmington (1997) induced calcium release using Ag^{+} and measured [Ca^{2+}] with Fura-2 after loading of the vesicles from muscle homogenate in rats during the uptake process in the fast-twitch (EDL) muscles. Caffeine has been shown to induce Ca^{2+} release from SR in skinned fibres and fragmented SR (Weber and Herz 1968, Stephenson 1981, Su and Hasselbach 1984). Actions of caffeine on fast- and slow- twitch muscle were different. A threshold caffeine concentration for an observable increase in force is detectable at about 0.5mmol.l^{-1} for soleus muscles and 5.0 mmol.l^{-1} for EDL muscle (Fryer et al. 1989). Thus SR in slow-twitch muscle is more sensitive to caffeine than in fast-twitch muscle (Fryer et al. 1989). Therefore, using caffeine to measure Ca^{2+} release may have some limitations when comparing individuals with a differing muscle fibre type.

2.1.6 Ca^{2+}ATPase Enzyme and SR Ca^{2+} Uptake

The ATP-driven calcium pump in the SR is also referred to as the Ca^{2+} ATPase enzyme (Toyoshima et al. 1993). In early the 1960s, the skeletal muscle SR ATPase was shown to have a calcium pump activity (Hasselbach and Makinose, 1962). They later suggested that a calcium pump was present in the SR membrane, and these removed Ca^{2+} from the cytoplasm during skeletal muscle relaxation (Hasselbach and Makinose, 1964). The Ca^{2+}ATPase enzyme was purified by Stewart and MacLennan in 1974, and was cloned by Brandl et al. (1986).

The Ca^{2+}ATPase enzyme accounts for about 80% of the integral membrane protein in the SR and occupies nearly half its surface area. The density of the pump in SR membrane is

about 25,000 per μm^2 (Stewart and MacLennan 1974). The SR lumen contains calsequestrin, a calcium-binding protein rich in carboxylate side chains, which serves to chelate and store calcium ions. Calsequestrin can bind up to 40 moles of Ca^{2+} per mole of protein. Consequently, the SR Ca^{2+} content may be as high as 10 mM (Stewart and MacLennan 1974).

The Ca^{2+}ATPase from sarco/endoplasmic reticulum (SERCA) is essential for the maintenance of a low cytosolic Ca^{2+} concentration. Three distinct genes encode SERCA isoforms. The SERCA 1 (1a and 1b) gene is expressed in fast- twitch skeletal muscle (MacLennan et al. 1985, 1997), whereas the the SERCA 2 gene gives rise to SERCA 2a and SERCA 2b isoforms by alternative splicing (Lytton et al. 1988, 1989). The SERCA 2a isoform is expressed in cardiac and slow-twitch skeletal muscle, while SERCA 2b is the dominant isoform found in the cerebellum (Lytton et al. 1987, Brandl et al. 1987, Miller et al. 1991); SERCA 3 is expressed in non-muscle tissue (Burk et al. 1989, Wu et al. 1995). The Ca^{2+}ATPase of the SR is also distributed in a fibre type specific manner (Pette and Staron 1997).

Ca^{2+} ATPase proteins in the SR belong to the P-type ion active pump, and have a very similar structure with the Ca^{2+} pump in the sarcolemma (Carafoli, 1991). The head region (H) contains a nucleotide-binding domain, phosphorylation domain, and a β sheet domain (Fig.2.7). The nucleotide-binding domain (which binds ATP) is a compact globular structure composed of one section of a polypeptide chain that constitutes a recognizable unit of the tertiary structure of a protein (Toyoshima et al. 1993). Domains may fold up independently and maintain their native conformation when the connecting sections of the chain are broken. A 25-Å stalk (S) joins the head to the transmembrane domain (M), which contains 10 helical segments that traverse the bilayer. Only a small part of the Ca^{2+} pump (L) is located in the lumen of the SR (Toyoshima et al. 1993).

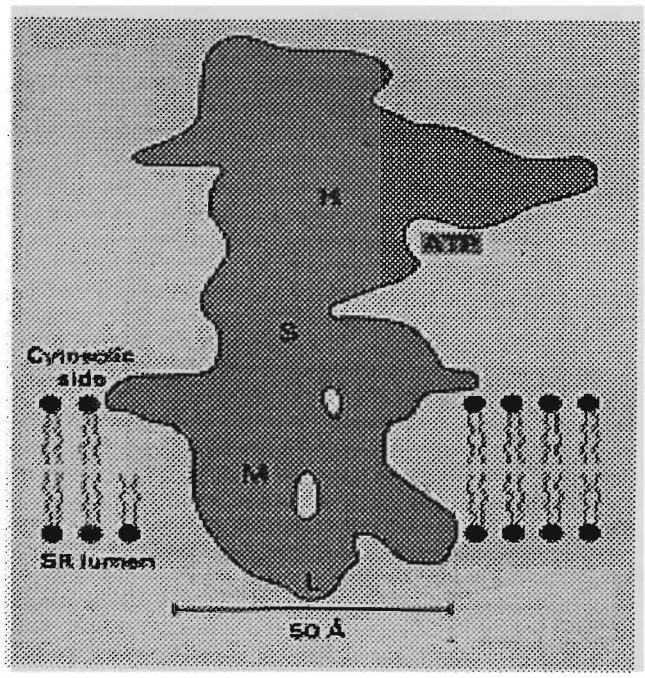


Figure. 2.7 Schematic diagram of the sarcoplasmic reticulum Ca^{2+}ATPase based on an electron crystallographic analysis at 14-Å resolution (Toyoshima et al. 1993).

A cycle of conformational changes driven by phosphorylation and dephosphorylation transports two Ca^{2+} ions for each ATP hydrolyzed by the SR pump (Toyoshima et al. 1993). The Ca^{2+} ATPase has two major conformational states, named E_1 and E_2 (Fig. 2.8). The process of Ca^{2+} uptake in the SR, initially, involves two Ca^{2+} ions binding to the Ca^{2+}ATPase (E_1) in the cytosolic side. This promotes the phosphorylation of a β-carboxylate of a specific aspartyl group on the enzyme by ATP. This causes a conformational change in the enzyme from the E_1 to the E_2 state. Two Ca^{2+} ions are then liberated inside the SR lumen, the acylphosphate is hydrolyzed in an exergonic reaction and the enzyme is converted back from the E_2 to the original E_1 state. This process can be reversed under condition of high concentrations of Ca^{2+}, ADP, and inorganic phosphate, resulting in the synthesis of ATP (Klug and Tibbits, 1988, MacLennan et al. 1997).

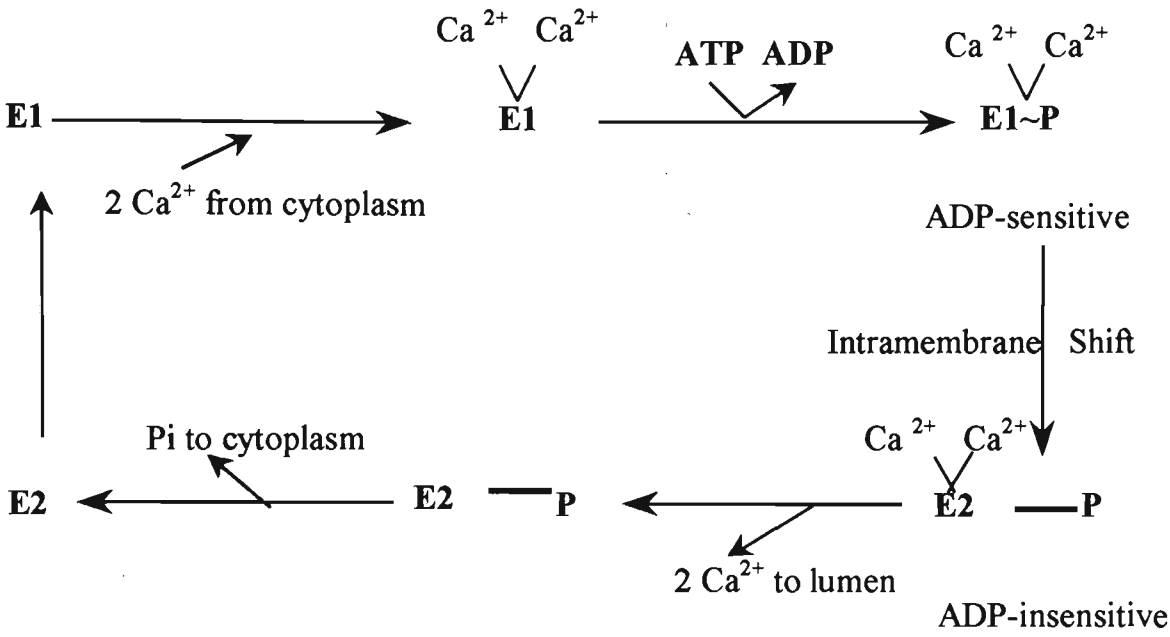


Figure 2.8 Schematic diagram of the mechanism of Ca^{2+} uptake by SR. Modified from Klug and Tibbits, (1988).

2.1.7 Factors Influencing SR Ca^{2+}ATPase Activity and Ca^{2+} Uptake

The capacity of the SR to sequester calcium is essential for the proper regulation of cytoplasmic calcium concentrations (Campbell et al. 1980, Lüttgau & Stephenson 1986, Laver & Curtis 1996a). The following section discusses some factors influencing SR Ca^{2+}ATPase activity and Ca^{2+} uptake.

2.1.7.1 Cytosolic Ca^{2+} concentration

The Ca^{2+} pump is active at the 0.1μM [Ca^{2+}] in resting muscle, and it reaches maximal activity at about 1 μM [Ca^{2+}] (Rüegg 1992). The increased extent of Ca^{2+} and carrier protein complex formation with a raised [Ca^{2+}] enhances Ca^{2+} uptake (Hasselbach and Makinose 1963, Weber et al. 1966, 1971). Both Mg^{2+} and ATP are essential for this process.

2.1.7.2 Mg^{2+}

Krause (1991) reported that an increased free [Mg^{2+}] in muscle can inhibit the SR Ca^{2+} uptake process by reducing the Ca^{2+} pump activity. Engelender and De-Meis (1996) studied the Ca^{2+}ATPase isoforms of sarco/endoplasmic reticulum (SERCA) derived from cerebellum, cardiac muscle, and skeletal muscle. The Mg^{2+} dependence varied among the three enzyme preparations. The Ca^{2+} transport was activated by free Mg^{2+} concentrations varying from 0.1 to 0.3 mM in skeletal muscle vesicles, but not in cerebellar or cardiac vesicles. Concentrations of Mg^{2+} in excess of 1 mM inhibited Ca^{2+} transport in all three vesicle preparations but with a more pronounced effect in cerebellar and cardiac vesicles.

2.1.7.3 Muscle energy supply and substrate depletion

ATP

It is well known that the process of Ca^{2+} uptake by SR requires ATP hydrolysis (see Figure 2.8). The pumping of 2 mol Ca^{2+} requires the hydrolysis of 1 mol ATP with an enthalpy of 46 kJ mol^{-1} (Lüttgau and Stephenson 1986). The intracellular [ATP] also effects SR Ca^{2+} ATPase activity and thus, Ca^{2+} uptake (Toyoshima et al. 1993). Further, as opposed to the average intracellular [ATP], the local ATP concentration may affect the Ca^{2+}uptake and Ca^{2+} ATPase activity (Han et al. 1992, Owen et al. 1996, Dawson et al. 1980).

Using mutations of Ca^{2+} ATPase to study Ca^{2+} and ATP binding

Mutation is a stable, heritable change in the nucleotide sequence of genetic nucleic acid, which typically results in the generation of a new allele and a new phenotype. The Ca^{2+} ATPase is predicted to contain ten trans-membrane α-helices, that are consistent with a variety of labeling and antibody binding sites. Mutations of Ca^{2+} ATPase can modify ATP binding and have been used for SR Ca^{2+} ATPase binding studies (Vilsen et al. 1991, Adams et al. 1998). Vilsen and Andersen (1992) have reported CrATP (gamma-bidentate

chromium III complex of ATP) induced Ca^{2+} occlusion in mutants of the Ca^{2+} -ATPase of SR. This provided a new approach to identification of amino acid residues involved in Ca^{2+} binding and in the closure of the gates to the Ca^{2+} binding pocket of the Ca^{2+} ATPase.

Glucolytic and Glycogenolytic metabolism

A glycogenolytic-SR complex has been demonstrated in rat skeletal muscle, this complexed SR accumulates Ca^{2+} upon stimulation of glycogen phosphorolysis in the absence of added ATP (Nogues et al. 1996). It is shown that an efficient Ca^{2+} uptake involves the sequential action of glycogen phosphorylase, phosphoglucomutase and hexokinase, which generate low concentrations of ATP (~ 1-2 μM) compartmentalized in the immediate vicinity of the SR Ca^{2+} ATPase. The Ca^{2+} uptake supported by glycogenolysis in this subcellular structure is strongly stimulated by micromolar concentrations of AMP, showing that the glycogen phosphorylase associated with this complex is in the dephosphorylated b form. The flux through this compartmentalized metabolic pathway should be enhanced in physiological conditions leading to increase AMP concentrations around the SR, such as long-lasting contractions and in ischemic muscle (Nogues et al. 1996). The mutant Gly-310-->Pro, previously classified as ADP-insensitive phosphoenzyme intermediate type (Andersen et al. 1992), was unable to occlude Ca^{2+}, even though Ca^{2+}-activated phosphorylation from MgATP took place in this mutant. Vilsen and Andersen (1992) have been reported CrATP-induced Ca^{2+} occlusion in mutants of the Ca^{2+} -ATPase of SR.

2.1.7.5 Free radicals inhibit SR Ca^{2+}ATPase and Ca^{2+}uptake

Free radical accumulation in muscle has been suggested as a possible cause of SR damage by lipid peroxidation (Davies et al. 1982). However, Scherer and Deamer (1986) have shown that a decrease in SR Ca^{2+}ATPase activity due to oxidation of SH group was not

due to lipid peroxidation. SR protein photooxidation may results in inhibition of Ca^{2+}ATPase activity and of Ca^{2+} uptake, and the oxidation involves histidyl and tryptophanyl residues (Yu et al. 1974). SR Ca^{2+}-ATPase activity was decreased by nitric oxide (NO) which may be elevated by SH groups oxidation (Byrd 1992). When SR microsomes were oxidized by exposure to the free radicals peroxydisulfate and hydrogen peroxide, Ca^{2+}ATPase activity and Ca^{2+} transport were decreased and the Ca^{2+} permeability were increased (Hess 1981, Kondo et al. 1994).

Castilho et al. (1996) have shown that oxidative stress induced by Fenton's reaction (Equation 2) alters SR function.

$$Fe^{2+} + H_2O_2 \rightarrow HO\bullet + OH^- + Fe^{3+} \qquad \text{Equation 2}$$

ATP hydrolysis by both SR vesicles and purified ATPase was inhibited in a dose-dependent manner in the presence of 0-1.5 mM H_2O_2 plus 50 μM Fe^{2+} and 6 mM ascorbate. Ca^{2+} uptake carried out by the Ca^{2+}ATPase in SR vesicles was also inhibited in parallel. The inhibition of ATP hydrolysis and Ca^{2+} uptake was not prevented by butylhydroxytoluene (BHT) at concentrations which significantly blocked formation of thiobarbituric acid-reactive substances (TBARS), suggesting that inhibition of the ATPase was not due to lipid peroxidation of the SR membrane. In addition, dithiothreitol (DTT) did not prevent inhibition of either ATPase activity or Ca^{2+} uptake, suggesting that inhibition was not related to oxidation of ATPase thiols. The passive efflux of $^{45}Ca^{2+}$ from pre-loaded SR vesicles was greatly increased by oxidative stress and this effect could be only partially be prevented (~ 20%) by addition of BHT or DTT. Trifluoperazine, which specifically binds to the Ca^{2+}ATPase, causing conformational changes in the enzyme fully protected the ATPase activity against oxidative damage. These results suggest that the alterations in function observed upon oxidation of SR vesicles are mainly due to direct effects on the Ca^{2+} ATPase (Castilho et al. 1996).

Xu et al. (1996) reported hydroxyl radical (•OH) (0.2 mmol/L Fe^{3+}-NTA+ 1 mmol/L H_2O_2) exposure completely inhibited Ca^{2+} ATPase activity and SR $^{45}Ca^{2+}$ uptake for both cardiac and skeletal muscles in rabbits. The •OH radical denatures the SR Ca^{2+}ATPase by directly attacking the ATP binding site.

2.1.8 Ca^{2+} -Binding Proteins

The regulation of Ca^{2+} within the cell involves additional proteins to the DHPR, RyR and Ca^{2+}ATPase. Important Ca^{2+}-binding proteins include calmodulin, parvalbumin and calsequestrin.

2.1.8.1 Calmodulin

Calmodulin is a binding protein with a molecular weight of 16.8 kD and contains four Ca^{2+}-binding domains. Once $Ca^{2+}$is bound by calmodulin, the calmodulin molecules can attach themselves to a wide variety of enzymes in the cytosol and thereby activate them. One of the enzymes activated by calmodulin is the Ca^{2+}ATPase in the surface membrane; by increasing the extrusion of Ca^{2+} from the cell, the enzymes complete a negative feedback loop across the membrane (Clarke et al. 1989).

2.1.8.2 Parvalbumin

Parvalbumin is a soluble, 12 kD Ca^{2+}-binding protein and contains two Ca^{2+}-binding pockets. Parvalbumin is present in high concentration (up to millimolar levels) in skeletal muscle fast -twitch fibres, but parvalbumin is virtually undetectable in slow- twitch fibres (Heizmann, et al. 1983; Gillis, 1985). The SR was able to remove Ca^{2+} bound to Parvalbumin (Blum, et al. 1977) and most of the Ca^{2+} released from the SR was localized in the cytoplasm possibly bound to it (Gillis, et al. 1979). Thus, parvalbumin is considered

as a relaxing factor. Parvalbumin concentration is very low in human muscle, but high in muscle of mouse and small animal.

2.1.8.3 Calsequestrin

Calsequestrin has a MW of approximately 40 -50 kD. It is found in highest concentrations in the TC of the SR and is attached to the part of the membrane facing the transverse tubules. A single molecule can capture ~970 nmol of Ca^{2+} and responsible for collecting Ca^{2+} ions in the SR when the fibre is not contracting (MacLennan and Wong 1971). When the muscle fibre becomes activated, the calsequestrin protein gives up its Ca^{2+} ions for release through the Ryanodine channels of the SR.

2.2 SR Function In Different Muscle Fibre Types

Skeletal muscle comprises several different muscle fibre types and these possess different physiological, biochemical, morphological and functional characteristics. These fibre types respond differently to training, fatigue, disuse and disease. Muscle fibres are dynamic structures capable of altering their molecular composition and contractile properties in response to altered functional demands (Guth and Yellin, 1971, Pette 1990). The following section reviews the different characteristics of muscle fibres, focussing on mammalian muscle and emphasises any differences in SR characteristics and function.

2.2.1 Skeletal Muscle Physiological and Morphological Characteristics

Skeletal muscle fibres can be classified into two main types on the basis of different contractile, biochemical and histochemical characteristics. These are referred to as Type I and Type II, which can be further subclassified into Type IIa, IIB and IIc (Brooke and Kaiser 1970, Saltin et al. 1977). Type IIa fibres are intermediate in most respects between the Type I and Type IIB fibres (Clamann and Broecker 1979, Brooke and Kaiser 1970, Bárány 1967, Saltin and Gollnick 1983, Thompson 1994, Stienen et al. 1996, Sahlin et al. 1998). The most commonly used histochemical technique utilizes differences in the acid /alkaline stability of myosin ATPase to differentiate between Type I, IIa and IIB fibres (Brooke and Kaiser 1970). The major contractile and metabolic differences between these types are shown in Table 2.2. Adapted from Saltin and Gollnick (1983); includes data from Stienen et al. (1996) (b), Sahlin et al. (1998) (a).

Table 2.2 Fibre type characteristics in skeletal muscle.

Characteristics	Type I	Type II	Type IIa	Type IIB
Contractile				
ATP expenditure (μmol/g wet wt) (b)	6.5		17.6	26.6
Myosin ATPase (mmol $L^{-1}s^{-1}$) (a)	0.10		0.27	0.41
Time to peak tension (ms)	80	30		
Substrate contents in human VL muscle				
ATP(μmol/g wet wt)	4.1	5.1	5.3	4.9
Creatine Phosphate (μmol/g wet wt)	12.6	14.7	14.5	14.8
Glycogen (Glucose units)	78	84.7	83.1	89.2
Triglycerides (μmol/g wet wt)	7.1	4.2		
Enzyme activities in human VL muscle				
Creatine kinase (mmol/min.g protein)	13.1	16.6		
Phosphorylase (mmol/kg.min)	2.8	7.3	5.8	8.8
Phosphofructokinase (mmol/kg.min)	7.5	15.4	13.7	17.5
Lactate dehydrogenase (mmol/kg.min)	59	257	221	293
Succinate dehydrogenase (mmol/kg.min)	7.1	4.6	4.8	2.5
Citrate synthase (mmol/kg.min)	10.8	7.5	8.6	6.5
3-Hydroxyacyl-CoA dehydrogenase (mmol/kg.min)	14.8	9.3	11.6	7.1

Using immunocytochemistry, it is possible to identify different molecular forms (isoforms) of key muscle proteins in skeletal muscle and the myosin protein has been extensively studied in recent years. Myosin heavy chains can reflects the rate of cross-bridge reaction with the actin filaments and hence the speed of muscle shortening (Reiser et al. 1985). The myosin heavy chains sensitive to the pH of the ATPase reaction are

responsible for the depth of histochemical staining at the various pH in the procedure of Brooke and Kaiser (1970b). There are at least four major heavy chain isoforms and seven light chain isoforms in adult mammalian muscle (Staron & Pette, 1990). Human muscle fibres express three pure myosin heavy chain isoforms (MHCI, MHCIIA, MHCIIB) (Billeter et al. 1981). These myosin heavy chains isoforms correspond to I, IIa, IIB fibre types classified histochemically, and the IIC fibres contain both the MHCI and MHCII isoforms (Table 2.3).

The other isoform may be responsible for distinguishing a further subtype of the type II fibre (IIX or IID) (Bär and Pette, 1988, Schiaffino et al. 1990). Only two fast MHC isoforms have been identified in adult human limb and trunk musculature, initially identified as MHCIIB and MHCIIA. However, the human MHCIIB isoform is homologous to the rat MHCIID/X (Smerdu et al. 1994, Ennion et al. 1995).

Table 2.3 Myosin heavy chain content of human muscle fibres. Adapted from Billeter et al. (1981).

DESIGNATION	FIBRE TYPE (Histochemical)	MYOSIN HEAVY CHAINS (Immunocytochemistry)
Slow-twitch	Type I	MHCI
Fast-twitch	Type IIA	MHCIIA
Fast-twitch	Type IIB	MHCIIB
Fast-twitch & slow-twitch	Type IIC	MHCI + MHCIIA

2.2.1 SR Ca^{2+} Content in Different fibre Typres

A study in resting skinned skeletal muscle fibres demonstrated that the steady-state SR Ca^{2+} content of FT fibres was 1.32mM which was only one-third of the maximal saturated, whereas the SR Ca^{2+} content in ST fibre was totally saturated at 1.35mM (Fryer and

Stephenson 1996). The ability to increase the SR Ca^{2+} content was at least 3-fold greater in Type II fibres, consistent with their greater calsequestrin content compared with ST fibres (Fryer and Stephenson 1996). It is well accepted that a larger volume of the SR (9%) is contained in fast-twitch fibres compared with slow-twitch fibres (4.5%) (Eisenberg 1983, Feher et al. 1988).

2.2.2 DHPR and RyR in Different Fibre Types

Type II fibres also contain a greater number of DHP receptors than Type I fibres (Franzini-Armstrong et al. 1988). For example, there is a greater level of DHPR in EDL than SOL in rabbit (Lamb & Walsh 1987), with nearly 2.4 fold higher DHPR levels in rat EDL than in SOL (Saborido et al. 1995). Type I fibres also contain 2-4 fold lower number of calcium release channels (Appelt et al. 1989) and a exhibit lower calcium current in T-tubule (Lamb & Walsh 1987). These differences are indicated in Table 2.4.

Table 2.4. Comparative measurements of transverse tubule DHPR and SR RyR in muscles comprising mainly Type I (slow-twitch) and Type II fibres (fast-twitch).

Component	Species	Slow-twitch	Fast-twitch	Reference
DHP binding	Rabbit	22	69	Lamb & Walsh (1987)
	Rat	9	22	Saborido, et al. (1995)
DHP binding	Guinea-pig & Rat	(24-27)	(79-91)	Franzini-Armstrong et al. (1988)
RyR		12-14	27-33	Appelt et al. (1989)
RyR	Rat	(15)	(36)	Schwartz (1985) & Lamb and Walsh (1987)
Feet	Rat	86-126	172-204	Cullen et al. (1984)

Units are pmol g^{-1} muscle; number per μm^2 of surface and t-system membrane shown in brackets. Adapted from Lamb (1992).

2.2.3 *Ca^{2+} Uptake and Ca^{2+}ATPase Activity in Different Fibre Types*

The rates of SR Ca^{2+} uptake and Ca^{2+}ATPase activity differ in ST and FT fibres of mammalian muscle. The major differences that exist between the rate of relaxation in different muscles suggest that their SR may vary with regard to its capacity to sequester Ca^{2+}. Immunohistochemical studies on rat and rabbit muscles have demonstrated markedly higher Ca^{2+}ATPase contents in fast compared with slow fibres (Jorgensen et al. 1979, Maier et al. 1986, Krenacs et al. 1989, Benders et al.1992). In addition, some proteins related to the Ca^{2+} regulatory system are expressed specifically in fast and slow muscles (Table 2.5).

Table 2.5 Distribution of Ca^{2+} regulatory proteins and isoforms in extrafusal muscle fibres of small mammals. From Pette and Staron (1997).

	Slow-twitch	**Fast-twitch**
SR Ca^{2+}ATPase	SERCA2a	SERCA1a
Calsequestrin	CaS_{fast} + CaS_{card}	CaS_{fast}
Calreticulin	Low amount	High amount
Phospholamban	Yes	No
Parvalbumin	Extremely low	High (IIB>IID/X>IIA)

On the basis of the fibre type composition of vastus lateralis and SOL being 60% and 20% fast- twitch fibres, respectively, it was deduced that Type II fibres have 4-6 fold greater rates of SR Ca^{2+} uptake and Ca^{2+}ATPase activity in human muscle (Benders et al. 1994, Kim et al. 1982, Feher et al. 1988,). The differences between animal and human skeletal muscles are indicated in Table 2.6.

Table 2.6. SR Ca^{2+}uptake and Ca^{2+} ATPase activity in different muscle fibre types.

Measurement	Species	Method	Muscle			Reference
			Slow	Mixed	Fast	
Ca^{2+}uptake	Rat	Millipore	1.29 SOL	3.68 VL	5.35 SVL	1. Kim et al. (1982)
	Guinea-pig	Millipore	3.8 SOL	7.5 WVL		2. Fiehn & Peter (1971)
	Rat	Homog. ^{45}Ca	33 SOL		124 EDL	3. Feher et al. (1988)
	Rat	Homog. Fura-2	97 SOL		197 EDL	4. Warmington (1997)
	Human	Homog. Indo-1		10.42 VL		5. Booth et al. (1997)
	Human	Homog. Indo-1		1.70 VL		6. Ruell et al. (1995)
Ca^{2+} ATPase activity	Rat		0.14 SOL	0.58 VL	0.95 SVL	7. Kim et al. (1982)
	Rat	Homog.		74.3 VL		6. Ruell et al. (1995)
	Rat	MFI	0.33 SOL		5.18 EDL	7. Saborido et al. (1995)
	Human	Homog.		41.4 VL		11.Benders et al.(1993)
	Human	Homog.		15.3 VL		6. Ruell et al. (1995)
	Human	Homog.		88.8 VL		8. Booth et al. (1997)
Densities	Guinea-pig	MVI	32000SOL	25000VL		9. Ferguson et al. (1988)
Concentration	Human	Homog.		6.74		10. Madsen et al.(1994)

Abbreviations: Homog. Homogenate, MVI microsomal vesicles isolated, MFI microsomal fractions isolated, Muscles: SOL Soleus, VL vastus lateralis, WVL white vastus lateralis. References and Unit: 1. $\mu mol.mg^{-1}$; 2. 7.$\mu mol\ .min^{-1}\ mg^{-1}$protein;. 4. $nM.min^{-1}\ mg^{-1}$; 5. 8.$nmol.min^{-1}\ mg^{-1}$protein; 3. 6. $\mu mol.min^{-1}\ g^{-1}$; 9. μm^2; 10, nmol g wet wt^{-1}; 11. munits/mg of protein.

2.3 Role of the SR in Muscle Fatigue

2.3.1 Overview of Fatigue

Muscle fatigue is defined as an inability to maintain the required or expected force or power output, resulting from muscle activity under load and which is reversible by rest (Edwards 1981, NHLBI 1990). Fatigue is characterised by reductions in maximal force and the peak rate of force development and by a marked slowing of muscular relaxation (Vøllestad et al. 1988). Muscle fatigue is a complex phenomenon, and no single mechanism can account for all of these components of muscular fatigue (Westerblad et al. 1991, McKenna 1992, Fitts, 1994, Allen et al. 1995, Stephenson et al. 1995). Fatigue also depends on an individual state of fitness, muscle fibre type composition and the type of activity that the person is performing. The mechanisms responsible for fatigue are also likely to be highly specific, based on the exercise intensity and duration.

2.3.2 Sites of Fatigue

Muscle fatigue can be divided into central and peripheral components. Central fatigue is related to events of neural input to the higher brain centers, recruitment of the alpha motor neurone pool, and the alpha motor nerves themselves. Recently, the corticospinal output to muscle during maximal voluntary contractions, and during fatigue have been studied by the transcranial magnetic stimulation technique (Gandevia et al. 1990). Using this technique, Gandevia et al (1995) demonstrated a progressive reduction in voluntary drive to motoneurones during isometric contractions, therefore indicating central fatigue. This confirms earlier measurements of central fatigue using the interpolated twitch technique during repeated isometric contractions (McKenzie et al. 1992). However, during voluntary isokinetic contraction of knee extensors, James et al. (1995), found central fatigue only contributed to a small effect (< 1/5) to the total loss of power elicited by electrical

stimulation in a human leg muscle. Thus the major proportion of fatigue occurs at peripheral sites. These may include the activation of the sarcolemma and T-Tubular membranes, the activation of the voltage sensor, induction of SR Ca^{2+} release, activation and inactivation of the contractile elements, and at various sites in the metabolic pathways. The following section details the possible role of SR dysfunction in skeletal muscle fatigue and examines the molecular mechanisms involved in skeletal muscle fatigue.

2.3.3 Types of Muscular Fatigue

Recently, Allen et al. (1998) and Lamb (1998) reviewed fatigue mechanisms in skeletal muscle using single, intact fibre and skinned muscle fibre models and based their discussion of fatigue on three categories: T-tubular fatigue, metabolic fatigue and long lasting fatigue. These three categories are briefly outlined in the following section, with detailed discussion of ionic and metabolic effects on SR function presented in a later section.

2.3.3.1 T-tubular (high frequency) fatigue

T-tubular fatigue is also termed high frequency fatigue, or T-tubular failure. This is characterised by a rapid decline in force during continuous stimulation at high frequency, followed by rapid recovery of force within only several seconds of cessation of stimulation (Jones 1996). A progressive depolarisation of the T-system seems the logical cause of this type of fatigue. Recently Clausen et al. (1998) reported that during high frequency stimulation, virtually all Na^+-K^+ pump into action within a few seconds. Despite this muscle cells lose K^+ and gain intracellular [Na^+], suggesting that the sarcolemmal Na^+-K^+ pump is unable to maintain the ionic gradients for K^+ and Na^+ (Bigland-Ritchie et al. 1979, Jones et al. 1979, Clausen et al. 1998, Nielsen and Harrison 1998).

2.3.3.2 Metabolic fatigue (intermittent)

Fatigue due to metabolic failure occurs during repeated, intermittent stimulation and force declines in several distinct phases, at least in single fibres. This timing of these phases is dependent on the fibre type and force fully recovers again over 10 minutes (Allen et al. 1995). In the final phase force declines quite rapidly because depolarisation-induced Ca^{2+} release is reduced (Allen et al. 1995, Nagesser et al. 1992, Westerblad 1998b). There are marked changes taking place in the chemical composition of the muscle fibre cytoplasm, each of which are likely to exert some regulatory role on muscle SR function, and these are detailed in a later section.

2.3.3.3 Long -lasting fatigue.

Long -lasting fatigue, also called "long- lasting failure of Ca^{2+} release"or low frequency fatigue occurs following prolonged low frequency stimulation and the muscle fibre is unable to produce the original level of force until a day or more later (Allen et al. 1998). The potential reason in this type of fatigue may link to a complete failure of T-tubular DHPR activation or to reduce SR Ca^{2+} release as a result from a short periods of elevated cytosolic $[Ca^{2+}]$. For example, exposure of skinned muscle fibres to 10-23μM $[Ca^{2+}]$ for 10 seconds at 23°C abolished depolarization-induced force responses in muscle fibres of the toad and rat (Lamb et al. 1995). This also occurred in parallel with structural changes at the triad junction that were detectable with electron microscopy (Lamb et al. 1995). Chin and Allen (1996) induced infrequent tetanii in single muscle fibres, which alone did not cause fatigue. However, when the tetanic $[Ca^{2+}]$ was increased with caffeine and 2,5-di (terbutyl)-1,4-benzohydroquinone (TBQ), calcium pumps inhibitor during stimulation, this induced long-lasting fatigue.

2.3.4 SR Ca^{2+} Regulation and Muscle Fatigue

Impairment of excitation-contraction coupling is now widely regarded as a major causative factor in skeletal muscle fatigue, consistent with the central role of Ca^{2+} in muscle contractile function (Fitts and Metzger 1988, Vøllestad and Sejersted 1988, Westerblad et al. 1990). Decreases in the amount of Ca^{2+} released and the rate of Ca^{2+} uptake by SR would theoretically reduce force development and prolong relaxation, respectively (Sembrowich et al. 1977, Dawson et al. 1980).

Numerous different experimental approaches have investigated the role of SR Ca^{2+} regulation in muscle fatigue. For simplicity, these experimental approaches have been divided into two sections in this review. The first section examines those experiments utilising single intact or skinned muscle fibres in animal studies. These models have the advantage that mechanisms of fatigue might be studied in great detail, by manipulating a single variable at a time. The second section examines those experiments using muscle homogenate techniques, in whole body exercise studies using animals or humans. These studies are important since they allow investigation of mechanisms of fatigue during whole body exercise, when multitudes of changes are occurring.

2.3.4.1 Single muscle fibre models in animal studies

Skeletal muscle Ca^{2+} regulation during muscle fatigue has been investigated using intact, living single fibres (Allen et al. 1989, Lee et al. 1991), and isolated, skinned single fibres (Lamb and Stephenson 1991, Nosek et al. 1987, Godt and Nosek 1989). These methods allow direct observation of the main features of muscle fatigue under a single condition. Thus, the importance of discrete events in fatigue can be directly tested. In the intact single fibre, Ca^{2+} indicator dyes are used to assess the myoplasmic $[Ca^{2+}]$ and can therefore directly indicate the extent and time course of the release, binding and re-uptake of Ca^{2+} within the fibres (Allen et al. 1989). The skinned muscle fibre, in which the

surface membrane of muscle is chemically or mechanically removed to expose the contractile proteins, uses measurements of muscle force to examine the role of SR Ca^{2+} regulation and fatigue (Owen et al. 1997).

Allen et al. (1992) proposed that there are three possible mechanisms leading to fatigue involving free intracellular [Ca^{2+}]. These were: 1). During fatigue Ca^{2+} release might decline, leading to reduced intracellular [Ca^{2+}] and thereby muscle tension; 2). The sensitivity of the contractile proteins to Ca^{2+} might decline; and 3). The maximal Ca^{2+}-activated tension is reduced.

Allen et al. (1989) measured the aequorin light signal to indicate myoplasmic $[Ca^{2+}]_i$ in single muscle fibres from Xenopus lumbrical muscles during fatigue. They showed that muscle fibre fatigue produced a steady decline in tetanic tension and a slowing of relaxation. These were associated with an initial increase and then a marked decline in $[Ca^{2+}]_i$, as well as a slowing of the decrease in $[Ca^{2+}]_i$ after each tetanus. They suggested that fatigue in their single fibre had two stages, classified as early fatigue and late fatigue. The early reduction in tetanic force with a significant increase in tetanic [Ca^{2+}] occurred in Xenopus and in mouse fibres, and was probably caused by reduced SR Ca^{2+} uptake. The latter rapid decline in tetanic [Ca^{2+}] and muscle force, may be due to a severe decline in SR Ca^{2+} release (Westerblad and Allen 1989, 1991, 1993a, 1993b, 1996b). Micro-injection of Fura-2 to measure $[Ca^{2+}]_i$ also demonstrated that the reduced tension in Xenopus muscle fibres with fatigue was due to reduced tetanic $[Ca^{2+}]_i$ and reduced sensitivity of the contractile elements (Troponin C) to Ca^{2+} (Lee et al. 1991). The reduction in maximal force generating capacity develops early during fatigue and is probably related to cellular metabolic changes (Westerblad and Allen 1991). Thus each of the three mechanisms proposed by Allen et al. (1989) to contribute to fatigue have been confirmed. Muscle fatigue in single fibres is also characterised by a reduced decline in post-tetanic [Ca^{2+}], reflecting a reduced rate of Ca^{2+} removal by the SR (Allen et al. 1989

Westerblad and Allen 1991, Westerblad et al. 1997b). This has been suggested to be one factor contributing to the slowing of muscle relaxation evident with fatigue (Allen et al. 1989, Dawson et al. 1980, de Haan et al. 1989). However, no relationship was found between the depression in SR Ca^{2+} uptake and the slowing of relaxation with fatigue, in either mouse (Westerblad and Allen 1993), or in human muscles (Booth et al. 1997). This suggests that the slowing of relaxation in fatigue result from a reduced rate of cross bridge detachment.

2.3.4.2 Muscle homogenate studies in animal and human exercise induced fatigue

Several recent studies have investigated the effects of fatigue on muscle SR function in animals and humans, using crude muscle homogenates (Byrd et al, 1989a, 1989b, Gollnick et al. 1991, Favero et al. 1993, Chin et al. 1995, 1996, Booth et al. 1997, Hargreaves et al. 1998). Details of these experiments and their findings are shown for animals and humans, in Tables 2.7 and 2.8, respectively. Using this preparation it is also possible to examine the relationship between the *in vitro* SR Ca^{2+} ATPase activity and Ca^{2+} uptake measured in whole muscle homogenates, with the *in vivo* muscle contractile function or performance (Fitts et al. 1982, Booth et al. 1997).

Animal Studies.

Bonner et al. (1976) first investigated the effects of fatigue on SR Ca^{2+} uptake, finding no significant changes after treadmill running. Fitts et al. (1982) studied alterations in the contractile and biochemical properties of fast- and slow- twitch skeletal muscle in rats following a prolonged swim to exhaustion. The isometric contractile properties were altered in the soleus (~84% Type I fibres) and the EDL muscle (~60% Type IIa fibres), but not in the superficial region of the vastus lateralis (SVL, 100% Type IIB fibres). Peak tetanic tension (Po) and the rate of tension development were decreased after prolonged exercise in both the SOL and the EDL. Furthermore, the Ca^{2+} sensitivity of the myofibrils was unaffected by exercise in both fast and slow muscle. The Ca^{2+} uptake capacity of the

SR was reduced in both the SOL and the fast-twitch type IIa deep region of the vastus lateralis, whereas the SR Ca^{2+} ATPase activity was unchanged. The uncoupling of Ca^{2+} uptake and Ca^{2+} ATPase activity did not depend on muscle fibre type.

Byrd et al. (1989b) examined the effects of acute high-intensity exercise on the rate and capacity of SR Ca^{2+} uptake and Ca^{2+} ATPase activity and the reversibility of these effects. Thoroughbred horses were run at maximal O_2 uptake on a high-speed treadmill until fatigued. Muscle temperatures and biopsy samples were collected at rest, immediately after exercise, and at 30 and 60 min after exercise. Muscle and blood [Lac-] were three to five-fold greater than pre-exercise values. Muscle temperature and muscle homogenate pH immediately post-exercise were 43°C and 6.55, respectively. The initial rate and maximal capacity of Ca^{2+} uptake of muscle homogenates and isolated sarcoplasmic reticulum were significantly depressed immediately after exercise. However, Ca^{2+} uptake and Ca^{2+} ATPase activity, as well as muscle temperature and pH had returned to normal by 60 min after exercise. These findings suggest that changes in SR function after high-intensity exercise may be induced, but not sustained by, local changes in muscle pH and/or temperature. Also McCutcheon (1992) have similar findings in the middle gluteal muscle after fatiguing exercise in horses.

SR isolated from the red gastrocnemius muscle of rats after a single bout of prolonged exercise was shown to have depressed Ca^{2+} ATPase activity over a temperature range of 15 to 42.5°C, when compared to SR obtained from control muscle (Luckin et al. 1991). FITC is a fluorescent molecule that has been shown to bind specifically to the ATP binding site of SR Ca^{2+} ATPase enzyme (Luckin et al. 1991). SR vesicles prepared from exercised muscle showed a 40% reduction in the ATP- binding capacity, as shown by reduced fluorescein isothiocyanate, with no apparent change in Kd. These results indicate that a single bout of exercise induces a structural change in the Ca^{2+}ATPase protein of rat

muscle but this is not a direct result of gross lipid alterations or increased muscle temperature.

Chin et al. (1996) hypothesized that the differences in SR Ca^{2+} ATPase activity and Ca^{2+} uptake in SR fractions observed in different studies are due to different SR isolation techniques. To investigate this possibility, rat white and red gastrocnemius muscles from control and run animals were studied by using two conventional isolation techniques to obtain a crude microsomal fraction and an isolated SR vesicle (SRV) fraction. These were compared with measurements from whole muscle homogenates. Treadmill running to exhaustion did not alter SR protein yields, percent SR extraction, or basal or Ca^{2+}ATPase purification in either fraction. SR Ca^{2+} ATPase activity was not altered by exercise in any of the fractions examined, but Ca^{2+} uptake was reduced by 27% and 40% in the homogenates and SRV fractions, respectively from the red gastrocnemius at free [Ca^{2+}] of 600-700 nM. These data indicate that reductions in SR Ca^{2+} uptake are dissociated from changes in Ca^{2+}ATPase *in vitro* and occur only in a specific population of vesicles. The mechanisms may involve a reduction in the number of Ca^{2+}ATPase enzymes or a selective sedimentation of damaged vesicles in the SRV fraction.

The reasons underlying the large variability found in various studies in rats remains unclear. However, most studies have shown reduced SR Ca^{2+} uptake and SR Ca^{2+} ATPase activity with fatigue and coupling between these two changes (Tables 2.7 and 2.8). These are most likely explained by structural alterations to the Ca^{2+} ATPase with fatigue, which may be induced by increases in the concentration of free radical species in fatigued muscle, elevations in temperature and/or metabolic perturbations, as discussed later.

Human Studies

In human studies, fatigue induced by intense knee extensor exercise reduced vastus lateralis homogenate Ca^{2+} uptake by 42% (Table 2.8) and this was associated with a two-

fold prolongation of twitch 1/2 RT (Gollnick et al. 1991). With prolonged cycling exercise to fatigue, both SR Ca^{2+} uptake and Ca^{2+} ATPase activity were reduced by 17% and 21% respectively, but without prolongation of twitch or tetanic 1/2 RT (Booth et al. 1997). In that study, muscle ATP, CP, glycogen and pH were significantly reduced and lactate significantly elevated at fatigue. By 60 min post-exercise all but muscle glycogen had returned to resting levels, but SR Ca^{2+} uptake and Ca^{2+}ATPase remained depressed. In one subject, both SR Ca^{2+} uptake and Ca^{2+}ATPase were still depressed 6 hr after exercise. Under physiological conditions it is more likely that a more permanent physical alteration in the SR Ca^{2+} ATPase or its environment is responsible for the decreased Ca^{2+} uptake, rather than some metabolic factors. These findings and the reduction in maximal contractile force suggest that structural changes to the Ca^{2+} ATPase enzyme, and possibly to the RyR, are possible mechanisms contributing to long -lasting fatigue in human muscle (Booth et al. 1997). More recently, Hargreaves et al. (1998) found that a reduction in peak power develops during fatigue in high intensity exercise along with reduced Ca^{2+} uptake (28%), ATP, CP, Glycogen and pH and lactate, IMP and creatine. The mechanisms for impaired SR Ca^{2+} uptake was not discussed but it is likely that impaired SR function, along with the metabolic alteration results contributed to the reduced exercise performance during fatiguing exercise (Hargreaves et al. 1998). Warmington (1997) found that the rate of SR Ca^{2+} uptake was significantly depressed in 8 subjects after exercise at work rates corresponding to 140% $\dot{V}O2_{peak}$ (1.4 ± 0.2 min) and 72% (111.4 ± 20.6 min), by 65% and 68%, respectively. These most likely result from structural alterations to the Ca^{2+} ATPase enzyme, although the mechanisms responsible for this remain unclear. However, it is possible that changes to membrane lipids and accompanying proteins also may play a role during fatigue.

In conclusion, most studies with humans show a decreased *in-vitro* SR Ca^{2+} ATPase and Ca^{2+} uptake during fatigue. These most likely from structural alterations to the Ca^{2+} ATPase enzyme, although the mechanisms responsible for this remain unclear. Other membrane lipids and accompanying proteins also may play a role during fatigue.

SR Ca^{2+} release and fatigue

Despite the vital role of SR Ca^{2+} release in muscle contractile function, only one paper has investigated the effects of fatigue on this variable using the homogenate technique. The rate of Ag^{+}-induced Ca^{2+} release was investigated in SR vesicles isolated from rat muscles following prolonged running exercise (Favero et al. 1993). They reported a 20-30% decrease in the rate of Ca^{2+} release with fatigue. There have been no previous reports on the effects of fatiguing exercise on the rate of Ca^{2+} release in SR vesicles from human skeletal muscle. This is clearly worthy of investigation.

Table 2.7. Effects of exercise on sarcoplasmic reticulum reticulum function in animals#.

Ref	N	Mode	Fatigue protocol	Duration (min)	Muscle	Prep	Ca^{2+}ATP-ase Assay $[Ca^{2+}]$ (μM)	Ca^{2+}ATP-ase Activity (a)	Ca^{2+}Uptake method	Ca^{2+}Uptake Start $[Ca^{2+}]$ (μM)	Ca^{2+}Uptake Change (b)	U/C a & b
1	5/5	TM	Increm→53.6m/ min, then→10%	Utr~8 Tr~13	G,SO, PL,BF	CM	2.5mM	ns, ns	Ox., $^{45}Ca^{2+}$	Total= 330	ns↑, ns↑	No
2	5	TM	25m/min, 10%	55-67	G	SRV	0.75,1.25 2.5, 5	ns, ↓33% ~↓25,39%	Pi, Ox			
3	6-10	Swim	2%BW-tail	420	SOL	SRV	Total 20	ns	Ox,$^{45}Ca^{2+}$	NR	ns	No
					EDL			ns			ns	No
					SVL			ns			ns	No
					DVL			ns			*↓ 26%	Yes
4	6-10	TM	21m/min 10%	20;45;140	SG+V	HOM				1.26	Ns, ns, ns	NR
					DG+V						ns,↓48,↓52%	
					SG+V	CM		ns, ns,ns	Electrode		ns,ns,ns	No
					DG+V		0.2-2.0	↓52,55,45%	Ox.		ns;↓40,↓72%	No
5	8	TM	NR(Horse)	4.65	GLUT	HOM			Electrode Ox	0.65	↓45%; +60min ns	
6	>5	TM	21/min ,10%	105	RG	SRV	0.8-2.0	↓40%@37 C				
7		TM	70-75%VO_2		G	SRV	10, 1, 0.1	2min ↓13%, 15min↓118 % 45min↓18%	Ox., $^{45}Ca^{2+}$		ns	Yes

8	10	TM	Intermittent×2.5 min@52m/min	15-20	WG	HOM		ns	Indo-1,Ox.	0.25-	ns	No
					RG			ns		.35	ns	No
					SOL			ns			ns	No
9	9-11	Stim.	Intermittent10Hz	90	RG	HOM	10	ns	Indo-1,Ox.	0.48-	ns	No
					WG			ns		.51	ns	No
					RG	CM	100	ns			↓ at 0.3-0.5	Yes
					WG			ns		0.56-.60	ns	No
10	12	TM	8%, 1h each @21,25,28m/min	100	WG	HOM		ns	Indo-1,Ox.	0.1-0.8	Pooled↓at	Yes
					RG			ns			600,700nM	Yes
	10			141	WG	CM	pCa 3-8	ns			ns	No
					RG			ns			ns	No
	10			141	WG	SRV		ns			Ns	No
					RG			ns			↓at~700nM	Yes

All studies used rats except #5 (Horse), N number; U/C uncoupling, between activity and uptake; NR not reported, TM treadmill running, HOM homogenate, CM and SRV isolated sarcoplasmic reticulum vesicles. Muscles: RG/WG red/white gastrocnemius, PL plantaris, SOL soleus, BF, biceps lateralis, N number, GLUT gluteal, S/DG+V superficial/deep gastrocnemius and vastus, Pi phosphate production measure, Ox. Oxalate, Electr. Calcium sensitive microelectrode. Significance:* $P<0.05$,

References. 1. Bonner et al. (1976); 2. Belcastro et al. (1981); 3. Fitts et al. (1982); 4. Byrd et al. (1989a); 5. Byrd et al. (1989b); 6. Luckin et al. (1991); 7. Belcastro et al. (1993); 8. Dossett-Mercer et al. (1995); 9. Chin et al. (1995); 10. Chin and Green (1996).

Table 2.8. Effects of exercise on muscle sarcoplasmic reticulum reticulum function in humans.

Ref.	N	Mode	Fatigue protocol	Dura -tion min	Ca^{2+}ATPase		Ca^{2+}Uptake Rate			Unco-upling
					Assay $[Ca^{2+}]$	Activity (a)	method	Start $[Ca^{2+}]$	Change (b)	a & b
1	5	One leg Kick	1Hz, load NR	2.8	NR	NR	Oxalate, Electr.	NR	↓42%*; +30min↓7%	NR
2	10	Cycle	~75% $\dot{V}O_2$max	72	10μM	Exh. ↓17% +20min↓22	Oxalate, Indo-1	~1μM	Exh.↓21%*+20min↓ 28*	Yes
3	5	Cycle	140% $\dot{V}O_2$max	1.4	NR	NR	Oxalate, Fura-2	NR	Exh.↓65%*	NR
3		Cycle	70% $\dot{V}O_2$max	111	NR	NR	"	NR	Exh.↓68%*	NR
4	6	Cycle	2×30s, 4 min rest		NR	NR	"	~1μM	↓28%*	NR

Homogenates of vastus lateralis were used at all study; NR not reported; N number, VL, Electr. Calcium sensitive microelectrode, Exh. Exhaustion,* $P<0.05$, R references. **References:** 1.Gollnick, et al. (1991), 2. Booth, et al. (1997). 3. Warmington. (1997). 4. Hargreaves et al. (1998).

2.3.5 *Effects of Metabolic and Ionic Disturbances with Fatiguing Exercise on Skeletal Muscle SR Function*

ATP

It is known that local ATP activates the SR Ca^{2+} release channel in skeletal muscle (Smith et al. 1985, Han et al. 1992, Meissner 1994, Owen et al. 1996). Owen et al. (1996) reported that a reduction in total [ATP] from 8 to 2 mM did not affect depolarisation-induced Ca^{2+} release in amphibian muscle fibres. However, when the total [ATP] was only 0.5 mM, depolarisation-induced Ca^{2+} release was significantly reduced. Thus millimolar [ATP] activates SR Ca^{2+} release, but micromolar [ATP] does not activate SR Ca^{2+} release. It is often thought that low [ATP] could not be responsible for the decline in SR Ca^{2+} release and muscle fatigue because the total cellular ATP only drops by 30-70% (~2-4mM) in extreme fatigue (Nagesser et al. 1993, Fitts 1994). However, in such a region of high ATP usage, it seems likely that the [ATP] will drop considerably below the bulk level in the myoplasm (Lamb 1998). The [ATP] in the fibre does not indicate what happens in local regions of high ATP utilisation, such as those which have very high local density of ATPase enzymes such as the Ca^{2+} ATPase and Na^+ K^+ ATPase. In addition, a reduction in [ATP] causes raised [Mg^{2+}], which will cause further inhibition of Ca^{2+} release in skeletal muscle (Lamb et al. 1991, 1994, Westerblad and Allen 1992, Lamb and Stephenson 1996). Thus, the combined effects of reduced local [ATP] and raised [Mg^{2+}] can inhibit RyR activation.

Allen et al. (1997) tested the role of ATP in the regulation of SR Ca^{2+} release and fatigue in single mouse skeletal muscle fibres. Intracellular release of ATP was induced by ultraviolet (UV) illumination of a caged ATP compound, during the final stage of a fatiguing contraction. Release of ATP resulted in an increase in cytosolic [Ca^{2+}] and the partial restoration of force in the fatigued fibre. Their results suggest that with increased ATP turnover during fatiguing contractions, local ATP may be reduced in the triadic

clefts, resulting in inhibition of SR Ca^{2+} release, Ca^{2+}ATPase, Ca^{2+} uptake and a consequent force decline.

The CK-PCr system is directly linked to Ca^{2+} flux regulation during the excitation and relaxation phases of muscle contraction. The mutation analyses provide a unique model to examine altered Ca^{2+} homeostasis. Steeghs et al. (1997) demonstrated that blocking creatine kinase (CK) - mediated PCr, inhibited ATP transphosphorylation in skeletal muscle of mutant mice. They found impaired tetanic force output, increased relaxation times and pathological structure in SR when striated muscle was expressed without CK. Also, absence of CK influenced both Ca^{2+}release and Ca^{2+} reuptake in depolarized cultured myotubes. The changes in SR function and morphology could explain the impaired force development. Therefore, CK-PCr system may play a role in the attainted to regulation of Ca^{2+} in ECC.

ATP and Ca^{2+} ATPase activity

It is well known that the process of Ca^{2+} uptake by SR requires ATP hydrolysis. The intracellular [ATP] affects SR Ca^{2+} uptake via modulating Ca^{2+}-ATPase activity (Toyoshima et al. 1993, Han et al. 1992, Owen et al. 1996). Ca^{2+}uptake is achieved through the favoured use ATP synthesised of cell in the SR triads (Han et al. 1992). Moreover, due to the chain of glycolytic enzymes from aldolase onward being directly associated with SR membranes, ATP production with glycolytic could be directly fuelling SR Ca^{2+} uptake (Xu et al. (1995).

Phosphate

A rise in intracellular inorganic phosphate (Pi) results from the splitting of ATP by myosin ATPase in each cycle, and from the consumption of ATP by ionic pumps in the surface membrane (Na^+, K^+ ATPase) and in the SR (Ca^{2+} ATPase). Cook and Pate (1985) found that exposure of skinned muscle fibres to 10 mM Pi reduced force by one third. Although the [Pi] can rise to 30 –40 mM in fatigued cells, there was only a modest loss force (Cady

et al. 1989). An increase in diprotonated phosphate to 20mM was found to reduce force by one-half (Nosek et al. 1987). Since this concentration is similar to that observed in fatigue, it is quite possible that the production of diprotonated phosphates is an important factor in the loss of force. There was correspondence between the change in protonated phosphate and the loss of force in human adductor pollicis muscle examined by NMR spectroscopy (Miller et al. 1988). Reducing Pi also significantly increasesd cross-bridge force production in intact muscle (Phillips et al. 1993, Bruton et al. 1997). However, both of these two studies were performed at room temperature (22-25°C), and the Pi effect may be markedly smaller at body temperature. 2-20 mM of Pi decreased Ca^{2+} release and increased Ca^{2+} uptake in rat skinned skeletal muscle fibres. It is suggested that this is mechanism responsible for decrease in SR Ca^{2+} release seen during fatigue in mammalian skeletal muscle (Fryer et al. 1995). Westerblad and Allen (1996a) observed that Pi injection caused a temporary reduction in resting [Ca^{2+}], tetanic [Ca^{2+}] and force. The most likely explaination is that inorganic phosphate enters the SR where it precipitates with Ca^{2+} and thereby reduces release of Ca^{2+} from SR.

Acidosis

During vigorous physical activity many metabolites accumulate in skeletal muscle, such as lactate and H^+ ions, Pi, ADP, IMP and glycolytic intermediates. The effects of elevations in H^+ and $lactate^-$ ions on SR function have received increased attention.

A fall in cytoplasmic pH occurs during fatiguing contractions, primarily due to the formation of lactic acid (Sahlin et al. 1975). The pH observed in human muscle homogenates with fatigue is around 6.4 to 6.6 (Hermansen and Osnes 1972, Sahlin 1978). NMR spectroscopy also can determine the change in contracting muscle pH *in situ*, showing similar results (Dawson et al. 1978). When pH was reduced from 7.0 to 6.5 in rabbit skinned muscle fibre, both force and velocity were decreased. The reduction in force was 30% in fast-twitch fibres but only 10% in slow-twitch fibres (Donaldson and

Hermansen, 1978). More recent work shows that this is temperature dependent with little depression in force found at a physiological temperature (Allen et al. 1998).

Low pH was found to decrease the frequency and duration of RyR channel opening in isolated channel studies (Ma et al. 1994). However, in rat and toad skinned muscle fibres a reduction of pH to 6.2 did not effect Ca^{2+} release but did slow SR Ca^{2+} uptake (Lamb and Stephenson 1994). Slow SR Ca^{2+} uptake was hypothesised to lead to a reduced SR Ca^{2+} release capacity and hence to reduced force (Fitts and Balog 1996). Thus an increased $[H^+]$ may indirectly lead to reduced SR Ca^{+2} release.

Lactate

Research conducted two decades ago gave support for the concept that an accumulation of lactate plays a major role in the development of local muscular fatigue during high intensity, and short duration exercise, within the time frame of ~10 s to 15 min (Tesch et al. 1978, Holloszy, 1973). Since then, however, lactate accumulation per se has largely been discounted as a causal agent. Hogan et al (1995) recently demonstrated that lactate may have an important role in musclar fatigue independent of pH. At constant pH and ionic strength, physiological lactate concentration caused a significant depression of maximal calcium activated force in skinned striated muscle fibres of the rabbit (Andrews et al. 1996). Recently, however, Favero et al (1997) reported that elevated $[lac^-]$ may disrupt excitation-contraction coupling via reduced SR Ca^{2+} release. This suggested that intense muscle activity produces a high concentration of lactate, which may lead to decreases in tension development and contribute to muscle fatigue.

Mg^{2+}

Increased cytosolic $[Mg^{2+}]$ during fatigue will affect SR Ca^{2+} regulation and thus, may directly influence muscle function (Westerblad and Allen 1992a, Lamb and Stephenson 1991), as described in Section 2.17.

A diagrammatic summary of the main biochemical and ionic changes in the myoplasm effecting the SR in the development of fatigue is shown in Figure 2.10.

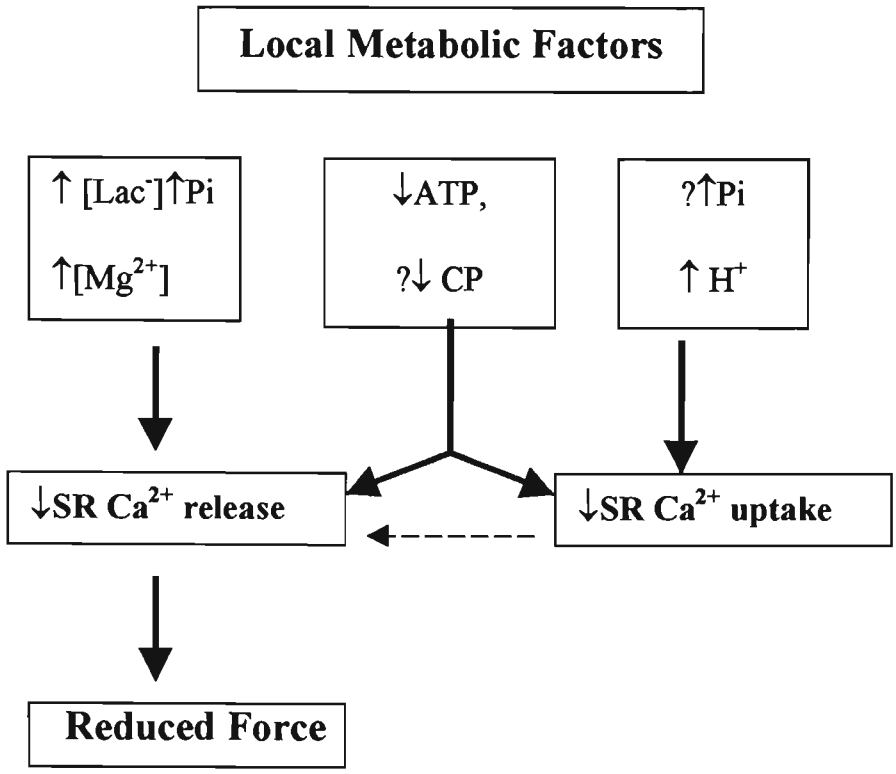


Figure 2.9 Main biochemical and ionic changes during development of fatigue.

2.3.6 Effects of Free Radicals in Skeletal Muscle Fatigue

Free radicals are most likely important factors contributing to muscle fatigue (Reid et al. 1992, Reid 1996). Cellular accumulation of free radicals has been associated with an impaired SR membrane integrity and were first indicated as a possible cause of exercise induced damage to skeletal muscle SR by Davies et al. (1982).

Reid et al. (1994) found that intravenous infusion of *N*-Acetylcysteine, which is a non-specific antioxidant of structural similarity to glutathione, reduced muscular fatiguability during electrically evoked contractions in human limb muscle. Fatigue induced by "high frequency" stimulation (40Hz) was unchanged by *N*-Acetylcysteine, whereas fatigue induced by "low-frequency" stimulation (10Hz) was reduced. This suggested that oxidative stress may play a causal role in long lasting fatigue and that antioxidant therapy was useful in attenuating this effect.

2.4 Effects of Training on Skeletal Muscle Function and SR

Individual skeletal muscles of humans and animals vary both in degree and nature in their response to different forms of acute and chronic exercise. The acute response to exercise and muscular fatigue may also be modified by a chronic increase (training) or decrease (disuse) in physical activity. The following section outlines the effects of chronically increased physical activity on muscle, focussing on SR structure and function. Important background information presented include alterations in muscle fibre type and metabolic characteristics with training, each of which may effect SR function.

2.4.1 Muscle Morphological Changes With Training

2.4.1.1 Training alters skeletal muscle fibre type proportions

Usually, the proportions of Type I and II fibres are nearly equal in human vastus lateralis, gastrocnemius and diaphragm muscles (Green et al. 1981, Bellemare et al. 1986). It is now well established that training may alter the proportions of muscle fibre types in humans (McKenna and Hargreaves, 1995).

Prolonged endurance training in humans causes an increase in the proportion of oxidative fibres, which may result in an elevation in Type I and / or IIa fibre proportions, and a decrease in Type IIB fibre proportions (Andersen and Henriksson 1977, Green et al. 1979, Howald 1982, Schantz et al. 1983). Endurance-trained athletes exhibit very few Type IIB fibres in their trained muscles (Fitts 1996).

Fibre type transformations between Type I and II fibres are not evident in either upper or lower limb muscles after resistance training (Thorstensson et al. 1976; Luthi et al. 1986; MacDougall 1986). Resistance training may increase the proportion of Type IIa fibres, at the expense of IIB fibres, without interconversion between Type I and II fibre pools (Klitgaard et al. 1990, Adams et al. 1994).

2.4.1.2 Training and muscle fibre cross sectional area

Endurance training appears to result in a smaller cross section of the myofibrils and the fibres themselves. It is likely that this adaptation allows better diffusion of metabolites and nutrients between the contractile filaments and the cytoplasm, and cytoplasm and the interstitial fluid (Saltin and Gollnick 1983). Resistance training programs are the most effective means of improving muscle bulk and strength. Such training induces an increase in contractile protein, with hypertrophy evident in both main muscle fibre types, but being particularly pronounced in the Type II fibre (MacDougall et al. 1980, 1986, Staron et al. 1990, Hather et al. 1991, Klitgaard et al. 1990).

2.4.2 Metabolic Changes in Skeletal Muscle with Training

The metabolic adaptations to endurance and resistance training have been studied using a wide variety of invasive and noninvasive techniques, including arterio-venous balance measurements (Henriksson and Reitman 1977, Jansson and Kaijser 1987, Turcotte et al. 1992), muscle biopsy sampling (Coggan et al, 1993b, Madsen et al. 1990, Favier et al. 1986), isotopic tracer infusion (Brooks and Donovan 1983, Lamont et al. 1990) and NMR spectroscopy (McCully et al. 1989, Coggan et al. 1993a).

2.4.2.1 Endurance training

Endurance training induces marked and well-documented adaptations in muscle which include an enhanced capacity for aerobic metabolism (Green et al. 1992, Holloszy et al. 1976). Endurance training increases muscle capillary density, mitochondrial number and volume and therefore, the maximal activity of oxidative enzymes (Anderson 1975, Anderson and Henriksson 1977, Holloszy and Booth 1976, Hoppeler et al. 1973). Increased muscle aerobic capacity is probably a vital adaptation in relation to the reduced muscular fatigability (Ingjer 1979, Green et al. 1992). The more extensive capillary bed after training would be expected to improve the delivery of oxygen and circulating energy

sources to the fibres, while the products of muscle activity, especially carbon dioxide, hydrogen, potassium and lactate ions, would be removed more efficiently. The greater mitochondrial density would enhance aerobic metabolism during exercise. Mitochondrial and capillary densities in muscle have been shown to correlate positively with both oxidative enzyme activity (Bylund 1977) and maximal oxygen uptake (Anderson and Henriksson 1977). Enzymes involved in the Krebs cycle, electron transport chain and beta oxidation are all increased in endurance training. These changes are reversible and may be decreased by only one week of detraining (Costill et al. 1985, Henriksson and Reitman 1977). Diminished metabolic disturbances (for example, the lesser rise in [Lac^-] and [H^+]) with exercise and enhanced lipid utilisation are found after endurance training (Saltin 1985, Saltin and Gollnick 1983). However, myoglobin stores in human skeletal muscle are not increased by endurance training (Jansson et al. 1982).

2.4.2.2 Resistance training

MacDougall et al. (1979) found that a six month resistance-training program induced marked hypertrophy, but reduced the muscle mitochondrial density and the mitochondrial number/myofibrillar volume ratio. The authors claimed that the reduction in mitochondrial density was the result of the increase in total contractile protein, without a proportional increase in the number of mitochondria.

A cross-sectional study by Tesch et al. (1984) compared the number of muscle capillaries and the muscle capillary density in weight lifters, endurance athletes, and nonathletes. The muscle capillary density in weight lifters did not differ from untrained men, but was much less than in endurance athletes. In contrast, an increased number of capillaries were found with resistance training, but no change in capillary density (Schantz et al. 1982).

Research supporting changes in the muscle aerobic capacity after resistance training is scarce. The resistance training model has important implications for muscle aerobic capacity. No changes were found in muscle succinate dehydrogenase (SDH) and malate

dehydrogenase (MDH) enzyme activities after six months of heavy resistance training (Tesch et al. 1987). Muscle SDH and MDH enzyme activity were increased after seven weeks of isokinetic training using 30 sec bouts of maximal contractions, but were unchanged when the duration was only six sec (Costill et al. 1979). Both 6 and 30 sec training duration models induced increases in the glycolytic enzymes phosphofructo kinase (PFK) and lactate dehydrogenase (LDH) (Costill et al. 1979). Conversely, the study by Tesch et al. (1987) reported a decreased activity of PFK and no change in LDH, suggesting that resistance training was not associated with increased activity of glycolytic enzymes.

2.4.3 Effects of Endurance Training on SR

Despite the importance of SR to muscle function, very few studies have investigated the effects of training on skeletal muscle SR and SR Ca^{2+}regulation. Any change in muscle fibre type has important implications for muscle Ca^{2+} release and Ca^{2+}ATPase activity, since fast-twitch muscle has a greater SR volume, density of RyR, Ca^{2+}ATPase concentration and therefore a greater Ca^{2+}ATPase activity (Rüegg 1986). In addition, the DHPR density might be altered with training which could also effect SR Ca^{2+} release. Thus, each of the following sections details any change in muscle fibre type proportions, DHPR as well as in SR characteristics.

2.4.3.1 Dihydropyridine receptors

Recently, Saborido et al. (1995) observed that 12 weeks of endurance training increased the number of DHPR in rat soleus (42%) and EDL (60%) muscles, and also increased the DHPR density in the microsomal fractions isolated from both skeletal muscles. Training induced an increase in the proportion of Type I muscle fibres in SOL (96 vs 98 % $P>0.05$) and a decrease in the proportion of Type IIB muscle fibres in EDL (62 vs 44%). The

increases in DHPR with training were opposite to those expected on the basis of the small changes in fibre type, since DHPR density is normally higher in FT muscle (Saborido et al. 1995). Therefore up-regulation of the DHPR in skeletal muscle as a result of endurance training may be an important adaptation to accommodate increased contractile activity. However, the ratio between DHPR and the RyR in muscle with training has not yet been investigated.

2.4.3.2 SR Ca^{2+} release

No published study has investigated the effects of endurance training on skeletal muscle SR Ca^{2+} release characteristics. Such an investigation is crucial for understanding adaptations in muscle function with training.

2.4.3.3 SR Ca^{2+} ATPase and Ca^{2+} uptake

A number of studies have investigated the effects of increased muscle contractile activity on SR Ca^{2+} ATPase and Ca^{2+} uptake. The tremendous adaptability of skeletal muscle SR Ca^{2+}ATPase is best demonstrated using chronic electrical stimulation models. For example chronic 10 Hz stimulation in rat skeletal muscle resulted in a decline in SR Ca^{2+}ATPase activity by 14 days, which preceded changes in myosin heavy chains (Lieber et al. 1988). Rabbit tibialis anterior muscle SR Ca^{2+}ATPase activity and Ca^{2+}uptake were decreased by 52% and 50% by muscle stimulation after only two days stimulation (24h/day with 0.15 ms impulses at a frequency of 10Hz, Heilmann and Pette, 1979). Their findings suggested that the changes in the SR occur at least simultaneously or even earlier than those of metabolic enzymes (Heilmann and Pette, 1979, Kraus et al. 1994, Hicks et al. 1997). The time course of changes in muscle SR characteristics relative to other changes in skeletal muscle is shown in Table 2.9.

Table 2.9. Time course of changes in muscle SR characteristics by chronic electrical stimulation. Other changes in skeletal muscle are also identified for comparative purposes. Data from: Heilmann and Pette, 1979 (1), Lieber, 1988 (2), Kraus et al. 1994 (3), Hicks et al. 1997 (4).

Time	Changes in muscle SR and other characteristics	(Ref.)
3 hours	Swelling of the SR	(2)
2 days	↓52% Ca^{2+}ATPase activity and ↓20-50% Ca^{2+} uptake in rabbit. ↓2 times DHPR, ↓ 5 times RyR	(1,3)
4 days	↑ number and size of mitochondria, leading to a rise in oxidative enzyme activity. Capillary formation, leading to rise in muscle blood flow.	(2)
14 days	↓ Ca^{2+}ATPase activity in rat; Increased width of Z-line. ↓ fractional volmes of T-tubules and SR.	(2)
20 days	↓ Ca^{2+}ATPase activity ↓DHPR, RyR, and trtriadin concentration in rabbit.	(4)
28 days	Appearance of slow-twitch isoforms of myosin heavy and light chains and troponin. Decrease in muscle bulk, associated with reduced muscle fibre cross-sectional area.	(2)
90 day	↓~90% Ca^{2+}ATPase activity and ↓~90% Ca^{2+} uptake	(1)

Relatively few studies have investigated muscle SR changes with physical training (Table 2.10). Kim et al. (1981) have shown that 14-16 weeks of endurance training decreased Ca^{2+} uptake V_{max} by 35% and K_m by 33% in the superficial vastus lateralis (v.l.s) muscle of the rat. No changes were found in the more oxidative deep vastus lateralis (v.l.d) and SOL muscles. Endurance training reduced Ca^{2+}ATPase activity in SR vesicles isolated from the plantaris muscle of young rats and this was reversible with cessation of training, (Belcastro 1987). In contrast, in the mature rat, no changes in Ca^{2+}ATPase activity were

seen with either training (Belcastro 1987, Saborido et al. 1995), or detraining (Belcastro 1987).

Only three studies have investigated changes in human skeletal muscle SR characteristics with training. Short term endurance training (4-6 weeks) studies using young healthy men found no change in SR Ca^{2+}ATPase activity (Green et al. 1995a) and SR Ca^{2+}ATPase concentration (Madsen et al. 1994). However, before and after training the Type II fibres had a positive relationship with SR Ca^{2+}ATPase concentration (Madsen et al. 1994). No differences were found in muscle SR Ca^{2+}ATPase activity between long-term (14 ± 2 years) endurance-trained and resistance-trained elderly men (Klitgaard et al. 1989). The effects of endurance training on skeletal muscle SR characteristics are summarized in Table 2. 10.

2.4.4 Resistance Training Effects on SR

Resistance training results in a decreased muscle mitochondrial density, due to a dilution effect (MacDougall et al. 1977). Therefore, a similar effect may be evident for muscle SR. Kandarian et al. (1994) reported decreases in both SR Ca^{2+}ATPase (12%) and SR Ca^{2+}uptake (15%) in rat muscle after 5 weeks resistance training. Twitch contraction times were prolonged in bodybuilders, suggesting that SR volume density may also be reduced after strength training (Alway et al. 1988, Sale et al. 1983). It may therefore be hypothesized that strength training may result in a reduction in SR function. Klitgaard et al (1989) compared the effect of strength training for aged men (70 years old), and demonstrated that in the vastus lateralis in aged controls there was a selective atrophy of Type IIa and IIB fibres, but unchanged fibre type distribution. The Type I myosin heavy chain composition was increased with age. The SR Ca^{2+}ATPase content was significantly lower in the aged group, but, not within a group of resistance-trained older subjects. Thom et al. (1997) recently studied muscle SR function after 3 weeks of strength training that

followed 10 days of cast immobilisation, (leg extension, 8RM × 3) in humans. They observed a 21% decrease in Ca^{2+} uptake after ten days of immobilisation, that was reversed by 7 days resistance training. Neither Ca^{2+} ATPase activity nor Ca^{2+} release were significantly altered by either immobilisation or strength training (Thom et al. 1997),Table 2.10.

Table 2.10 Effects of training on skeletal muscle sarcoplasmic reticulum Ca^{2+}ATPase content, Ca^{2+}ATPase activity and Ca^{2+} uptake.

Reference	Species	n	Age	Muscle	Training Duration (w)	Ca^{2+} ATPase Activity	Ca^{2+} Uptake Rate	Ca^{2+} ATPase Content
Endurance Training Studies								
Bonner et al. (1976)								
Kim et al. (1981)	Rat	8	NR	v.l.s.	14-16		V_{max} ↓35% K_m	
				v.l.d.			↓33%	
				soleus			ns	
							ns	
Green et al. (1984)	Rat	3	adult	v.l.s.	15			↓ CaCal†
		3		v.l.d.				↓ CaCal‡
Belcastro (1987)	Rat	30	6 weeks	plantaris	6, 12	↓38, ↓34%	↓20, ↓33%	
			12 weeks			ns		
Saborido et al. (1991)	Rat	24	adult	soleus	12	↓39%‡		
				EDL		ns		
Saborido et al. (1995)	Rat	16	adult	soleus	12	ns		
				EDL		ns		
Madsen et al. (1994)	Human	39	22-39 yr	v.l.	6			ns
Green et al (1995a)	Human	7	NR	v.l.	6-11			ns
Resistance Training Studies								
Kandarian et al. (1994)	Rat	12	Adult	plantaris	5	↓12%	↓15%	
Green et al (1995) b	Human	6	NR	v.l.	4, 7, 12	ns		
Cross-Sectional Studies					**Training Comparison**(vs Controls)			
Klitgaard et al. (1989)	Human	6	68-70 yr	v.l.	Resistance			ns
		6, 5			Endurance			ns

Abbreviations: n number; muscles: gastroc. gastrocnemius, v.l. vastus lateralis, v.l.s. superficial v.l., v.l.d. deep v.l.; ns no significant difference; NR not reported; CaCal, Ca^{2+} ATPase / calsequestrin (ratio of 115 kDa/30kDa) ↓79%(†) and ↓63% (‡); significantly less when expressed per mg protein, but ns per wet weight. Adapted from McKenna et al. (1996).

2.5 Effects of Disuse on Skeletal Muscle Function and SR

Disuse is characterised by skeletal muscle weakness, reduced muscle strength, and increased fatiguability. These functional changes appear due to muscle atrophy, alterations in skeletal muscle fibre type proportions, and deteriorated morphology and function of the SR. The following review examines each of these factors. Different models used to examine the effects of muscular disuse include complete or partial immobilization, hindlimb suspension, tetrodotoxin (TTX), and denervation. These disuse models all reduce muscle activity to different degrees and therefore are likely to exert different effects on muscle function and on muscle SR. The relative merits of these disuse models are indicated in Table 2.11. Denervation is not a good model to study inactivity effects due to the absence of normal neural supply, and the consequent effects may be different to those of immobilisation.

Table 2.11 Advantages and disadvantages of models which induce muscle atrophy. Adapted from Booth et al. (1983).

Model	Advantages	Disadvantages
Limb immobilization	Large data base of changes. Has been used on non-growing rodents. Muscle atrophy occurs if muscle not stretched.	No isotonic contractions. Muscles that are fixed at a stretched length cannot be studied.
Hindlimb suspension (Head-down tilt)	Isotonic contraction in absence of support of body weight. Muscle atrophy occurs.	Small data base.
TTX	Large data base. Muscle atrophy occurs if muscle not stretched.	Inability to reverse denervation.
Denervation	Large data base. Muscle atrophy occurs if muscle not stretched.	Absence of neural supply. Absence of small amount of neurally-induced muscle contraction Inability to reverse denervation. Surgical intervention.

The effects of disuse on human muscle biochemistry and function are not well known. Few studies have directly investigated this question by limb immobilisation in healthy human subjects and these have mostly focussed on muscle force and atrophy (Müller 1970, MacDougall et al. 1977, 1980, Appell 1990, Jokl 1990, Berg et al. 1997).

2.5.1 Muscle force in disuse

Both isometric and dynamic muscle functions are severely compromised after immobilisation (Table 2.12). These are time dependent as the duration effects the force loss (Gibson et al. 1987; Rutherford et al. 1990, Convertino et al. 1991, Hather et al. 1992, Berry et al. 1993, Adams et al. 1994, Berg et al. 1991, 1997).

The combined effects of surgical trauma and subsequent disuse also impair human isometric and dynamic muscle function (Sargeant et al. 1977, Imms et al. 1977, White and Davies 1984). Twenty weeks of leg immobilisation substantially altered triceps surae contractile properties, with a decrease in the twitch tension (25%), and (25-49%) submaximal tetanii (White and Davies 1984). The time to peak twitch tension was reduced 25% and the half relaxation time prolonged 17% (White and Davies 1984). Few studies have investigated the effects of disuse on muscular dynamic endurance. Maximal work output during a 6 minute cycling test was reduced 27% after 6 weeks immobilisation (Ingemann-Hansen and Halkjaer-Kristensen 1985), whilst one-legged cycling maximal oxygen consumption declined 15% after 19 weeks immobilisation (Sargeant et al. 1977).

A recent experiment showed that 37 days of head-down bed rest in humans diminished maximum isometric torque (25%), maximum concentric torque (29%) with the latter change being similar (27%) across knee angular velocities (Berg et al. 1997).

2.5.2 Muscle atrophy in disuse

The loss of muscular strength reported after unloading has mainly been attributed to a decrease in skeletal muscle mass. Patient undergoing lower limb immobilisation due to fractures, knee surgery, or disease suffer a marked musclar atrophy, with reduction in

thigh circumference and quadriceps cross-sectional area, as well as increased subcutaneous fat (Halkjaer-Kristensen and Ingemann-Hansen 1985, Ingemann-Hansen and Halkjaer-Kristensen 1977). Immobilization was associated with a significant atrophy of Type I fibres (Gibson et al. 1987, Häggmark and Eriksson 1979, Berg et al. 1997). However, Sargeant et al (1977) found substantial atrophy in both type I and type II fibers in the quadriceps muscle of patients with legs fractures, withthe cross -sectional areas (CSA) were reduced by 46% and 37%, respectively. The CSA of Type I and Type II fibres in the long head of the triceps were reduced by 25% and 30% respectively after 5-6 weeks of immobilisation (MacDougall et al. 1980). Young et al. (1982) showed that the muscle wasting is due to fibre hypotrophy, not hypoplasia.

The decrement in muscle mass during limb immobilization in man was due mainly to a substantial (25%) decrease in muscle protein synthesis (Gibson et al. 1987). The above studies suggested that the decline in force with disuse in skeletal muscle is primary due to muscle hypotrophy.

2.5.3 Disuse Effects on Skeletal Muscle Fibre Type Proportions

Muscle disuse may change muscle fibre composition from slow to fast twitch and thus, would also be expected to alter skeletal muscle SR function. Several studies show that muscle fibre type proportions can also be affected by muscular disuse in humans. There are now several reports that the percentage of human Type I fibres decreases after immobilisation. For example this change has been noted in vastus lateralis muscle 4-6 weeks after knee immobilization for acute ligamentous injuries (Young et al. 1982, Ingemann-Hansen and Halkjaer-Kristensen 1983, Halkjaer-Kristensen and Ingemann-Hansen 1985). Similarly, the proportion of Type IIB fibres was increased in the soleus muscle after a similar period of disuse (Haggmark and Eriksson 1979, Sargeant et al 1977). However, one study has reported no significant changes in fibre type proportions

after 6 weeks of bed-rest (Berg et al. 1997). Renstrom et al. (1983) reported an increased proportion of Type IIB and IIC (transitional) fibres in the quadriceps of the amputated limb versus the non-amputated limb in post 2 years below-knee amputees. If only a small amount of isometric contraction is allowed, this will better maintain the fast-twitch fibres (Sargeant et al. 1977). Studies have investigated the effects of disuse on fibre proportions in animal models, and show a tendency for shifts from Type I →Type IIa →Type IIB fibres (Martin et al. 1988, Pette and Staron 1997). In a recent study however, neither the percentages of Type I, IIa, IIB fiber, nor their relative proportions of myosin heavy chain (MHC) isoforms in vastus lateralis muscle, were changed after 6 weeks of bed rest (Berg et al. 1997). The effects of long term (years) disuse on muscle fibre type proportions in healthy humans is unknown.

2.5.4 Effects of Disuse on Muscle Metabolism

Relatively few studies have investigated the effects of disuse on muscle metabolic properties in human skeletal muscle. Immobilisation for 5 weeks reduced creatine phosphate (CrP) and glycogen contents in the triceps brachii muscle of healthy volunteers, by 25% and 40%, respectively (MacDougall et al. 1977). After 90 days immobilisation, reductions in ATP, CrP and glycogen contents in rat soleus muscle were 29%, 32% and 58%, respectively (Booth and Seider 1979). When expressed per unit of protein, no effects were found, suggesting that the decline in muscle substrates with immobilisation, was regulated with the decline in contractile tissue (Booth and Seider 1979). In contrast, human muscle myoglobin content was increased by 16% after 6 weeks of immobilisation (Jansson 1988).

Six weeks of lower limb immobilisation in humans reduced the regulatory oxidative enzyme activities succinate-dehydrogenase (SDH) and citrate synthase (CS) by 21-35% (Häggmark et al. 1981, Halkjaer-Kristensen and Ingemann-Hansen 1985, Wiggerstad-

Lossinet al. 1988, Jansson 1988). Human muscle immobilisation did not modify the regulatory glycolytic enzyme (phosphofructokinase, Halkjaer-Kristensen and Ingemann-Hansen 1985, Haggmark et al. 1981, Jansson 1988), but reduced by 24% the proportion of pyruvate dehydrogenase (PDH) in the active form (Ward et al. 1986). The effects of immobilisation on phosphorylase activity have not been determined. Changes in non-equilibrium glycolytic enzymes with immobilisation are contradictory and therefore difficult to interpret (Halkjaer-Kristensen and Ingemann-Hansen 1985, Wiggerstad-Lossinget al. 1988, Jansson 1988). Muscle creatine kinase (CK) activity was reduced 18% after immobilisation (Jansson 1988).

2.5.5 SR Function in Disuse

Very few studies have investigated the effects of muscular disuse on SR function in human skeletal muscle. The following review firstly examines the few studies to have examined disuse effects on SR in animal models and secondly human muscle studies. Following immobilisation and hindlimb suspension SR Ca^{2+} ATPase activity and the rates of SR Ca^{2+} uptake and release were initially increased, in association with slow-to-fast fibre transition, in rats and in guinea pigs (Kim et al. 1982, Leivseth et al. 1992, Arkhipenko et al.1993, Stevens and Mounier 1992, Table 2.13). The immunochemically assessed Ca^{2+}ATPase protein content of the SR increased in denervated and stimulated soleus muscle, to levels similar to those found in rat EDL muscle (Gundersen et al. 1988).

The effects of muscle disuse have been more extensively studied in animal skeletal muscles. Disuse diminishes total muscle contractile protein and SR Ca^{2+}ATPase activity in rat, guinea pig and pig skeletal muscle (Kim et al. 1982, Booth 1982, Cooper 1972, Krieger et al. 1980, Leivseth et al. 1992). Kim et al. (1982) studied the effects of 6 weeks of hindlimb immobilization on skeletal muscle SR in rats. They sampled the slow-twitch soleus muscle (84% Type I, SOL), the fast-twitch, oxidative, deep region of the vastus lateralis (70% Type IIA, DVL), and the fast-twitch, glycolytic, superficial region of the

vastus lateralis (100% Type IIB, SVL). Immobilization in the first 2 weeks, induced a 79% increase in the Ca^{2+} uptake rate (Vmax) of SR vesicles, in SOL. This was followed by 41% and 28% declines in activity at 4 and 6 weeks, relative to controls respectively (Table 2.13). Jakab (1987) showed 2-3 fold increased in both Ca^{2+} ATPase activity and Ca^{2+} uptake in the early period of atrophy (1-2 weeks). Leivseth et al. (1992) found gastrocnemius muscle Ca^{2+} ATPase activity was increased by 22% (NS), with no change in muscle fibre type proportions, after 3 weeks immobilization in guinea pigs.

Recently, Takekura et al. (1996) have observed morphological changes in the SR, T-Tubules and triads of rat slow and fast muscle fibres following denervation, as well as immobilization. The arrangement of the membrane systems was disordered in both slow and fast muscle fibres following both disuse conditions. Increases in the T-tubule network were apparent; there were more triads than in normal fibres, and pentadic and heptadic structures were frequently found following both denervation and immobilization. They appeared much earlier (1 week) in denervated than in immobilized (3 or 4 weeks) muscle fibres. Therefore disuse may invoke structural changes in muscle SR and T-Tubules. Whether this adversely effects muscle performance is unknown.

The capacity of the sarcoplasmic reticulum for Ca^{2+} sequestration is reduced in chronic diaphragm disuse in rats (Howell et al. 1997). The study was designed to evaluate a potential role for altered cellular Ca^{2+} metabolism in the adaptive response of the diaphragm to chronic disuse. To estimate Ca^{2+} contents and exchange fluxes for extracellular and intracellular compartments in the *in vitro* hamster hemidiaphragm after prolonged disuse caused by tetrodotoxin (TTX) and denervation (Dnv), an analytic method based on simulation and modeling of long-term $^{45}Ca^{2+}$ efflux data was used. Compartmental analysis of efflux data estimated that the Ca^{2+} contents and Ca^{2+} exchange fluxes of the largest and slowest intracellular compartment were reduced by approximately 50% in TTX and Dnv muscle groups. In addition, the kinetic model predicted significant decreases in total intracellular Ca^{2+} and total diaphragm Ca^{2+} in TTX and Dnv muscles.

Madsen et al. (1994) found no effect of detraining on Ca^{2+} ATPase after a period of 6 weeks detraining following an endurance training program. A recent abstract reported the

effects of ten days of a single leg cast immobilisation on the vastus laterals muscle SR function in eight women (Thom et al. 1997). They observed a 21% decrease in Ca^{2+} uptake, but no significant change in the maximal rate of Ca^{2+} ATPase activity or Ag^{+}-induced Ca^{2+} release. It is possible that the period of immobilisation was too brief and subject numbers too small, to detect a more severe deterioration in SR function.

In summary, muscle disuse results in marked muscle atrophy, with deterioration in muscle contractile function. The effects of disuse are more marked in animals than in humans and are opposite to those induced by training. Both Type I and Type II muscle fibres become smaller. Very little is known about the effects of disuse on skeletal muscle SR function. Several animal studies showed an increased Ca^{2+}ATPase activity in the initial weeks of immobilization, which were shifts toward fast-twitch muscle SR characteristics. However, this time course was too early to induce changes in fibre type. In most muscles decreases in Ca^{2+}ATPase activity were found in later weeks, indicating a shift towards slow-twitch SR characteristics. The effect of disuse on skeletal muscle SR function in humans remains virtually unexplored and is worthy of investigation.

Table 2.12 The effects of disuse on skeletal muscle size, fibre type proportions and strength in humans.

Ref	Muscle	n	Disuse model	Duration (weeks)	Limb CSA	Fibre CSA		Fibre Type Proportions		Isometric Force	Dynamic Force
1	Quad.	6	Leg fracture	8-30		I	↓46% *	I	↓3.3%ns	NR	↓17% $\dot{V}O_2$peak
						II	↓ 37% *	II	↑ ns		
2	VL	9	Immobilization	6	↓2.1%	I	↓26%*	I	↓ ns	NR	NR
						II	↓% ns				
3	Quad.	15	Immobilization	NR	*↓22%	↓28%		↓I 39 vs 46 %*		NR	NR
4	Triceps	5	Immobilization	5-6	NR	I	↓25%	ns		↓41%	
						II	↓33%				
5	VL	10	Amputation	20-456	↓14	MFA	↓26% *	IIA	↓19% *	NR	NR
								IIB	↑19.7*		
6	Quad.	84	Immobilization	0.4 - 1	NR	I ↓	40 %IL *	I	↓5.1% *	↓56% opera.	↓47% opera
						II ↓	26% IL*	IL		↓43% nonopera	↓26% nonpera
7	Quad.	6	Immobilization	4-6		I	↓15%*	I	ns	NR	NR
						II	ns	II	ns		
8	Quad.	7	Immobilization	52 - 260	↓ 16%*	NR		NR		↓31% MVC	NR
9	VL	7	Bed rest	6	NR	I	↓19%	I	ns	↓25%-30%	↓27%
						MFA	↓18%%	II	ns		
10	Lower limb	NR	Suspension	4	↓7%			NR		PT-↓11% AST-↓7%	16*
11	Quad	8	Bed rest	~ 4	↓10%	NR		NR		NR	NR
12	Quad	10	Suspension	2	↓8% *	ns		NR		NR	↓12% *

Abbreviations. Quad. Quadriceps,VL vastus lateralis, PT peak torque, AST angle specific torque, I Type I muscle, II Type II muscle fibre and IIA, IIB. NR not reported, opera. Operated group, nonopera. No operated group, IL injured leg, MFA mean fibre area, *$P < 0.05$.
References. 1. Sargeant et al. 1977; 2. Häggmark et al 1981; 3. Young et al. 1982; 4. MacDougall et al. 1980; 5. Renström et al. 1983; 6. Halkjær-Kristense & Ingemann-Hansen 1985; 7. Gibson et al. 1988; 8. Rutherford et al. 1990; 9. Berg et al. 1997; 10. Berg et al. 1991; 11. Convertino et al. 1989; 12. Adams et al. 1994.

Table 2.13 Effects of disuse on skeletal muscle SR Ca^{2+}release, Ca^{2+}ATPase activity and Ca^{2+} uptake.

Ref.	Species	N	Muscle	age/ body mass	Disuse model	Duration (weeks)	Ca^{2+} uptake	Ca^{2+}ATPase activity	Ca^{2+} release
1	Rat	14	SOL	NR	immobilization	1	NR	↑ 63%*	NR
						2	NR	↑ 79% *	
						4	NR	↓ 41% *	
						6	↓17% ns (3)□	↓ 28% *	
			DVL			1	NR	↑ 53% *	
						2	NR	↓ 11% ns	
						4	NR	↓ 6% ns	
						6	↑31% (3)	↑ 3% ns	
			SVL			1	NR	↑ 12% ns	
						2	NR	↑ 9% ns	
						4	NR□	↑ 13% ns	
						6	↑28% (3)	↑ 9% ns	
2	Rat	NR	Gastroc.	NR	DNV	1-2	↑167%	↑115%	NR
3	Rat	10	SOL	300g	Hind limb suspension	2	↑*	NR	↑25%
4	Rat	NR	Gastroc.		Hind limb suspension	3-4	↑41%	NR	NR
						5-6	↑58%		
5	Guinea pig	42	Gastroc.	700-1000g	immobilization	3	NR	↑22% ns	NR
6	Human	8	VL	22.6year	immobilization	1-2	↓21% *	↓7% ns	↓14% ns

Abbreviations: n number; muscles: gastroc. gastrocnemius, v.l. vastus lateralis, v.l.s. superficial vastus lateralis, v.l.d. deep vastus lateralis; ns no significant difference; *p<0.05, NR not reported. **References** 1. Kim et al. (1982); 2. Jakab et al. 1987; 3. Stevens and Mounier 1992; 4. Arkhipenko, et al. 1993; 5. Leivseth et al. 1992; 6.Thom et al. 1997.

2.6 Aims & Hypotheses

2.6.1 General Aims

This thesis examines several factors effecting SR Ca^{2+} regulation in human skeletal muscle, including muscle fibre composition, the acute and chronic effects of physical exercise. The techniques used to measure SR function involve measurement of the rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity in crude skeletal muscle homogenates.

The first study investigates the acute effects of fatiguing maximal intensity exercise on SR function. This involved muscle biopsies at rest and immediately after 50 repetitions of maximal knee extensor contractions. This study was of particular interest since no previous published studies have investigated the effects of intense fatiguing exercise on SR Ca^{2+} release or Ca^{2+}ATPase activity in human skeletal muscle.

The second study investigated the influence of muscle fibre composition, as well as the effects of chronic elevation in physical activity, by examining skeletal muscle SR Ca^{2+} regulation in resistance- and endurance-trained human subjects. This involved muscle biopsies at rest and fatigue in both groups. This investigation was important since very few studies have investigated either the role of fibre type, or the effects of training on SR function in humans. The rationale for this study was that chronic endurance training may invoke a reduced fatiguability and increased proportion of Type I muscle fibres, with a consequent reduction in the SR density in resting muscle. Resistance training may decrease fatiguability and as consequence of hypertrophy, lead to a lower SR density in resting muscle.

The third study explored the possible effects of chronic disuse on skeletal muscle SR Ca^{2+} regulation in humans. The study investigated SR function in skeletal muscle at rest in lung transplant patients (LTx), who exhibit a poor exercise tolerance. The advantage of

this group of patients is that a previous ventilatory limit to exercise is removed through lung transplantation, potentially allowing the effects of chronic disuse on muscle function and SR characteristics to become evident. It is known that heart transplant recipients have a lower proportion of Type I and a higher proportion of Type IIB muscle fibres than controls. However, skeletal muscle characteristics have not been studied in lung transplantation recipients. Further, no published studies have investigated the effects of disuse on muscle SR function in humans. However, use of this patient group to study disuse per se is complicated since the exercise performance and muscle characteristics of these patients may be adversely effected by each of disuse, malnutrition, disease and the medications taken.

A fourth study was therefore undertaken to investigate the effects of one of these factors on SR function in skeletal muscle. High concentrations of Cyclosporine A (CsA), an immunosuppressive drug taken by organ recepients patients, have been shown to depress SR Ca^{2+} ATPase activity in rat cardiac muscle, whereas no studies have examined possible skeletal muscle toxicity. This study therefore examined the effects of CsA on SR function in rat skeletal muscle at rest. This involved added CsA at different CsA concentrations (1, 25, 50 μg/ml) to SOL and EDL muscle homogenates of rats and measuring SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity in homogenates.

2.6.2 Hypotheses.

The hypotheses tested in this thesis are:

1 Sarcoplasmic reticulum function in vastus lateralis muscle in healthy, untrained individuals will be depressed by intense fatiguing knee extension exercise; where SR function is assessed by *in-vitro* measures of the rates of Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity in a crude homogenate preparation (Chapter 4).

2 Human skeletal muscle SR characteristics will be dependent upon muscle fibre composition (Chapter 5).

3 Endurance-trained athletes will exhibit a high proportion of type I muscle fibres in the vastus lateralis muscle, with corresponding low rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity in resting muscle, compared to controls (Chapter 5).

4 Resistance-trained athletes will exhibit low rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity in resting vastus lateralis muscle compared with control subjects, despite having similar proportions of type II muscle fibres (Chapter 5).

5 Resistance and endurance training will be characterised by reduced fatigability during repeated maximal contractions of the knee extensors, coupled with smaller fatigue-induced depressions in the rates of skeletal muscle SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity (Chapter 5).

6 Lung transplant recipients will exhibit a high proportion of type II muscle fibres in the vastus lateralis muscle and correspondingly high rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity, compared to controls (Chapter 6).

7 The immunosuppressive drug Cyclosporine A will depress the *in-vitro* rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity in rat skeletal muscle (Chapter 7).

CHAPTER III GENERAL METHODS

OVERVIEW

The first two studies in this thesis examind the effects of fatigue, physical training status and muscle fibre composition on skeletal muscle SR function. The third study explored whether muscle SR function was altered in lung transplant recipients, a group of patients characterised by extremely low levels of physical activity. The final study investigated whether the altered SR function in these patients was of iatrogenic origin and involved the use of a rat model.

3.1 Human Studies

3.1.1 Subjects

Prior to commencing the study, each subject completed a detailed medical questionnaire and gave written informed consent (Appendix A). All protocols and procedures were approved by the Victoria University of Technology Human Research Ethics Committee (Chapters 4, 5, and 6). A total of 24 healthy subjects participated in the first two studies. The effects of fatigue was investigated in 8 untrained controls (UT, Chapter 4). Control subjects were not well trained and did not participate in regular sporting activities, but were recreationally active. Data from these subjects was also used in the second study which also included 8 resistance-trained (RT) and 8 endurance-trained (ET) athletes (Chapter 5). The RT and ET subjects were recruited through the university, local competitive clubs and representative teams, and had been training continuously for at least 2 years. The RT subjects were selected on the basis of being able to lift at least 1 to 1½ times their bodies mass during squatting exercise with free weights. The RT group had

typically trained 4-5 times per week with ~1 hour per session. The ET subjects were selected on the basis of their peak oxygen consumption, which must have at least equalled 60 ml min^{-1} kg^{-1}. The ET group had performed running and/or cycling endurance training for 4-5 hours per day. No differences in age, height or body mass were found between the three groups. Physical characteristics of the UT, RT and ET subjects are shown in Table 3.1.

The third study comprised 7 successful lung transplantation recipients and 7 healthy control subjects who were matched for age and gender (Chapter 6). The thoracic transplantation patients were studied in collaboration with the Department of Respiratory Medicine, and The Heart & Lung Transplantation Medical Team, The Alfred Healthcare Group. All protocols and procedures for this study were in addition approved by The Alfred Group of Hospitals Ethics Committee review (Chapter 6). The mean duration post-transplantation was 11.6±8.1 (mean ± SD) months, ranging from 3-24 months. Physical characteristics of patients and controls are shown in Table 3.2, there were no significant difference between controls and lung transplant patients.

Table 3.1 Subject physical characteristics

	UT	**RT**	**ET**
n	8	8	8
Sex (M/F)	8 Male	7 Male/1 Female	8 Male
Age (year)	26.4 ±3.9	26.8 ± 7.9	26.4 ± 3.1
Body mass (kg)	80.4 ±6.8	81.6 ± 3.3	70.6 ± 9.9
Height (cm)	183.3 ±5.7	176.1 ± 4.7	177.2 ± 7.1

Mean ± SD. Untraned (UT), resistance-trained (RT) and endurance-trained (ET).

Table 3.2 Physical characteristics of lung transplantation recipients and controls (Chapter 6).

	Controls	Lung Transplant
Subject	7	7
Male/Female	3/4	3/4
Age (year)	37.0 ± 11.4	34.9 ± 4.3
Height (cm)	169.3 ± 7.8	170.3 ± 12.5
Body mass (kg)	67.7 ± 94	67.3 ± 13.8

Mean ± SD.

The diagnosis, operation method and relevant medication records of the patients are shown in Table 3.3.

Table 3.3 Clinical characteristics of lung transplant patients

Patient	Diagnosis	Opera -tion	Duration Post-operation (mo)	Immunosuppressive Treatment [CsA] (ug/L)	CsA (mg/d)	Prednisone (mg/d)	Imuran (mg/d)
1	IPF	SLTx	12	210	350	224	100
2	PL	SLTx	9	336	300	15	NA
3	PPH	HLTx	24	393	450	10	25
4	PH	DLTx	8	313	400	15	100
5	BA	DLTx	21	198	500	15	100
6	CF+A-a	DLTx	3	222	300	20	75
7	CF	DLTx	4	221	450	15	100

Diagnosis: PPH, primary pulmonary hypertension; PL, pulmonary lymphangiolieomatosis; IPF, idiopathic pulmonary fibrosis; CF, cystic fibrosis; PH, pulmonary hypertension; A-a, al-antitrypsion deficiency; BA, bronchiolitis abtilerans.

Medications: 1. Immunosuppressive medications used in transplant patients to prevent graft rejection were: CsA, Cyclosporine A; Imuran, Azathioprine; Prednisone, Corticosteroid. NA, details not unavailable.

2. Other major medications included: Antihypertension drugs: calcium channel blockers, Verapamil and Codezyme; angiotensin-converting enzyme inhibitors, Zestril; H^+-K^+-ATPase inhibitors: Omeprazole; H_2 receptor antagonist Zantac; calcium and magnesium supplements.

[CsA] = CsA concentration in blood.

3.1.2 Anthropometric Measurements

Anthropometric measurements were conducted in the second study to confirm differences between the different groups.

All subjects were measured for height using a stadiometer (Novel Inc, Addison Illinois, U.S.A) and body mass recorded with a sensitivity of 0.005 kg on an electronic scale (Sauter E1200, West Germany). Subcutaneous skinfold thickness was measured at 8 sites: tricep, bicep, subscapular, mid-axilla, suprailiac, abdominal, anterior thigh and medial calf using Harpenden calipers (British Indicators, W. Sussex, Great Britain). Sum of 8 skinfold thickness was determined and body density was estimated using the following regression equations (Jackson & Pollock 1978, Jackson et al. 1980; Siri, 1956). The percent body fat was calculated using equation 3.1 (Siri, 1956).

Equation 3.1. Percent Body Fat = [(4.95/BD) – 4.50] *100

The regression equations for men and women are shown in Equations 3.2 & 3.3 respectively.

Equation 3.2 Regression equation for men:

$$BD = 1.112000 - 0.0004399\ (X_1) + 5.5\ 10^{-7}(X_1)^2 - 0.00028826(X_2)$$

Where BD = body density, X_1 = sum of triceps, biceps, mid-axilla, subscapular, abdominal thigh and calf skinfolds, X_2 = age (years)

Equation 3.3 Regression equation for women:

$$BD = 1.096095 - 0.0006952\ (X_1) + 1.1*10^{-6}(X_1)^2 - 0.0000714(X_2)$$

where BD = body density, X_1 = sum triceps, abdominal supra-iliac, and thigh skinfolds, X_2 = age (years)

An estimate of thigh volume was determined by water displacement. Thigh volume was obtained by subtracting the volume of the lower leg measured at the lateral and medial epicondyles of the femur, from the volume of the whole leg, which was measured up to the gluteal furrow in the horizontal plane. The limb segment volume was also determined by the use of limb lengths and girths (Jones & Pearson 1969). With subjects standing erect and feet slightly apart, girths were measured with a flexible tape measure while segmental lengths were determined with an Holtain anthropometer to an accuracy of 1 mm.

3.1.3 Lung Function Testing

All subjects performed spirometry to determine their vital capacity (VC) and forced-expired volume in 1 second (FEV_1). Healthy subjects were tested at Victoria University of Technology on a spirometer (Minato Osaka, Japan) whilst transplant patients were tested at the Alfred Hospital on a Jaeger Masterlab 3.30 spirometer (Wuerzburg, W. Germany). The results for all groups and comparison to predicted values (Crapo et al. 1981) are shown in Appendix A.

3.2 Exercise Tests.

All subjects in the first two studies performed three exercise tests, each separated by at least several days, but by no longer than 2 weeks (Chapters 4 and 5). The first two tests comprised an incremental exercise test on a cycle ergometer, muscle isometic and dynamic contractile function assesment and fatiguability tests. These were designed to contrast exercise performance and muscle fatiguability of the different subject groups. The final test examined muscle fatiguability in association with a sampling of blood and muscle tissue.

3.2.1 Maximal Aerobic Power

Each subject refrained from exercise, alcohol and caffeine consumption for 24 hours and then performed an incremental exercise test on an electrically braked cycle ergometer (Lode N.V. Groningen, Netherlands) to determine their peak oxygen consumption ($\dot{V}O_2$ peak). Subjects cycled at 60 rpm (except endurance trained, 80rpm) and workrate was increased by 25 $W.min^{-1}$ until volitional exhaustion, defined as an inability to maintain the desired pedal cadence. Subjects breathed through a Hans-Rudolph 2-way non-rebreathing valve, with expired air passed through flexible tubing into a mixing chamber; expired volume was measured using a ventilometer (KL Engineering Sunnyvale, California, USA); mixed expired oxygen and carbon dioxide contents were analysed by rapidly responding gas analysers (Applied Electrochemistry S-3A O_2 and CD-3A CO_2, Ametek, PA, USA.). The gas analysers were calibrated immediately prior to each test, and rechecked after the test, using commercially prepared gas mixtures. The ventilometer was calibrated prior to each test using a standard 3 l syringe. ECG was measured to determine the heart rate and monitor heart rhythm (Mortara, X- Scribe, Muoaukee.WI, USA.).

Arterialised venous blood samples were collected from a dorsal hand vein at rest, each minute during exercise and at 1, 2, 5, 10, 20 and 30 min in recovery. Arterialisation was obtained by heating the hand as described below (Section 3.3).

3.2.2 Muscle Function Assessment

Maximal muscle strength and fatiguability of the knee extensor muscle group were determined using two separate tests on a Biodex isokinetic dynamometer (Medical Systems Inc., USA). Prior to testing, subjects completed a 3 min warm up on a cycle ergometer at 50W. Subjects were then strapped to the Biodex chair using belts across the hips and chest, to restrict upper body movement and across the thigh to stabilise the leg.

Muscle torque-velocity Relationship. The first test measured peak muscle torque at a series of limb velocities. The right leg was tested only. Subjects performed 2 practice maximal repetitions, followed by 1 min rest for each speed. Then 3 maximal repetitions were performed at each of 60, 120, 180, 240, 300 and 360 °/s, each separated by a 2 min rest. After 3 minutes rest all subjects were tested for isometric peak torque (0°/s). Peak torque at each velocity was recorded and used to construct a torque-velocity relationship. Peak torque was expressed in Nm, and relative to body mass (Nm/kg).

Muscle fatigue test. The second test was performed after a recovery period of 30 min and was designed to rapidly induce muscular fatigue of the knee extensor muscles. Subjects completed 50 repetitions of maximal knee extension at 180°/s 0.5 Hz as modified from Thorstensson et al. (1976a). The peak torque during the test was calculated as the mean of the 5 strongest in the first 10 contractions. The final peak torque during the test was calculated as the mean of the 5 weakest in the final

10 contractions The fatigue index was also was calculated as the percent decline in power (FI_{torque}) and work (FI_{work}) from the five highest initial to the five lowest final contractions (Equation 3.4).

Equation 3.4: Fatigue Index = 100 x (Peak Torque-Final Torque)/Peak Torque

3.2.3 Muscle Fatigue Test with Invasive Measures

Subjects performed a second muscle fatigue test on a Cybex isokinetic dynamometer (Cybex II, Lumex Inc., Ronkoukowany, New York, USA). Separate dynamometers were used for the muscle fatigue test for practical reasons. The Biodex dynamometer was preferred for characterisation of muscle function of the different subject groups but was located at a different campus of the University to the Exercise Physiology Laboratory, where the invasive testing could take place. A Cybex dynamometer sited in the Exercise Physiology Laboratory was used for the invasive muscle fatigue test. Each subject refrained from exercise, alcohol and caffeine consumption for 24 hours prior to the test. Muscle biopsies were taken at rest and following exercise and arterialised venous blood samples were drawn at rest, during and following the fatigue test (Figure 3.1).

Subject posture, stabilisation and exercise requirements on the Cybex were identical to those described earlier for the Biodex dynamometer. Data were expressed in Nm, and the fatigue index based on the percentage decline in peak torque (FI_{torque}), was calculated as in Equation 3.5. There were no significant differences in FI_{torque} between the fatigue tests on the two isokinetic dynamometers.

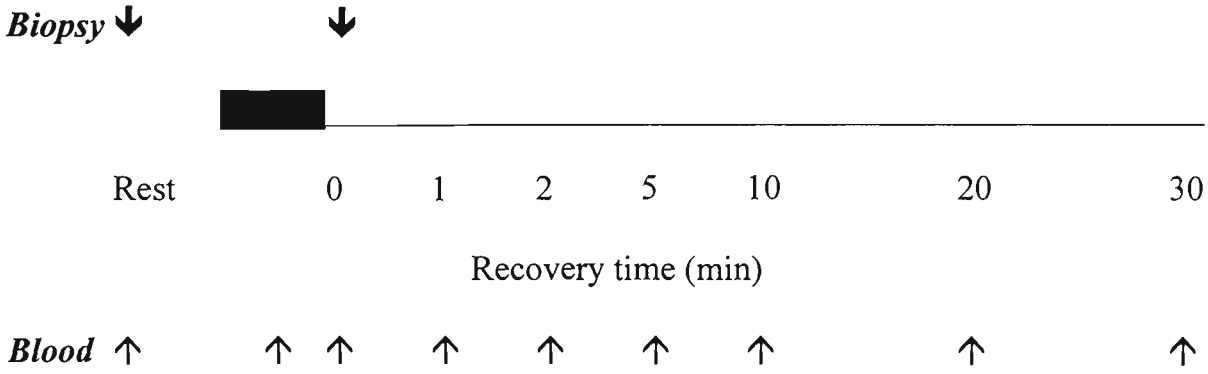


Figure 3.1 Muscle and blood sampling at rest, during and following muscle fatigue test, comprising 50 maximal knee extensions (■, 180°/s, 0.5Hz) on a Cybex isokinetic dynamometer.

3.3 Blood Analyses

3.3.1 Blood Sampling

Venous blood samples were drawn from a superfical dorsal hand vein at rest during and following the fatigue test and $\dot{V}O_2$ peak test. All blood samples were arterialised by heating the hand in a hot (45°C) water bath for 10 min (M^{c}Loughlin et al. 1992). Subjects maintained a seated posture for all samples. Two syringes of blood were drawn at each sampling time, the first for plasma gas and electrolyte concentrations (1.5 ml, Rapidlyte, Ciba Corning Diagnosics Corporation, Medfield, MA, USA), the second for haematocrit and lactate measurements (5ml, Terumo Medical Corporation, Elkton, MD, USA). Periodic infusions of isotonic saline (1- 2 ml) were used to keep the catheter patent.

3.3.2 Blood Processing

The blood was mixed well, air bubbles were expelled from the syringe. The blood gas syringe was capped tightly and placed on ice for subsequent analysis of plasma pCO_2, pO_2, pH (865 Ciba Corning, Medfield, MA, USA). From the second syringe 1ml of blood was portioned into an eppendorf tube and analysed in duplicate for haematocrit (Hct) with a microcentrifuge (D-7200 Haematukrit, Tuttlingen) and triplicate for haemoglobin concentration ([Hb]) by the cyano-methaemoglobin method (Radiometer OS2, Copenhagen, Denmark). Whole blood (250 µl) was deproteinised in 500 µl of 0.6 M perchloric acid, centrifuged and the supernatant drawn off and stored at -60°C for later analysis of lactate in triplicate (Annan, 1975). Plasma lactate was analysed in triplicate, using an enzymatic spectrophotometric technique (Lowry and Passonneau, 1972). All analytical instruments were calibrated before and during the analyses with precision standards.

3.4 Muscle Needle Biopsy Sampling

3.4.1 Muscle Biopsies.

In the first two studies, three muscle biopsies were taken for each subject, comprising two biopsies at rest and one immediately at the point of fatigue (Chapters 4 and 5); in the third study two resting biopsies only were taken for each subject (Chapter 6). Resting muscles also were using for two other investigations (Na^+-K^+ pump activity and mitochondrial function). After injection of a local anaesthetic into the skin and fascia (2% Xylocaine), a small incision was made in the mid-portion of the vastus lateralis muscle of the right leg. Muscle samples (40 - 120 mg) were taken at a constant depth using the needle biopsy technique (Bergstrom, 1962). Resting biopsies were analysed for muscle fibre type, SR

function, substrate and metabolite contents. The fatigue biopsy sample was analysed for muscle SR function, substrate and metabolite contents. Immediately after excision, the muscle was rapidly separated into portions, with one portion rapidly frozen and stored in liquid nitrogen for subsequent metabolite, substrate and pH determinations. The remaining portion was immediately weighed, homogenised and frozen in liquid nitrogen for later SR function analyses.

3.4.2 Muscle Morphology.

A portion of the resting biopsy sample was mounted using an embedding medium (Jung Embedding medium, Nussloch, Germany), quick-frozen in isopentane precooled in liquid nitrogen, and stored at -80° C until analysis. Muscle fibre types were analysed using the myofibrillar ATPase method described by Brooke and Kaiser (1970). Fibre typing was performed on 10μm thick sections of muscle cut on a cryostat at -20° C (Microm GMBH D-6900 500, Heidelberg, Germany). Fibres were classified into Types I, IIA or IIB, according to their myofibrillar ATPase staining patterns after pre- incubation at pH 4.3, 4.6 and 10.3.

3.4.3 Muscle SR Function.

Skeletal muscle SR function was investigated in muscle homogenates using previously described methods (Ruell et al. 1995, Booth. et al. 1997).

3.4.3.1 Muscle Homogenisation.

Approximately 30-40 mg of muscle was weighed, diluted 1:10 (wt/vol) in a cold buffer containing Tris-HCl (40mM, pH 7.9), sucrose (0.3 M), L-Histidine (10μM), EDTA (10mM), Sodium Azide (10mM), and then homogenised on ice for 3 x 15

seconds. The homogenate was then rapidly frozen in liquid nitrogen for later analyses of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity.

3.4.3.2 Muscle Ca^{2+} uptake/release assay.

The Ca^{2+} uptake and Ca^{2+} release measurements were conducted in a standard buffer containing Hepes (20 mM, pH 7.0), KCl (150 mM), Mg-ATP (4.5 mM), Indo-1 (1 μM, Calbiochem), Oxalate (7.5 mM), Sodium Azide (10 mM) and TPEN (5 μM). No Ca^{2+} was added to the buffer, since buffer [Ca^{2+}] was typically already ~1μM, due to slight, but consistent contamination in the buffer. The buffer was magnetically stirred and maintained at 37 ^{O}C. The reaction was initiated by addition of 30-50 μl (human muscle) or 15-30 μl (rat muscle) of homogenate to the buffer. All measurements of Ca^{2+} uptake and release were performed in duplicate and were completed within 50 mins after thawing of the sample.

Homogenisation results in formation of vesicles with intact and normally functioning membranes. The rates of Ca^{2+} uptake into, and release from these vesicles were then monitored using a fluorescent (extra -vesicular) dual emission dye, Indo-1 (Figure 3.2). As Ca^{2+} is sequestered within SR vesicles in the homogenate preparation, free [Ca^{2+}] decreases, producing spectral changes of the dye, Indo-1. The Ca^{2+} uptake reaction was then allowed to stabilise before Ca^{2+} release was initiated by addition of $AgNO_3$ (141μM) to the reaction buffer. It is known that heavy metal ions such as Ag^{+}, Hg^{2+}, Cd^{2+}, and Cu^{2+} can oxidise sulfhydryl (SH) groups on proteins (Abramson & Salama, 1988). It has also been shown that the Ca^{2+}-release protein of the SR (RyR) contains critical SH groups which are in a hydrophilic environment and are thus able to bind to these heavy metals (Abramson & Salama, 1988). When these heavy metals bind to the SH groups on the RyR, Ca^{2+}-release is initiated. Addition of $AgNO_3$ initiated Ca^{2+}-release, leading to an increase

in extravesicular [Ca^{2+}], which was monitored via an increase in the fluorescence ratio of Indo- 1. This method was as described by Ruell et al. (1995) and an example is given in Figure 3.3. At the conclusion of the assay, EGTA (3.5 mM) and then saturating $CaCl_2$ (5 mM) were added for calibration purposes.

Spectral changes of Indo-1 were monitored by fluorometry using a luminescence spectrometer (AB2, SLM-Aminco, Urbana, Illinois, USA) (Figure 3.4). The sample was excited by a Xenon lamp at 349 nm with a bandpass of 1 nm. The emission was measured at 410 nm for Ca^{2+}-bound (F) and at 485 nm for Ca^{2+}-free (G) forms of dye, with 8 nm bandpasses. Data were collected every 0.5 s giving a ratio metric data point each second. Maximum and minimum Ca^{2+}dependent fluorescence was determined at the completion of the assay by the addition of 3.5mM EGTA to chelate residual Ca^{2+}, followed by addition of 5mM $CaCl_2$ to saturate all Ca^{2+} - binding sites of EGTA, muscle proteins and dye.

The ionized Ca^{2+} concentration of assay medium was calculated from the equation of Grynkiewicz et al., (1985):

Equation 3.6 $$[Ca^{2+}] = Kd \times (G_{min}/G_{max}) \times [(R-R_{min}) / (R_{max}-R)]$$

Where Kd is the effective dissociation constant for the interaction between Ca^{2+} and Indo-1 and was determined to have a value of 164 nM at 37° C; R is the ratio of the two fluorescence intensities F (410 nm) and G (485 nm), R_{max} is the maximum R value at saturating [Ca^{2+}] and R_{min} is the minimum R value at zero [Ca^{2+}].

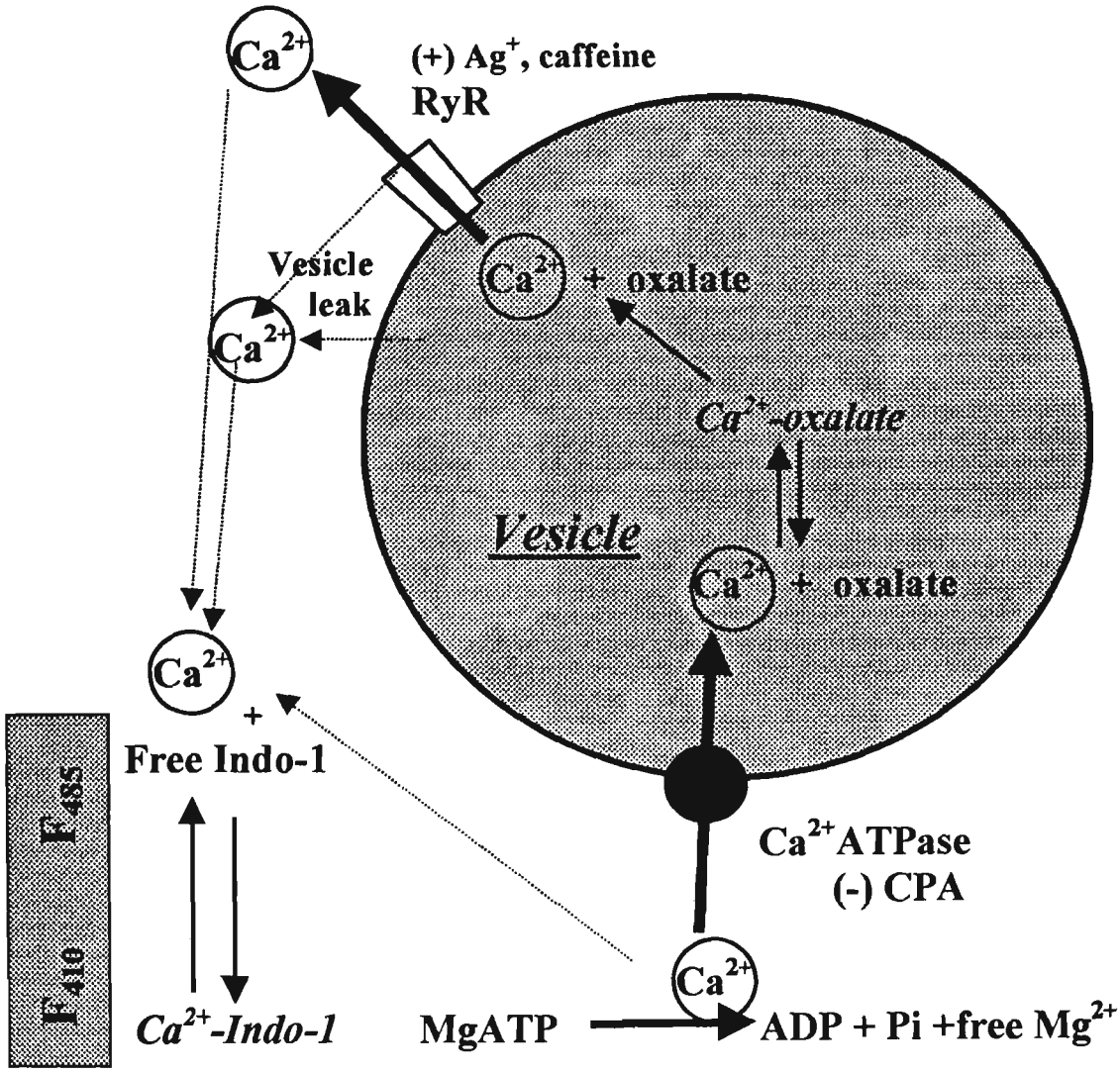


Figure 3.2 Schematic representation of methods used to monitor the rates of Ca^{2+} uptake and Ca^{2+} release in vesicles in a muscle homogenate.

The Ca^{2+} ions are initially actively transported into vesicles via Ca^{2+}ATPase and bound by intravesicular oxalate. Ca^{2+} ions are then released via the RyR (Ryanodine receptor, Ca^{2+} release channel). Extravesicular free Ca^{2+} ions (Ca^{2+} in circle) bind to the free fluorescent indicator, Indo-1, with emission measured at 410 nm for Ca^{2+}-bound (F_{410}) and at 485 nm for Ca^{2+}-free (F_{485}) forms of the dye. Figure adapted from Warmington et al. (1996).

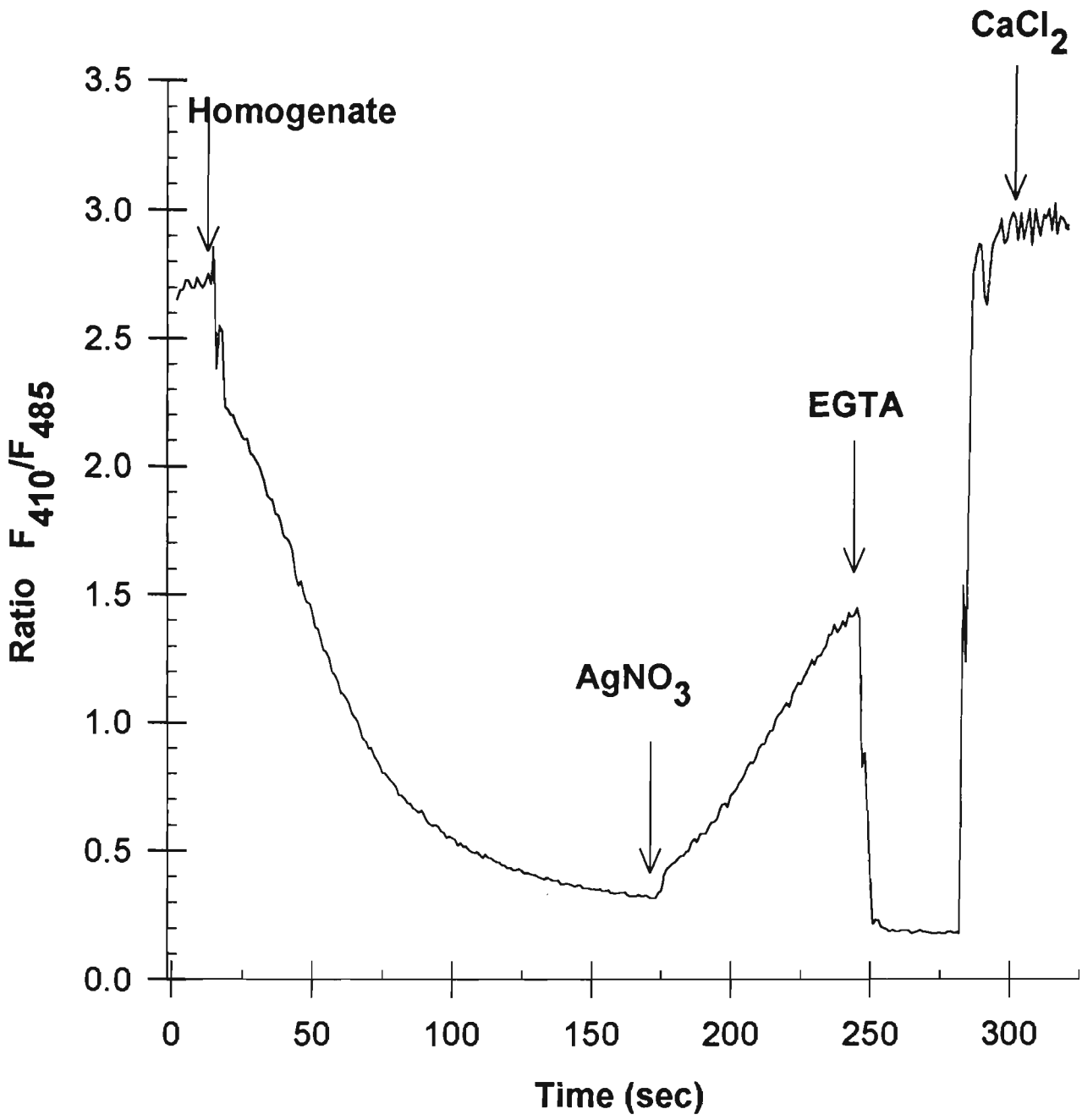


Figure 3.3 An example of the muscle homogenate sarcoplasmic reticulum Ca^{2+} uptake and Ag^{+} - induced Ca^{2+} release assay using Indo-1.

Ca^{2+} uptake was initiated by addition of homogenate and as the ratiometric signal approached a plateau, Ca^{2+} release was initiated by addition of $AgNO_3$ to the buffer. The extra-vesicular $[Ca^{2+}]$ was calculated from the ratio of emission fluorescence at 410 and 485 nm wavelengths, with calibration signals for zero $[Ca^{2+}]$ and saturating $[Ca^{2+}]$ obtained by addition of EGTA and $CaCl_2$, respectively. (See Appendix B - method and calculation)

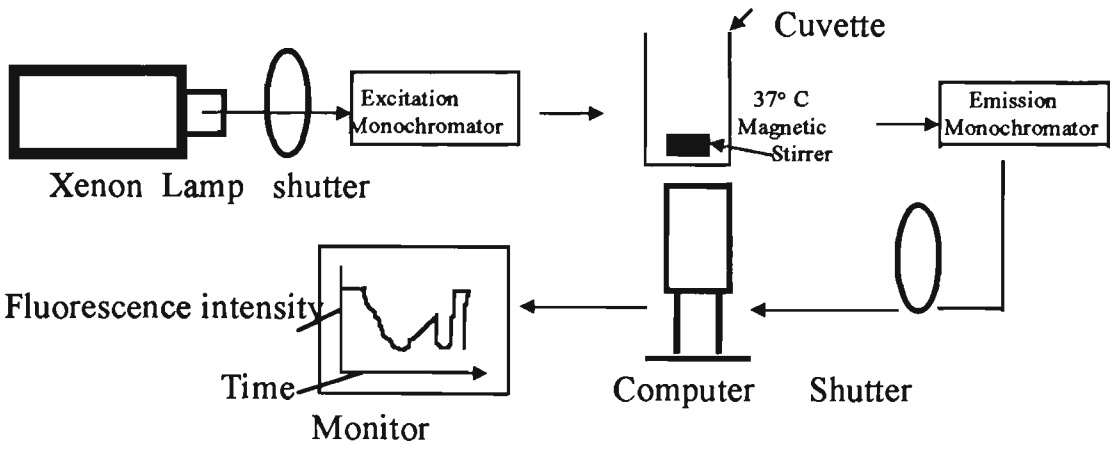


Figure 3.4 The experimental set-up used to measure fluorescence.

3.4.4 Muscle SR Ca^{2+}ATPase activity.

The SR Ca^{2+}ATPase activity was determined in triplicate spectrophotometrically, using 15µl for rat EDL muscle, and 30 -50 µl of homogenate for rat SOL and for human muscles. Different volumes were required since SR Ca^{2+}ATPase activity in SOL and in human muscles was less than in rat EDL muscle (Chapter 2.2). Separate measures were made of the Mg^{2+}-dependent, basal and total ATPase activities (Simonides & van Hardeveld, 1990, Ruell et al. 1995, Booth et al. 1997). The ATPase reaction was measured at 37 °C, in a 20mM Hepes buffer (pH 7.5). The measurements were performed in a cuvette holder using a spectrophotometer. NADH absorbance was measured at 340 nM and the rate of change was recorded every 5s for 1-2min. The total ATPase activity and basal (Mg^{2+}ATPase) were measured after addition of 10 µl of 100 mM $CaCl_2$ giving a final concentration of 0.6mM $CaCl_2$. The basal ATPase activity (Mg^{2+}ATPase) was measured after addition of 20 µl of 2M $CaCl_2$ giving a final concentration of 40mM $CaCl_2$. At the

addition of 20 µl of 2M $CaCl_2$ giving a final concentration of 40mM $CaCl_2$. At the 2M $CaCl_2$ the SR Ca^{2+}ATPase activity is selectively inhibited. ATPase activities were determined the greatest rate of change.

3.4.5 Protein Content

The muscle homogenate protein content was also determined using the method described by Lowry et al. (1976). Albumin was used as a standard.

3.4.6 Muscle Metabolites and pH.

Muscle was freeze dried, dissected free of any connective tissue, weighed, powdered and extracted (Harris et al. 1974). Approximately 2mg of freeze dried muscle was extracted in 0.5 M PCA and neutralized in 2.1 M $KHCO_3$. Muscle extracts were analysed for ATP, CP, glycogen and creatine contents using fluorimetric techniques (Model 112 Turner Fluorometer California, U.S.A) and for lactate contents using spectrophotometric techniques (Shimadzu UV-120-02), according to the methods of Lowry and Passonneau (1972). Reverse-phase high performance liquid chromatography (HPLC) was used to quantify muscle ATP, ADP, AMP, and IMP contents (Wynants & Van Belle 1985). A portion of the muscle extracts was assayed in 250 µl 2M HCl at 100 ^{O}C for 2hr and neutralised with 75µl 0.76M NaOH for measurement of glycogen using an enzymatic method (Lowry and Passonneau, 1972). Muscle homogenate pH was determined in freeze dried tissue (2-4 mg, 1mg/100µl dilution) with a pH microelectrode at 37 ^{O}C (MI-410 microelectrode Inc., U.S.A.). The homogenate buffer contained 5mM Sodium iodacetate, 145mM KCl and 10 mM NaCl (Spriet et al. 1989).

3.5 Animal Study

Eight female Sprague-Dawley rats (224±21 g) were used in the final study (Chapter 7). All procedures were approved by the Victoria University of Technology Animal Research Ethics Committee. Animals were killed by an overdose of anaesthetic (diethylether) and the extensor digitorum longus (EDL) and soleus (SOL) muscles were rapidly excised. Each muscle was homogenised and then frozen in liquid N_2 for later analyses of the maximal rates of calcium release and as described above, after incubation in either Cyclosporine A (CsA) at 0, 1, 25 or 50 μg/ml.

3.6 Statistical Analyses.

Results for two variable comparisons within the same subject were analysed by paired t-tests (e.g. Rest vs. Fatigue, Chapter 4), whilst for non-paired data, an independent t-test was employed (e.g. Chapter 6, LTx vs control). A 2 way analysis of variance (ANOVA) with repeated measures on one variable was used where more than 2 variables compared (eg. Chapter 5, Trained and fatigue). For the rat CsA study, a one-way ANOVA was applied (Chapter 7). When a significant F-ratio was obtained in ANOVA procedures, a Newman-Kuels post-hoc analysis was used. A level of significance of $P<0.05$ was accepted for all analyses. All experiment data are presented as mean ± standard error of the mean (SEM) except for population statistics (e.g. body mass) which are presented mean ± standard derivation (SD).

Chapter IV FATIGUE DEPRESSES SARCOPLASMIC RETICULUM CALCIUM RELEASE AND UPTAKE IN HUMAN SKELETAL MUSCLE

4.1 Introduction

Muscle fatigue compromises exercise performance in sporting and occupational activities, and may impair the quality of life in clinical populations. Recent evidence from isolated single fibres in animal experimental models suggests that a major cause of skeletal muscle fatigue is an impairment in the release of Ca^{2+} from the sarcoplasmic reticulum (SR). This causes a decline in muscle cytosolic [Ca^{2+}] during a tetanic contraction, which then leads to a decline in muscle force (Allen et al. 1989, Westerblad et al. 1990, Westerblad et al. 1995). Studies using SR vesicle preparations also showed a decreased rate of Ca^{2+} release in the red portion of the gastrocnemius muscles obtained from rats after prolonged treadmill running (Favero et al. 1993). However, no studies have investigated whether SR Ca^{2+} release is also depressed with fatigue in human muscle.

The rate of SR Ca^{2+} uptake also declines with fatigue, as shown in numerous studies with isolated single fibres, or intact muscle from mice, rats and horses (Chapter 2.4). A recent study demonstrated depressions in both skeletal muscle SR Ca^{2+} uptake and Ca^{2+}ATPase activity following prolonged exercise to exhaustion in humans, with these changes being highly correlated (Booth et al. 1997). Two other studies have reported a decline in SR Ca^{2+} uptake in human muscle after fatigue induced by intense exercise (Gollnick et al. 1991, Hargreaves et al. 1998). However, neither study measured the effects of fatigue on SR Ca^{2+}ATPase activity. Therefore whether an

impaired Ca^{2+}ATPase activity underlies the decreased Ca^{2+}uptake in this exercise model remains to be determined.

Thus, no studies have investigated fatigue effects on SR Ca^{2+} release, or on both Ca^{2+} uptake and Ca^{2+} ATPase activity, in human skeletal muscle. The recent development of fluorimetric techniques for measurement of SR Ca^{2+} release and Ca^{2+} uptake in homogenates using the Ca^{2+} indicator dye Indo-1 (Ruell et al. 1995) has created a unique opportunity for examining the effects of fatigue on these variables. This study therefore investigated the effects of fatigue induced by intense contractions on each of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity in human skeletal muscle. The hypothesis tested was that reductions would occur in each of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity after intensive fatiguing exercise.

4.2 Methods

Eight healthy untrained males signed informed consent and participated in this study. All subject details and experimental procedures for exercise tests, muscle biopsy and blood sampling and analyses have been fully described in Chapter III. Muscle biopsies were taken from the vastus lateralis muscle at rest and immediately after completion of 50 maximal knee exhaustive contractions. Muscle was analysed for SR function, fibre type proportions and metabolites.

4.3 Results

4.3.1 Fatigue Test.

Knee extensor muscle torque fell during the 50 maximal contractions on the Cybex isokinetic dynamometer, such that the calculated fatigue index was 43.4 ± 3.3 % (Figure 4.1). The total work was 10.68 ±0.32 kJ. There were no differences in the fatigue index between the muscle fatigue tests conducted on the Cybex and Biodex

dynamometers, with the fatigue indices being 43.4±3.3% and 53.6± 4.0%, respectively.

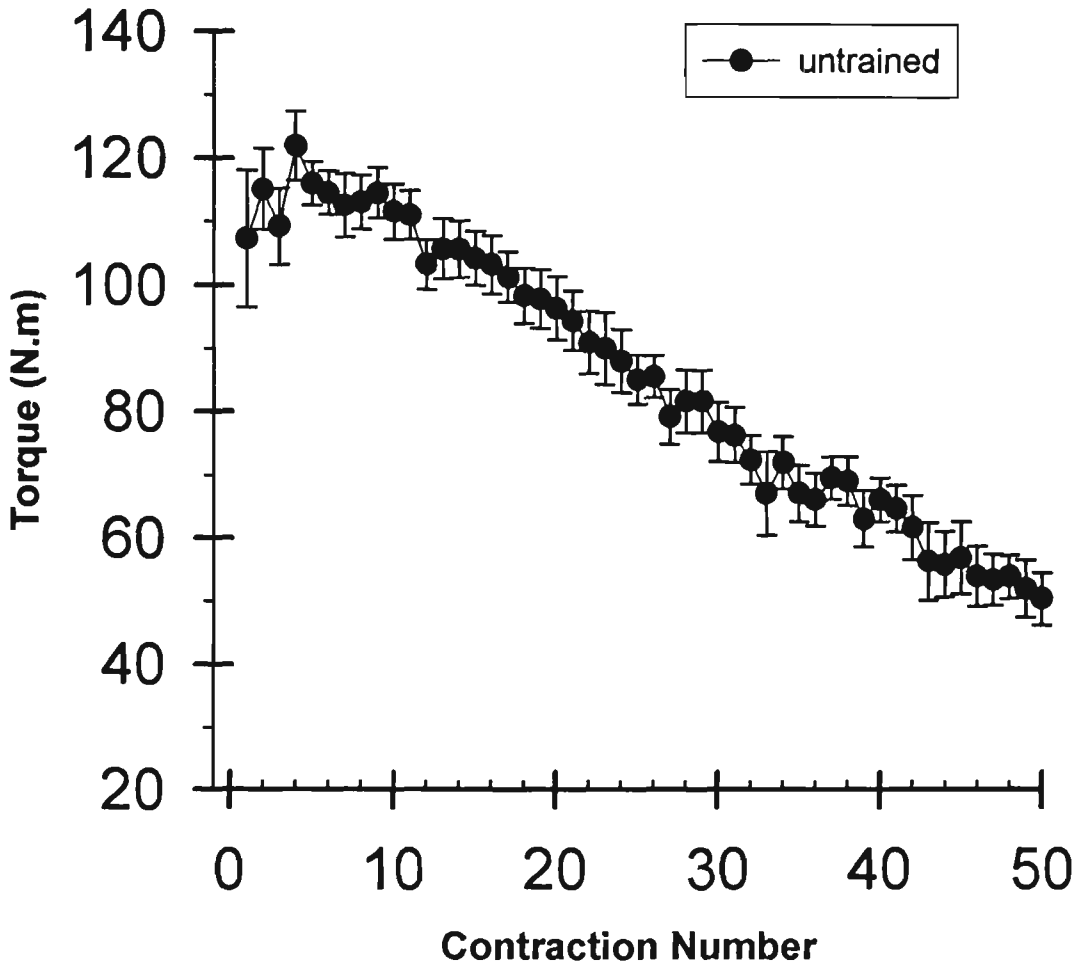


Figure 4.1. Knee extensor muscle torque during repeated maximal contractions on a Cybex isokinetic dynamometer. Mean ± SEM: n=8.

4 3.2 SR Function

Muscle SR function at rest and after fatigue, expressed per muscle wet weight, are shown in Figures 4.2 and 4.3. The resting rate of SR Ca^{2+} release was 1.98 ± 0.70 nmol. min^{-1}.mg $muscle^{-1}$. The resting rates of SR Ca^{2+} uptake and Ca^{2+}ATPase activity were 1.83 ± 0.65 nmol. min^{-1}.mg $muscle^{-1}$ and 15.05 ± 5.32 nmol. min^{-1}.mg $muscle^{-1}$, respectively. The SR Ca^{2+} release rate expressed per muscle wet weight was

depressed by 42.1% with fatigue (P<0.001, Fig. 4.2). The rates of SR Ca^{2+} uptake and Ca^{2+}ATPase activity were also decreased with fatigue by 42.9%, and 38.6 %, respectively (P<0.01, Fig. 4.3). The reduction in Ca^{2+} uptake following intensive exercise was paralleled by a reduction in Ca^{2+} ATPase activity. For pooled data, a significant association was found between the rates of SR Ca^{2+} uptake and Ca^{2+} ATPase activity (r =0.833, P <0.001, Figure 4.4).

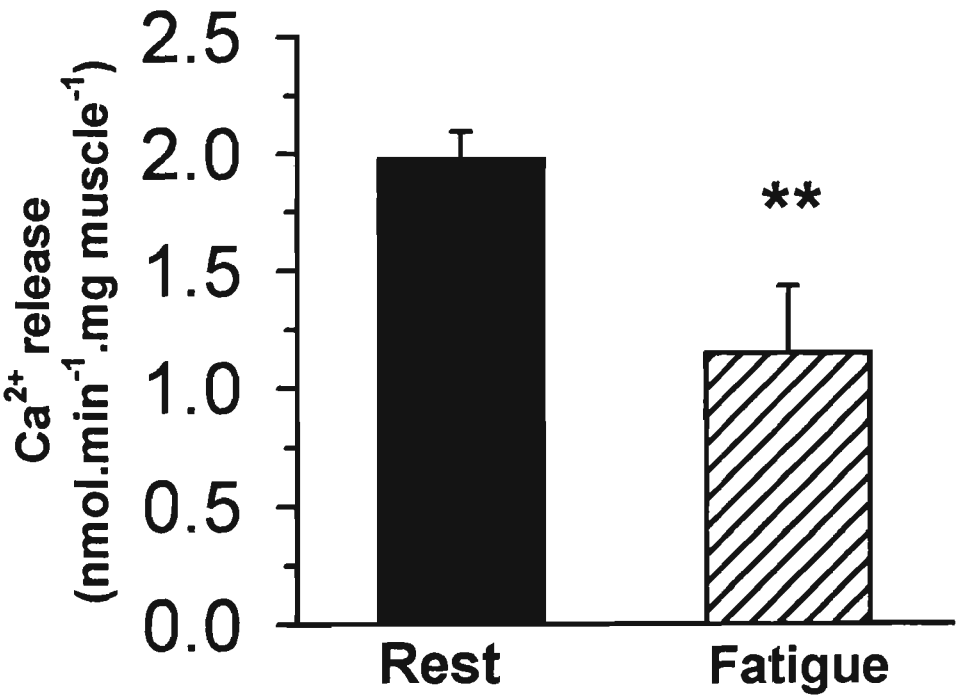


Figure 4.2 The maximal rate of SR Ca^{2+} release in muscle obtained at rest and at fatigue. Data are mean ± SEM, n=8; **P<0.01.

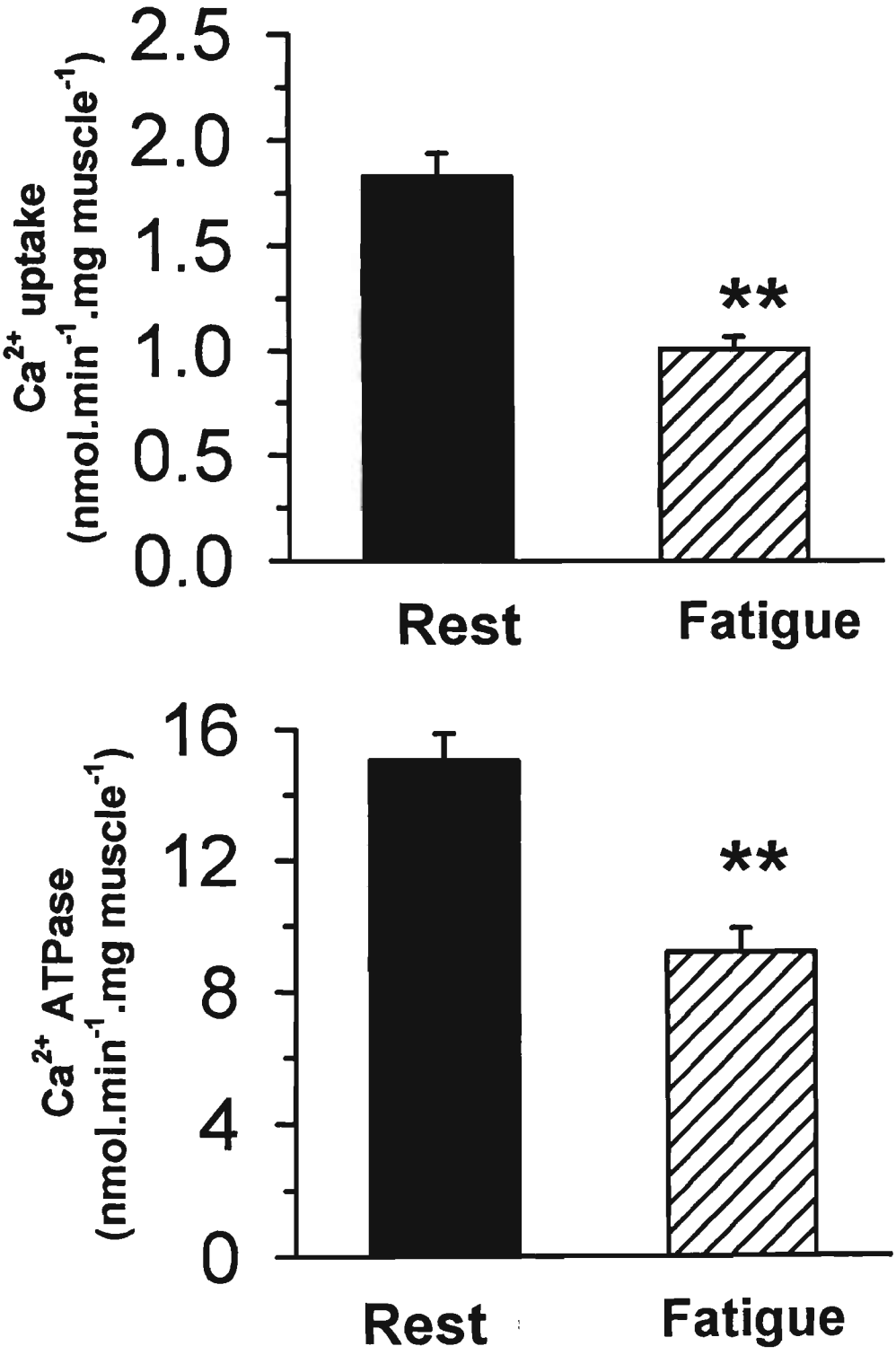


Figure 4.3 The maximal rate of SR Ca^{2+} uptake and Ca^{2+}ATPase activity in muscle obtained at rest and at fatigue. Data are mean ± SEM; n=8; $^{**}P<0.01$.

Similar depressions in muscle SR function were also apparent when expressed relative to muscle protein (Table 4.1). Thus, the maximum rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity expressed per unit protein were depressed in fatigued muscle by 41.0, 45.6 and 41.7 %, respectively ($P < 0.001$, Table 4.1).

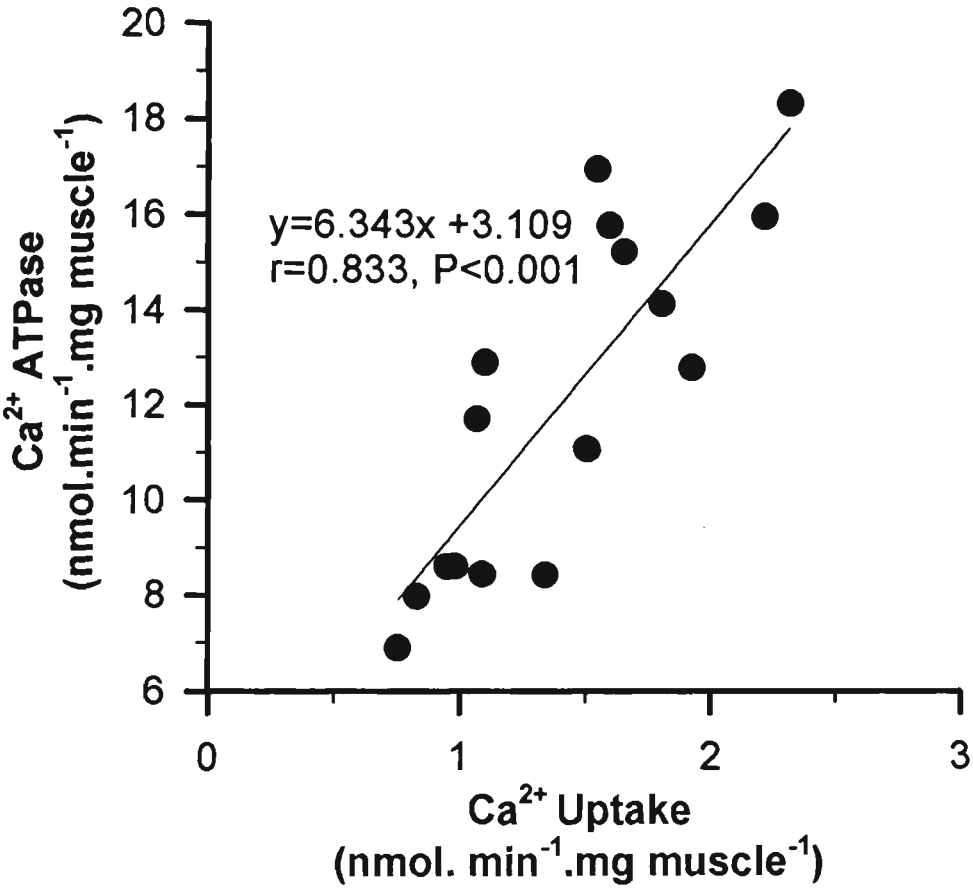


Figure 4.4 Relationship between SR Ca^{2+} uptake and Ca^{2+} ATPase activity, using pooled rest and fatigue data for 8 subjects.

Table 4.1 Effects of fatigue on SR Ca^{2+} release, uptake and Ca^{2+} ATPase activity in human skeletal muscle, expressed relative to muscle protein.

SR Characteristics	Resting	Fatigue
Ca^{2+}release	12.89 ±0.98	7.37 ± 0.60‡
Ca^{2+}uptake	11.89 ± 0.79	6.18 ± 0.40‡
Ca^{2+}ATPase activity	96.11 ± 2.89	55.98 ± 4.65‡

Date are mean ± SEM, n = 8; units: nmol.min^{-1}.mg $protein^{-1}$; ‡P<0.001.

4.3.2 Relationships Between Fibre Type, Fatigue and SR Function

The relationships between muscle Type II fibre proportions and fatigue indices are shown in Table 4.2. Only the FI based on the decline in work was significantly related to muscle fibre type (P<0.05).

Table 4.2 Relationships between muscle Type II fibre proportions and fatigue indices. n=8 for each regression.

Regression equation	r	P
Cybex test: FI_{torque} = (0.093 x Type II) + 41.447	0.211	ns
Biodex test: FI_{torque} = (0.4794 x Type II) + 23.637	0.530	ns
Biodex test: FI_{work} = (0.597 x Type II) + 15.241	0.762	P<0.05

ns not significant.

The relationships between muscle Type II fibre proportions and muscle SR function at rest and following fatigue, are shown in Table 4.3. The rate of SR Ca^{2+} uptake in resting muscle was positively related to the Type II fibre proportion (P<0.001).

However, no significant relationships were found between the resting rates of SR Ca^{2+} release or Ca^{2+} ATPase activity and the Type II fibre proportion (Table 4.3). The percentage depression in the SR Ca^{2+} ATPase activity in fatigued muscle was positively related to the Type II fibre proportion ($P<0.05$), but no significant relationships were found between the rate of SR Ca^{2+} release, or Ca^{2+} uptake and the Type II fibre proportion (Table 4.4).

No significant relationship was found between the SR Ca^{2+} ATPase activity and the proportion of Type II fibres. However the possibility of a Type II statistical error must be acknowledged due to the small sample size used (n=8 subjects). A later investigation showed significant association between these variables when a larger pool of subjects was investigated (n=24 subjects, Chapter 5), and is therefore consistent with the notion that SR Ca^{2+} ATPase activity is significantly related to muscle fibres type.

Table 4.3 The relationships between muscle SR function at rest and muscle Type II fibre proportions

Regression equation	r	P
Resting Ca^{2+}release = (0.019 x Type II) + 1.069	0.546	ns
Resting Ca^{2+}uptake = (0.027 x Type II) + 0.546	0.832	*P<0.001*
Resting Ca^{2+}ATPase activity = (0.103 x Type II) +10.222	0.416	ns

n=8 for each regression.

Table 4.4 The relationships between the percentage decline in muscle SR function at fatigue and muscle Type II fibre proportions. n=8 for each regression.

Regression equation	r	P
%Δ Ca^{2+}release = (0.005 x Type II) + 41.88	0.046	ns
%Δ Ca^{2+}uptake = (0.591 x Type II) + 15.21	0.376	ns
%Δ Ca^{2+}ATPase activity = (0.994 x Type II) - 8.078	0.791	$P<0.05$

4.3.3 Muscle Metabolites

In fatigued muscle, ATP content was decreased when analysed enzymatically ($P<0.01$) and tended to be less when analysed by HPLC ($P<0.08$, Table 4.5). Reductions were also found with fatigue in the muscle PCr content and pH ($P<0.01$), and in the glycogen content ($P<0.05$), whereas muscle IMP, lactate and creatine contents were elevated with fatigue ($P<0.01$, Table 4.5).

4.3.4 Relationships between Metabolites and SR Function

The relationships between SR function (nmol.min^{-1}.mg $muscle^{-1}$) and pooled metabolites (Rest plus Fatigue, mmol.kg^{-1} dw) are shown in Figures 4.5 to 4.9. SR Ca^{2+} release and Ca^{2+} uptake were positively related to the pooled ATP, PCr and glycogen contents, and pH ($P<0.005$, Figures 4.5, 4.7 and 4.9). The SR Ca^{2+} release and Ca^{2+} uptake were negatively related to the pooled muscle lactate, Cr and IMP contents ($P<0.05$, Figures 4.6 and 4.8). The relationships between SR Ca^{2+} ATP activity and these metabolites were similar to those found for Ca^{2+} uptake (data not shown).

Table 4.5 Muscle metabolites in rest and fatigue

Variable	Resting	Fatigue
ATP (Enzymatic)	25.67 ± 0.85	19.51 ± 1.71 **
ATP (HPLC)	24.71 ± 0.87	21.41 ± 1.43 *f*
ADP	2.77 ± 0.18	2.90 ± 0.47
AMP	0.13 ± 0.02	0.15 ± 0.02
TAN	27.62 ± 0.86	23.46 ± 1.54
IMP	0.08 ± 0.01	3.26 ± 0.84 **
PCr	93.3 ± 2.3	35.8 ± 3.5 **
Cr	42.0 ± 2.6	99.5 ± 4.6 **
Lactate	8.0 ± 0.8	91.6 ± 10.0 **
pH	7.17 ± 0.02	6.83 ± 0.03 **
Glycogen	469 ± 34	356 ± 31 *

Rest vs. fatigue: *f* $P<0.08$, * $P<0.05$; ** $P < 0.01$. Values are expressed in $mmol.kg^{-1}$ dry weight of muscle, except pH. Data are n=8, Mean ± SEM.

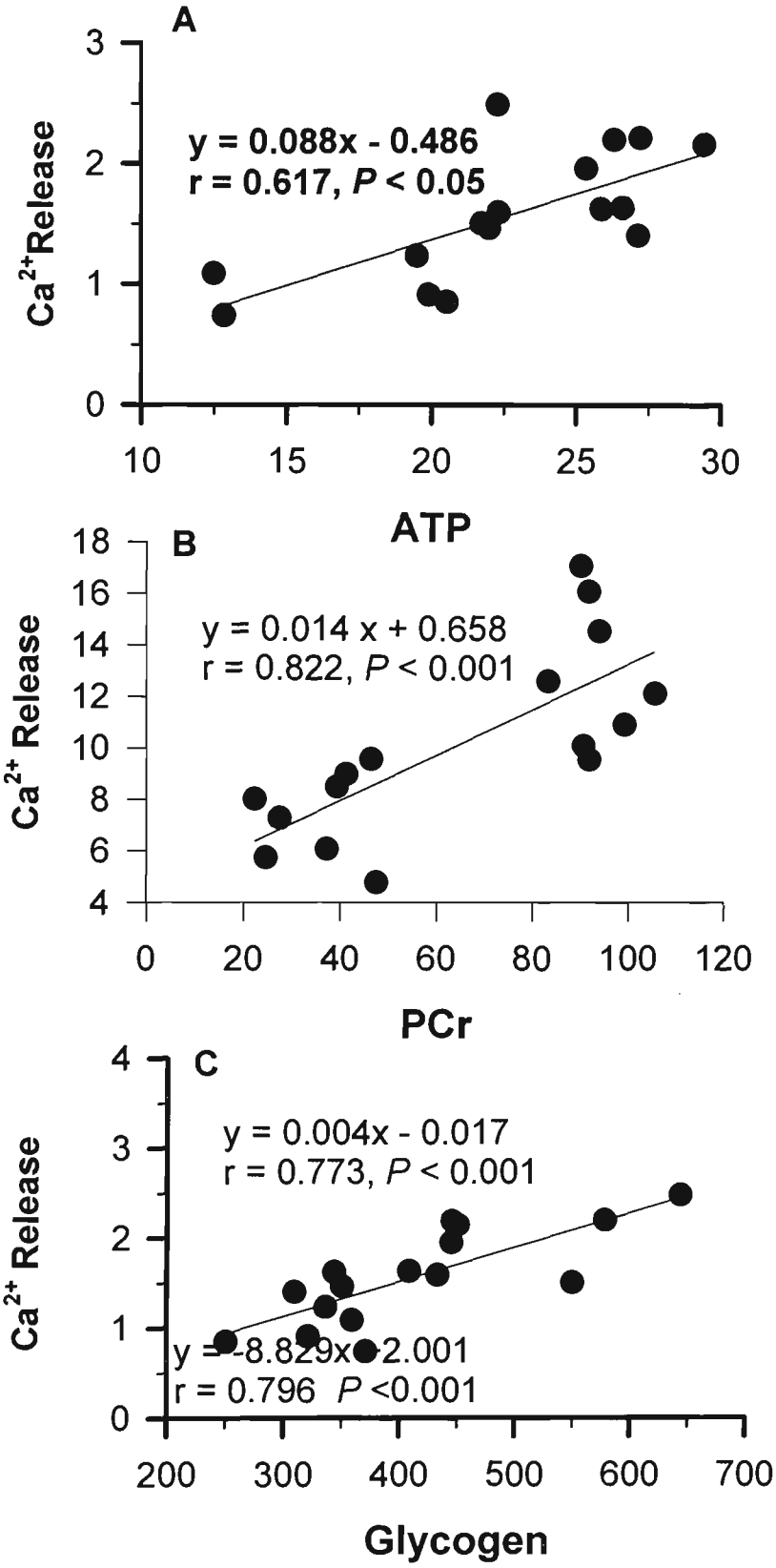


Figure 4.5 The relationships between SR Ca^{2+} release ($nmol.min^{-1}.mg\ muscle^{-1}$) and pooled (Rest, Fatigue) muscle ATP (A), PCr (B) and glycogen (C) contents ($mmol.kg^{-1}$ dm). n=8.

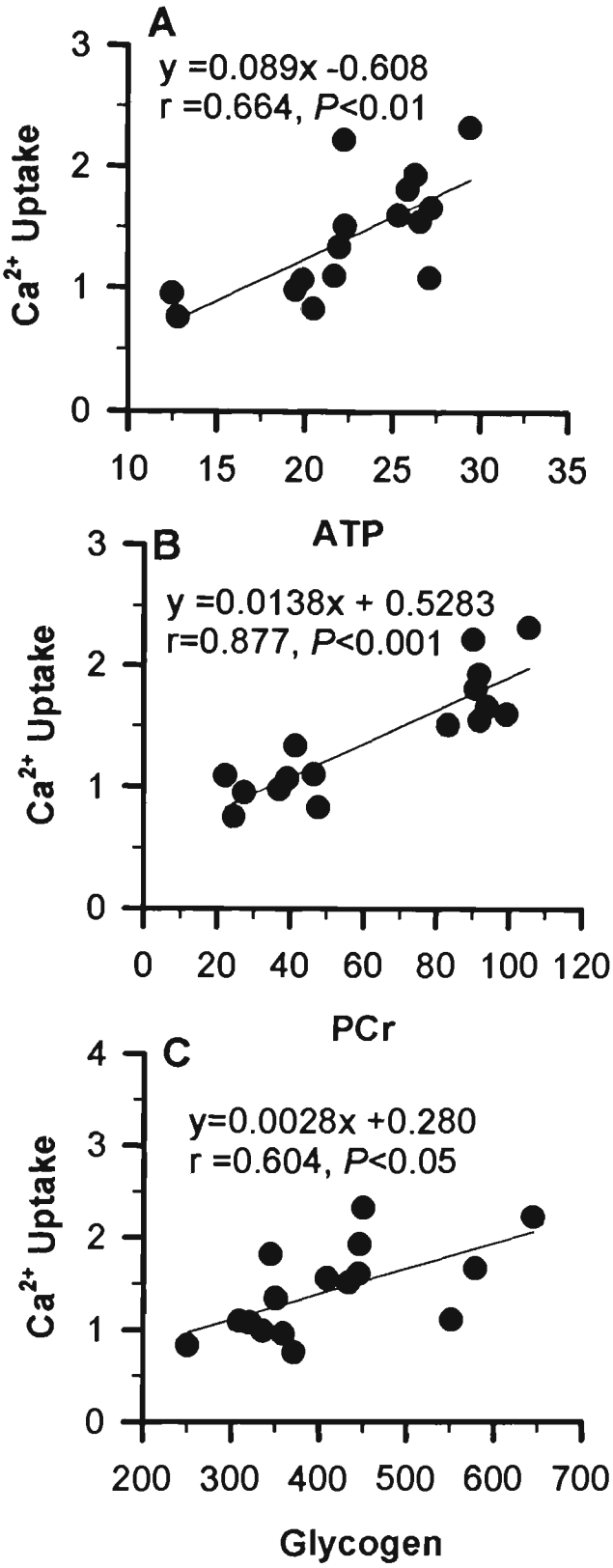


Figure 4.6 The relationships between Ca^{2+} uptake (nmol.min^{-1}.mg muscle^{-1}) and pooled (Rest, Fatigue) muscle ATP (A), PCr (B) and glycogen (C) contents (mmol.kg^{-1} dm) n = 8.

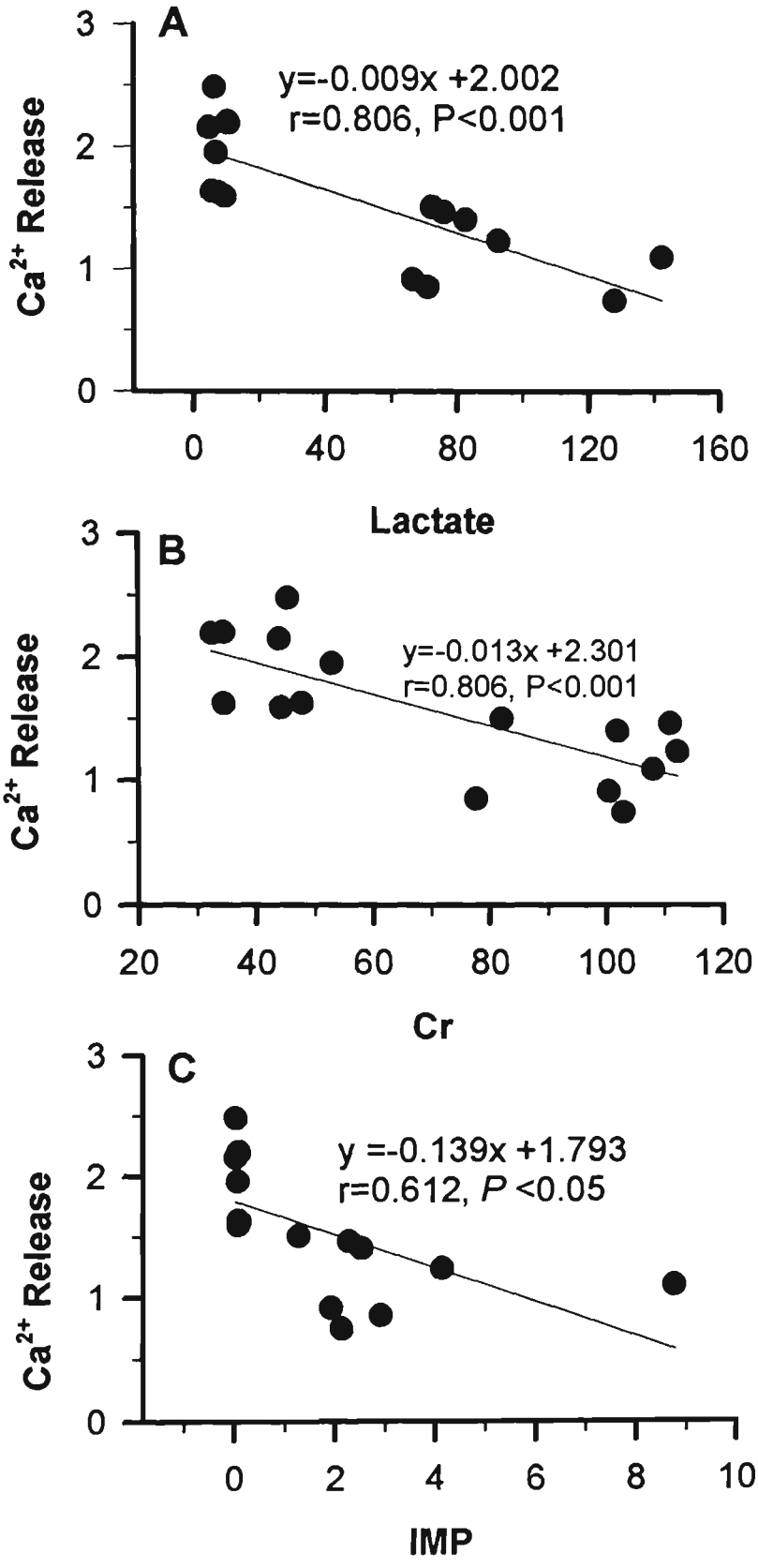


Figure 4.7 The relationships between Ca^{2+} release (nmol.min^{-1}.mg muscle^{-1}) and pooled (Rest, Fatigue) muscle lactate (A), Cr (B) and IMP (C) contents (mmol.kg^{-1} dm). n = 8.

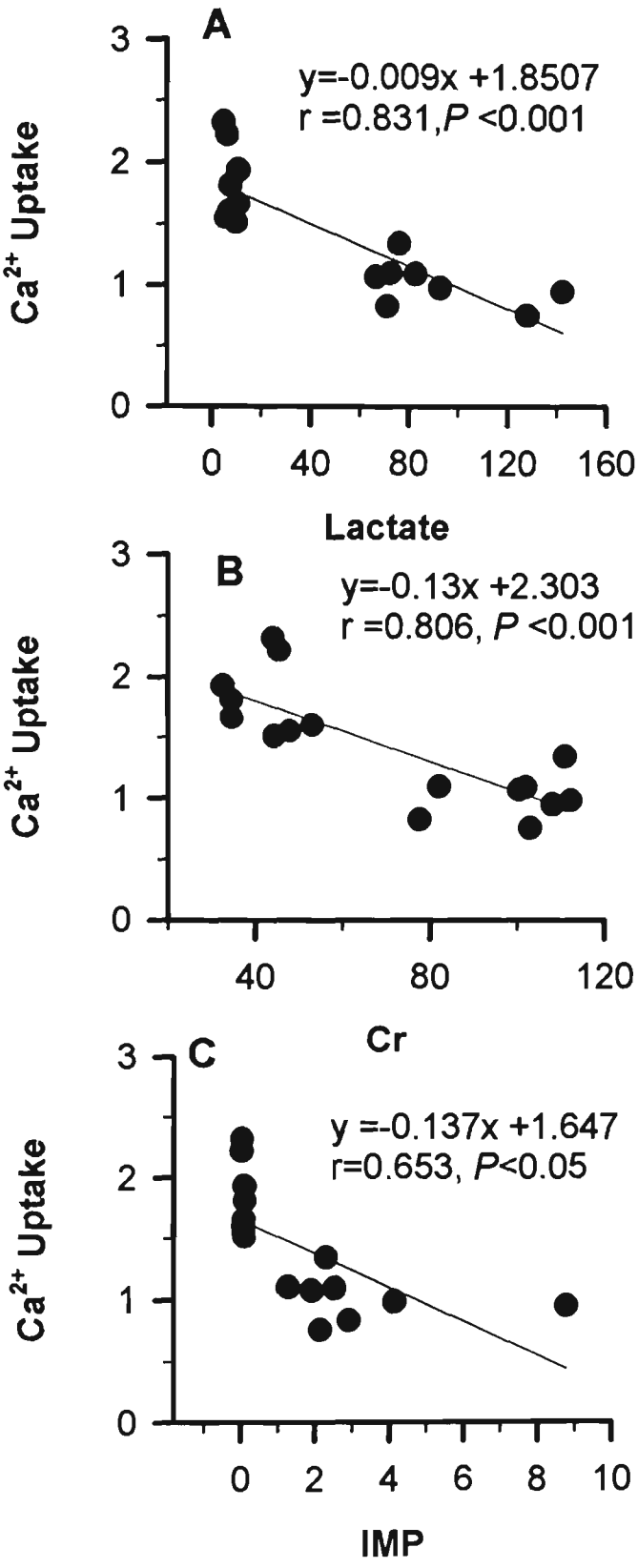


Figure 4.8 The relationships between Ca^{2+} uptake (nmol.min^{-1}.mg muscle^{-1}) and pooled (Rest, Fatigue) muscle lactate (A), Cr (B) and IMP (C) contents (mmol.kg^{-1} dm). n = 8.

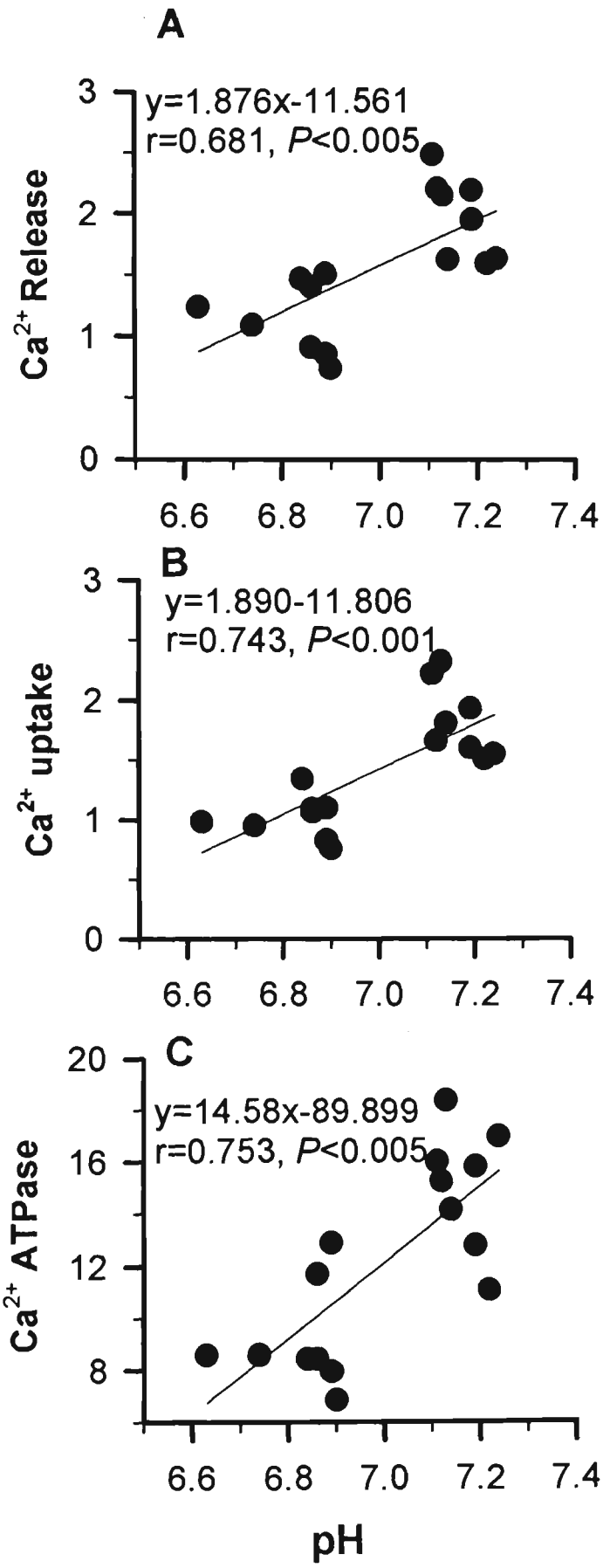


Figure 4.9 The relationships between Ca^{2+}release and Ca^{2+} uptake (nmol.min^{-1}.mg $muscle^{-1}$) and pooled (Rest, Fatigue) muscle pH n = 8.

4.4 Discussion

The major findings from this study were that marked depressions occurred with fatigue, in the maximal *in-vitro* rates of SR Ca^{2+} release (42%) and Ca^{2+} uptake (43%) in human muscle. These changes corresponded to a similar relative decline in knee extensor muscle torque during the repeated maximal contractions (43%).

4.4.1 Depression in SR Ca^{2+} Release After Intensive Fatiguing Exercise

This study showed for the first time a depression with fatigue in the *in-vitro* rate of SR Ca^{2+} release in a crude homogenate preparation from human skeletal muscle. SR Ca^{2+} release used was induced in this study by the addition of silver nitrate to the homogenate-buffer mixture, as previously documented in human skeletal muscle (Ruell et al. 1995). The 42% depression in human muscle SR Ca^{2+} release after fatiguing exercise is consistent with a previous report of decreased SR Ca^{2+} release (by 20-30%), also using silver nitrate as the releasing agent, in rat red portion of the gastrocnemius muscle SR vesicles following prolonged exercise to fatigue (Favero et al. 1993). In that study, the rate of rise in homogenates [Ca^{2+}] was measured by a Ca^{2+}-selective electrode and the depressed SR Ca^{2+} release was accompanied by a similar decline in the [^{3}H] Ryanodine binding capacity. This indicated that the reduced rate of Ca^{2+}release was due to a reduction in the number of functional Ca^{2+} release channels (Favero et al. 1993). Further, the present results are consistent with indirect measurements of depressed SR Ca^{2+} release in isolated animal single muscle fibres, as demonstrated by reduced cytosolic [Ca^{2+}] during repeated tetanii (see review by Westerblad et al. 1996c). In such experiments with mouse fibres, tetanic [Ca^{2+}] had fallen to ~50% of control levels when tension was halved with fatigue (Allen et al. 1989, Westerblad et al. 1990). Since the rate of Ca^{2+} uptake also declined with fatigue, and intra-SR stores could be liberated with caffeine or K^+-contractures, the findings of Allen, Westerblad and colleagues demonstrate a failure of SR

Ca^{2+} release. Similar results have been shown in amphibian muscle with fatigue (Westerblad and Lannergren 1993) Since the studies were performed with intact fibres, the failure in SR Ca^{2+} release may have been due not only to a failure in the RyR, but may also included inactivation of the DHPR and/or t-tubular membranes. Further, the reduced rate of SR Ca^{2+} uptake with fatigue partially compensated for the decline in Ca^{2+}release by maintaining an elevating cytosolic [Ca^{2+}]. In summary, the decline in SR Ca^{2+} release toward the end of the fatiguing contractions would have been larger than the 50% decline suggested by the fall in tetanic [Ca^{2+}] (Westerblad et al. 1990, 1991). In single frog fibres a marked reduction in SR Ca^{2+}release was also observed (Williams et al. 1997). Thus, the present results in human muscle are consistent with findings in other species, which have utilised different experimental methods. This suggests that a depression in SR Ca^{2+}release with fatigue is common in human, other mammalian and amphibian muscles.

A comparison of the decline in SR Ca^{2+} release with fatigue in this versus other studies is shown in Figure 4.10. The extent to which SR Ca^{2+}release is depressed with fatigue probably depends on both the exercise intensity and duration and thus may have highly specific underlying mechanisms. A lesser depression in SR Ca^{2+} release was found after prolonged exercise to fatigue (Favero et al. 1993) than after intensive short term exercise (present study).

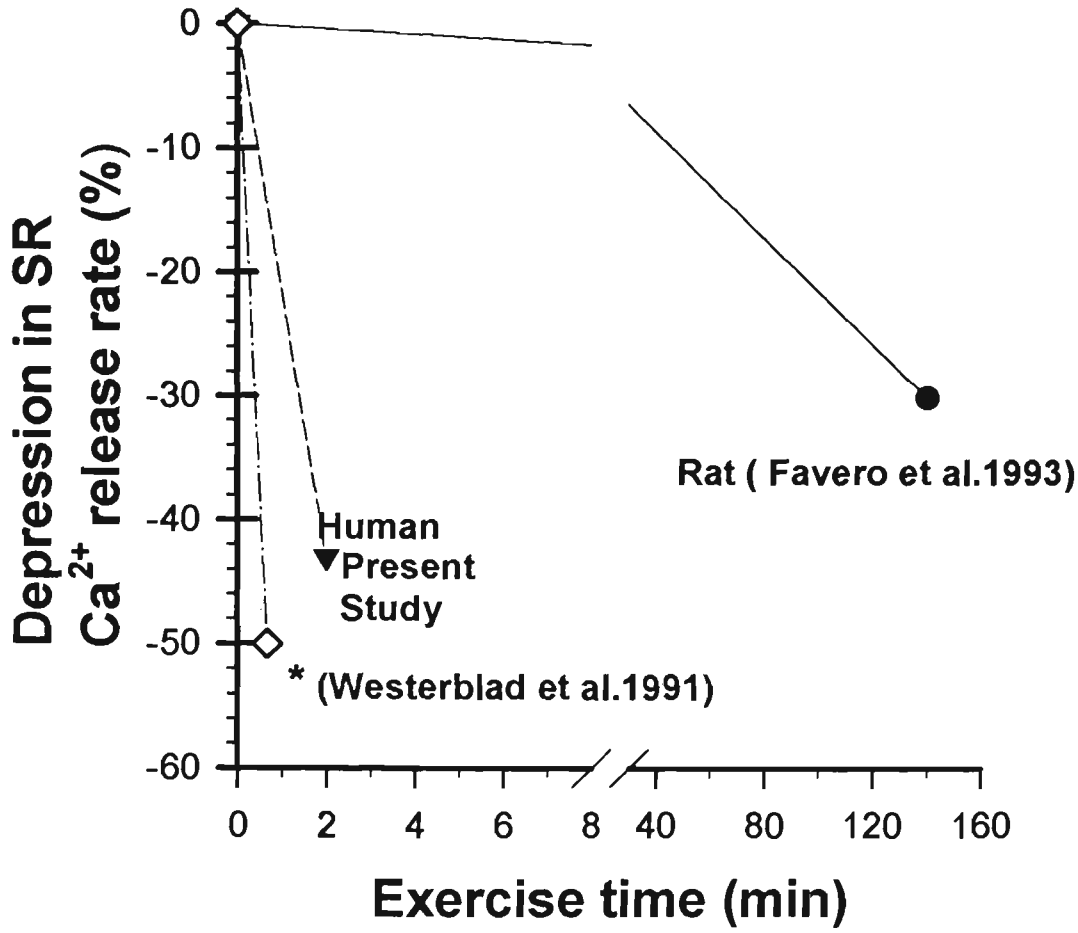


Figure 4.10 The percentage decline SR Ca^{2+} release in rats and human skeletal muscle with fatigue.

*The decline was estimated from the percent fall in cytosolic [Ca^{2+}] during tetanic contractions.

4.4.2 Critique of Method Used to Induce SR Ca^{2+} Release.

There are many possible causes for a reduction in SR Ca^{2+} release in fatigue. These include a possible failure of action potential propagation, most likely in the T-tubular membranes, a disruption to the mechanical linkage between the DHPR and the RyR (Ca^{2+} release channel), as well as a reduced Ca^{2+} content in the SR. The present method of

measuring release from SR vesicles in a crude homogenate cannot determine the function of T-tubular membranes, DHPR function, nor of SR Ca^{2+}content. Thus, the failure of SR Ca^{2+} release in this study reflects only a failure at the RyR. One criticism of the method used is that Ag^{+} is not a physiological trigger for SR Ca^{2+}release. Thus, the specificity of Ag^{+} for the physiological Ca^{2+} release channel, the RyR, and the validity of the Ag^{+} - induced Ca^{2+} release method, must be addressed. Several findings by others indicate that Ag^{+} probably interacts at the RyR (Salama and Abramson 1984). It has been shown that heavy metals (i.e. Ag^{+}) induce Ca^{2+} release from SR vesicles in the presence of catalysts in standard modulators of the RyR and sulfhydryl (SH) oxidizing agents (Hilkert et al. 1992, Salama et al. 1984). Conversely, sulfhydryl reducing agents reversed the effects of SH oxidizing agents and inhibited SR Ca^{2+} release (Trimm et al. 1986). The most important evidence supporting the direct affect of Ag^{+} on the RyR is that known RyR blocking agents inhibited Ag^{+}-induced Ca^{2+} release, when measured in purified 106 kD Ca^{2+} release channels isolated from rabbit white skeletal muscle (Hilkert et al. 1992). Each of dithiothreitol (DTT), [Mg^{2+}] (mM), ruthenium red, [Ca^{2+}] (mM) and Ryanodine (5nM) inhibited Ag^{+}-induced Ca^{2+} release. Further, Ca^{2+} release could be induced from these channels by known activators of the RyR (Zucchi and RoncA-Testoni 1997), including ATP (mM), Ca^{2+} (μM) and Ryanodine (0.5–2 nM). These purified 106 kD channels were shown to have similar functional properties to the larger 565 kD junctional foot protein (RyR) and were suggested to be a fragment or a subunit of the RyR, which retained the normal regulatory sites (Hilkert et al. 1992). Importantly, Ag^{+} has also been shown to cause a transient increase in the open time of purified 565 kD junctional foot proteins (Nagasaki et al. 1989). Other studies have found qualitatively similar effects on SR Ca^{2+} release between Ag^{+} and other channal modulators. For example, the depressive effects of lactate on SR Ca^{2+} release were demonstrable via Ca^{2+} release induced by Ag^{+} and by two other RyR activators, Doxorubicin and H_2O_2, all of which were consistent with a reduction

in [$^3H^+$] ryanodine (Favero et al. 1995). Therefore all of these findings strongly indicate that Ag^+-induced Ca^{2+} release is via the RyR.

It has been shown that the physiological mechanism for opening of the RyR is linked with mechanical interaction between the DHPR and the RyR (Chapter 2.1). Ag^+ opens the RyR via oxidation, but oxidation of the RyR does not appear to be physiologically important, at least in isolated skinned muscle fibres (Lamb et al. 1995). Despite these criticisms, it appears that Ag^+ can stimulate Ca^{2+} release from SR vesicles. However, it should be noted that similar "non-physiological" criticisms might be made of the caffeine method commonly used to induce SR Ca^{2+} release in isolated preparations.

After addition of the specific Ca^{2+} ATPase inhibitor cyclopiazonic acid to the assay medium, which inhibited Ca^{2+} uptake, a small rise in extravesicular [Ca^{2+}] occurred, reflecting a Ca^{2+} leak through the vesicle membrane or the Ca^{2+} release channels. This leak may result in an underestimate of the maximal rate of Ca^{2+} release in this preparation. However, this does not limit the interpretations of this study, since identical conditions were used pre- and post– fatiguing exercise (Appendix Table A3).

A further criticism of the Ag^+ -induced Ca^{2+} release method was recently made by Warmington et al. (1996). They concluded that Ag^+ could only induce Ca^{2+} release from SR vesicles from Type II fibres, since release could be obtained in the fast-twitch EDL muscle, but not in the slow-twitch soleus muscle in rats. They used a similar *in-vitro* methodology, but used Fura-2 to measure extravesicular Ca^{2+}. In this thesis Indo-1 was used to monitor extravesicular [Ca^{2+}], and Ag^+-induced Ca^{2+} release could be measured from SR vesicles in both soleus and EDL muscles in the rat (see Chapter 7). This finding suggests that Ag^+ can induce SR Ca^{2+} release in both Type I and II fibres. The different findings in two studies may be related to the different fluorescent dye used. The mechanism(s) underlying the possible differences in responses to Indo-1 and Fura-2 is not known.

4.4.3 *Mechanisms of Depressed SR Ca^{2+} Release with Fatigue*

Fibre-type effects. The decline in peak torque during repeated maximal knee extensor contractions was previously shown to be related to the proportion of Type II muscle fibres in the vastus lateralis muscle (Thorstensson et al. 1976b). In the present study, no significant relationship was found between the percentage decline in peak torque and the Type II fibre proportion. However, a significant relationship was found between the fatigue index calculated from the peak work decrement against fibre type. Therefore, it of interest to determine whether SR function was depressed to a greater extent in those subjects with a higher proportion of Type II fibres. No significant relationship was found between the percentage change in SR Ca^{2+} release and the proportion of Type II fibres, suggesting no clear fibre-type dependence on the deterioration in SR function with fatigue. However, the possibility of a Type II statistical error must be acknowledged with regard to the fibre-type dependency of the decline in peak torque with fatigue, as well as that of the depression in SR Ca^{2+} release, due to the small sample size used (n=8 subjects). A later investigation showed significant association between these variables when a larger pool of subjects was investigated (n=24 subjects, Chapter 5), and is therefore consistent with the notion that SR function is depressed to a greater extent in Type II muscle fibres. This possibility is also consistent with the reported greater susceptibility to fatigue in Type II fibres (Stephenson et al. 1998).

Metabolic Effects. Significant relationships were seen between the depression in SR Ca^{2+} release with fatigue and the availability of ATP, CP and glycogen, as well as the accumulation of metabolites, lactate and IMP and the depression in pH. Two different interpretations from this data are possible. Firstly, these relationships may merely reflect parallel physiological events, such that severe metabolic disturbances are found with fatiguing contraction and that SR Ca^{2+} release is depressed independent of these. The

second interpretation is that a causal relationship exists between these metabolic perturbations and the depressed SR Ca^{2+} release with fatigue.

The depression that we observed in SR function occurred whilst assayed under optional *in-vitro* conditions, with respect to muscle pH (7.0), ATP concentration (4.5 mM) and temperature (37 C). Thus, structural alterations are implicated in the observed SR changes, as previously concluded for the depression in SR Ca^{2+} uptake (Booth et al. 1997, Hargreaves et al. 1998). Therefore, any causal relationship between metabolic perturbations and SR Ca^{2+} release must be one that induced *in-vivo* structural alterations, that remained after muscle excision and were then detected in our *in-vitro* assay. Each of depressed ATP (and by implication CP and glycogen) and an accumulation of lactate within muscle have been shown to depress SR Ca^{2+} release in an intact cell (Allen et al. 1997), or in isolated vesicles (Favero et al. 1995). However, to date there does not appear to be evidence that these metabolic changes induce structural impairments in the RyR. Acidosis reduced RyR opening, when RyR were first isolated and then inserted into lipid bilayers (Ma et al 1994). In contrast, when an intact muscle preparation was used acidosis did not reduce muscle force and by implication, SR Ca^{2+} release (Lamb and Stephenson 1994). Therefore the most likely interpretation from this data is that the marked muscle metabolic changes measured and those of impaired *in-vitro* SR Ca^{2+} release reflect parallel rather than causal processes. Nonetheless, the differences in the severity of *in-vivo* more than *in-vitro* changes cannot be determine in this study, it is possible that the *in vivo* depression in SR Ca^{2+} release with fatigue may be greater than that observed *in-vitro*. For example Ca^{2+} release rate was decreased by at least 50% in single isolated mouse muscle with fatigue, when Ca^{2+} release was estimated from reductions in cytosolic [Ca^{2+}] (Westerblad et al. 1990). Therefore, the marked metabolic perturbations with fatigue most likely do contribute to decreased *in vivo* Ca^{2+} release and directly contribute to the decline seen in muscle torque. Consequently, the observed *in-vitro* reduction in Ca^{2+} release must

be due to other factors.

A possible mechanism of the depression in SR Ca^{2+} release with fatigue is an elevation of reactive oxygen species (ROS) in muscle. Cellular accumulation of free radicals was first implicated with an impaired SR membrane integrity and indicated as a possible cause of exercise-induced damage to skeletal muscle SR by Davies et al. (1982). Resting muscle produces free radicals and their production increases greatly in contracting muscle (Davies et al. 1982, Reid et al. 1992). Elevated levels of ROS can induce oxidation of proteins and lipid peroxidation (Neuzil et al. 1993, Ragaru et al. 1993), that conceivably could directly affect the RyR/ Ca^{2+} release channel. High concentrations of ROS inhibit Ca^{2+} release in skinned rat, as well as toad fibres (Brotto and Nosek 1996, Oba et al. 1996, Posterino and Lamb 1996) and in rabbit muscle homogenates (Meszaros et al. 1996). Exposure of rat muscle and single frog muscle fibres to 1-6 mM H_2O_2 for 5 -15 minutes dramatically inhibited SR Ca^{2+} release (Brotto and Nosek 1996, Oba et al. 1996). In contrast, $\geq$ 1mM H_2O_2 increased peak twitch stress, lengthened time-to-peak tension and half-relaxation time, in muscle fibre bundles from rat diaphragm (Reid et al. 1992). However, when the fibres were exposed to catalase (1 to 10^5 U/ml), which scavenges H_2O_2, the above twitch characteristics and the twitch-to-tetanus force ratio were decreased in a dose dependent manner (Reid et al. 1993). Also 5 mM H_2O_2 increased ryanodine binding in SR vesicles isolated from fast-twitch skeletal muscle and caused a rapid SR Ca^{2+} release in rabbits (Favero et al. 1995). The major difficulty in interpreting these studies is that the H_2O_2 concentration in skeletal muscle is not known. The role of nitric oxide (NO) in fatigue during voluntary exercise has not yet been directly tested, but skeletal muscle NO release is increased with electrical stimulation (Mészáros et al. 1996). Also, NO depresses Ca^{2+} release and contractile force in SR vesicles and striated muscle (Kobzik et al. 1994, Mészáros et al. 1996). NO reduced by 60% the rate of Ca^{2+} release from isolated skeletal muscle SR and skeletal muscle homogenates in the rabbit (Mészáros et al. 1996).

Alterations in muscle SR membrane structure and protein rotational mobility, using electron spectroscopy, was ruled out as possible factors in the depression in Ca^{2+} uptake after treadmill exercise in horses (McCutcheon et al. 1992). Of possible importance, SR area was increased ~1.6 fold after exercise at intensities greater than 40 % $\dot{V}O_2$ peak, with notable changes in the lateral sacs of the SR terminal cisternae. Smaller increases also occurred in response to exercise at 40% of the $\dot{V}O_2$ peak. An implication of these findings is that increased ROS in muscle leads to structural changes in SR membranes, causing depressed SR Ca^{2+} release with fatigue. As discussed in a later section, SR Ca^{2+} uptake is sensitive to increased muscle temperature, which might also adversely affect SR release. However, whether muscle temperature impairs SR Ca^{2+} release remains to be clarified.

Several studies have investigated whether an increased intracellular [Ca^{2+}] might play an important role during fatigue in animal muscle (Lamb et al. 1995, Chin and Allen 1995, Willams 1997, Allen et al. 1998). These studies have shown that a raised intracellular [Ca^{2+}] for even a short duration of several seconds, impairs RyR function, Ca^{2+} release and force production."

4.4.4 Depression in SR Ca^{2+} Uptake and Ca^{2+} ATPase activity with Fatigue

Human muscle homogenate SR Ca^{2+} uptake and Ca^{2+} ATPase activity were depressed after intense fatiguing exercise by 43% and 39%, respectively. These variables were highly correlated, consistent with previous findings after prolonged exercise in human skeletal muscle (Booth et al 1997). Similar SR Ca^{2+} uptake methods have been employed in numerous studies investigating the effects of voluntary exercise on SR function in animal muscles (Byrd et al. 1989a and 1989b). These studies have reported 13% to 57% depressions in SR Ca^{2+} uptake and Ca^{2+} ATPase in different muscles (see Chapter 2, Table

2.7 and Figure 4.11). Similar methods have also been employed in studies with humans, yielding 27-68% depressions in the rate of Ca^{2+} uptake with fatigue (Gollnick et al. 1991, Warmington, 1997, Hargreaves et al. 1998). The mechanisms underlying the reduction in SR Ca^{2+} uptake with fatigue do not appear to have a simple dependency on exercise duration or intensity. Interestingly, Ca^{2+} uptake was similarly reduced after exercise to fatigue at very different intensities and durations, declining by 65% after 1.4 min at 72% $\dot{V}O_2$peak and by 68% after 111.4 min at 140% $\dot{V}O_2$peak (Warmington, 1997). However, using the same technique, after 3 maximal 30 s sprint bouts Ca^{2+} uptake was reduced by 28 % (Hargreaves et al. 1998). In contrast, the reduction in the present study was more than 2-fold greater than after prolonged fatiguing exercise (43% vs 17%, Booth et al. 1997).

4.4.5 Critique of SR Ca^{2+} Uptake Method.

The method used to determine SR vesicle Ca^{2+} uptake was very similar to that previously described (Ruell et al. 1995). Some minor modifications employed were the use of a higher oxalate concentration which allowed increased vesicle Ca^{2+} loading and the deletion of dithiothreitol (DTT) from the homogenate medium, since DTT can inhibit SR Ca^{2+} release. The specificity of the Ca^{2+} uptake method is demonstrated by the near-complete inhibition of Ca^{2+} uptake after addition of the specific Ca^{2+} ATPase inhibitor cyclopiazonic acid to the assay medium (Ruell et al. 1995). This inhibition was also found using this slightly modified method (data not shown). An artifactual drop in extravesicular $[Ca^{2+}]$ occurs when homogenate is added to the assay medium, which partially obscures vesicle Ca^{2+} uptake (Ruell et al. 1995). This may result in an underestimate of the maximal rate of Ca^{2+} uptake in this preparation. However, this does not limit the interpretations of this study, since identical conditions were used pre- and post– fatiguing exercise.

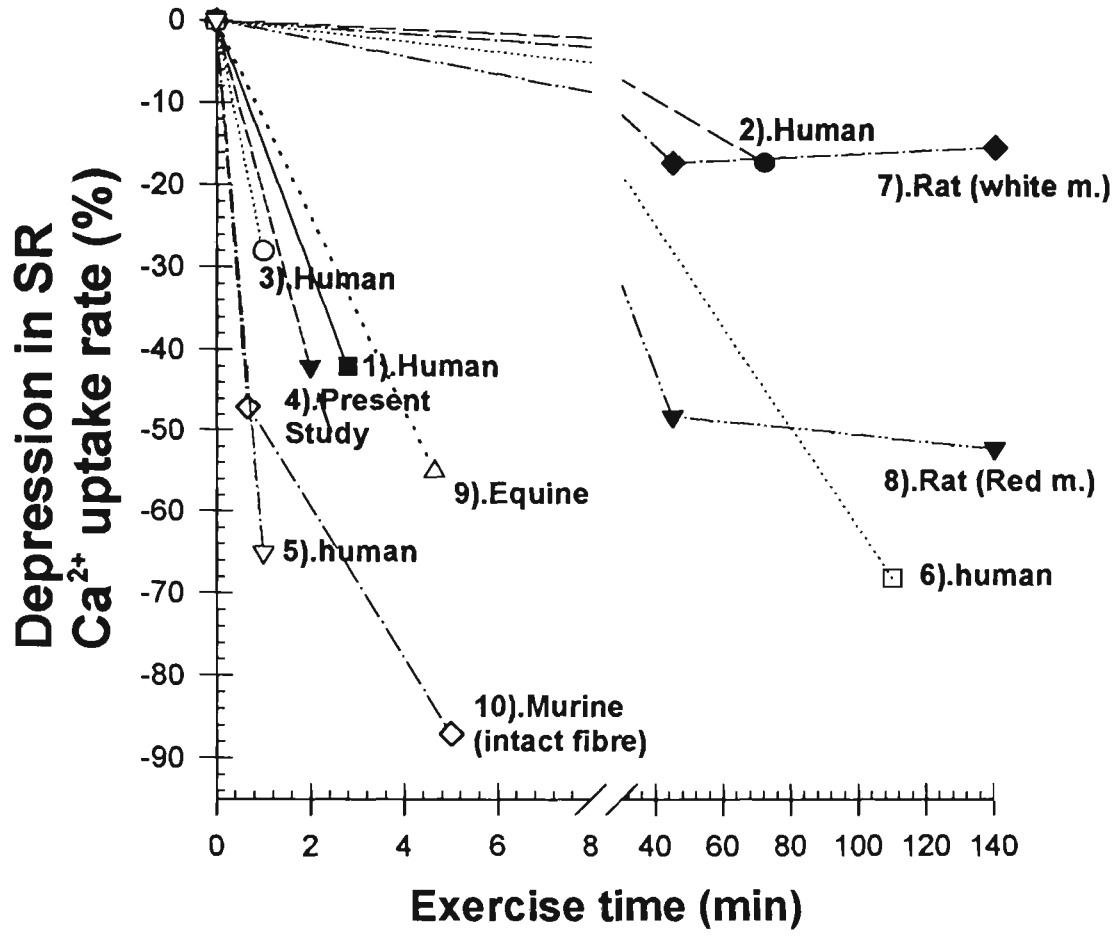


Figure 4.11 The effects of fatigue on the decline in SR Ca^{2+} uptake and Ca^{2+}ATPase activity in human and animal skeletal muscle.

References: 1. Gollnick et al. (1991); 2. Booth et al. (1997); 3. Hargreaves et al. (1998); 4. Present study; 5, 6, Warmington (1997) after 72% and 140%VO_2 peak, respectively; 7, 8. Byrd et al. (1989a) red and white gastrocnemius in rat muscle, respectively; 9. Byrd et al. (1989b); 10. Westerblad and Allen (1993).

4.4.6 *Mechanism of Impaired SR Ca^{2+} Uptake with Fatigue*

The reduction in SR Ca^{2+} uptake following intense exercise was paralleled by a reduction in Ca^{2+} ATPase activity, supporting previous studies in humans and in most animals (Chapter 2, Table 2.7). Each of the studies investigating the effects of voluntary exercise on SR Ca^{2+} uptake in humans have shown a depression (Gollnick et al. 1991, Booth et al. 1997, Warmington 1997, Hargreaves et al. 1998). Some conflicting findings exist however, with several studies reporting an uncoupling between SR Ca^{2+} uptake and Ca^{2+} ATPase activity in rat muscle (see Table 2.7). In contrast, an increased Ca^{2+} ATPase activity was found in prolonged exercise or ischaemic rat muscle (Ferrington et al. 1996, Green et al. 1996). These findings cannot be explained by differences in fibre type and methods used to determine SR function, but may reflect differences in the responses of SR to exercise in different species. The effects of differences in the exercise model are still unclear.

Possible causes of reduced Ca^{2+} ATPase activity after voluntary exercise are changes to the membrane environment surrounding the enzyme, or conformational changes in the Ca^{2+} ATPase structure (Byrd et al.1989, Luckin et al. 1991, Byrd 1992, McCutcheon et al. 1992). Increased muscle temperature can markedly affect SR Ca^{2+} uptake and Ca^{2+} ATPase activity. When muscle temperature was increased to 40 °C for only one min, SR Ca^{2+} uptake was reduced in both EDL and SOL muscles in the rat (Warmington, 1997). Passive heating of the leg to 35.8 °C in humans did not reduce vastus lateralis SR Ca^{2+} uptake (Warmington, 1997). Increased Ca^{2+} ATPase activity occurred when muscle temperature was increased from 15 °C to 42.5 °C (Inesi et al. 1973, Luckin et al. 1991), but inactivation of Ca^{2+} ATPase activity resulted when the temperature reached 50 °C (Inesi et al. 1973). Uncoupling between Ca^{2+} uptake and Ca^{2+} ATPase activity occurred at temperatures in excess of 40 °C (Inesi et al. 1973). Davidson and Berman (1996) recently showed that 7-15% reductions in ATP hydrolysis and steady-state phosphorylation could

cause complete loss of Ca^{2+} uptake. They concluded that a Ca^{2+} leak pathway in a small fraction of pump units was sufficient to uncouple the entire vesicle with less than 15% loss of ATPase activity. However, no uncoupling was found between Ca^{2+} uptake and Ca^{2+} ATPase activity at fatigue in the present study. This finding is consistent with previous findings after prolonged exercise (Booth et al. 1997), suggesting that thermal inactivation of Ca^{2+} ATPase did not occur.

Fibre-type effects. Conflicting findings were found on the relationships between fibre type and the percentage decline in SR Ca^{2+} uptake and Ca^{2+} ATPase activity with fatigue. Thus, the percentage depression in SR Ca^{2+} ATPase activity with fatigue was positively related to the Type II fibre proportion, but that of SR Ca^{2+} uptake was not. Therefore, no clear fibre-type dependence on the deterioration in SR Ca^{2+} uptake with fatigue was apparent, which may be related to the small sample size.

Metabolic effects.

Although significant relationships were found between *in vitro* SR Ca^{2+} uptake and the marked *in-vivo* metabolic perturbations, these probably do not reflect causal events, for similar reasons as described earlier for SR Ca^{2+} release. Thus, whilst a reduction in muscle pH can reduce Ca^{2+} uptake and Ca^{2+} pump activity *in* single fibre preparations (Lamb and Stephenson 1994), acidosis cannot explain the present *in vitro* depressions in SR Ca^{2+} uptake and Ca^{2+} pump activity, unless acidosis induces long-lasting effects.

The process of SR Ca^{2+} uptake requires ATP hydrolysis and therefore a decline in the intracellular [ATP] may adversely affect SR Ca^{2+} ATPase activity and Ca^{2+} uptake. The local ATP concentration is probably most important in this regard (Toyoshima et al. 1993, Han et al., 1992, Owen et al. 1996). A glycogenolytic-SR complex exists and efficient Ca^{2+} uptake involves the sequential action of glycogen phosphorylase, phosphoglucomutase and hexokinase, which generate low concentrations of ATP compartmentalized in the immediate vicinity of the SR Ca^{2+} ATPase (Nogues et al. 1996).

The present study showed reductions with fatiguing exercise in the metabolic fuels ATP, PCr and glycogen. It is possible that these metabolic alterations result in an *in-vivo* impairment in SR function. However, these metabolic perturbations most likely cannot explain the *in vitro* depression of SR Ca^{2+} pump activity in this study, unless persistent conformational changes occur. As with SR Ca^{2+} release, the impairment in SR Ca^{2+}-ATPase with fatigue may be linked with an intracellular accumulation of ROS, which induces structural damage and thus, disrupts the normal Ca^{2+} handling kinetics in the cell. The effects of ROS on skeletal muscle SR have been examined in some studies (Yu et al. 1974, Kondo and Kassai 1974, Scherer and Deamer 1986, Castilho et al. 1996, Xu et al. 1996, Byrd 1992). SR Ca^{2+}-ATPase activity was decreased by NO which may be elevated by SH groups oxidation (Byrd 1992). Recent studies have shown that the hydroxyl radical (•OH), H_2O_2, , AAPH-derived peroxyl radicals and HOCI (hypochlorous acid) inhibited skeletal muscle SR Ca^{2+} ATPase activity (Xu et al. 1996, Castilho et al. 1996, Viner et al. 1997, Favero et al. 1998).

4.4.7 Implications of Depressed SR Ca^{2+} Release and Ca^{2+} Uptake with Fatigue

Fatigue is characterised by reductions in maximal force and in the peak rate of force development, as well as by a marked slowing of muscular relaxation (Vøllestad et al. 1988). In the present study a large decline in muscle torque was found during 50 repeated maximal contractions. The decline in SR Ca^{2+} release with fatigue in the current study would most likely cause a reduction in the tetanic cytoplasmic $[Ca^{2+}]$. In isolated single fibres, a large fall in cytoplasmic $[Ca^{2+}]$ with fatigue explained much of the reduction in muscle isometric force (Westerblad et al. 1990, Westerblad et al. 1993). The decrease in the cytoplasmic $[Ca^{2+}]$ transient during fatigue may also reduce the maximal velocity of shortening (Julian et al. 1986, Moss et al. 1995). Thus, depressed SR Ca^{2+}release most

likely induced reductions in both the peak muscle force and the maximal velocity of shortening, leading to the severe reduction in peak torque observed with fatigue.

Fatigue decreased SR Ca^{2+} uptake, which was previously suggested to cause a slowing in muscle relaxation (Gollnick et al. 1991). However, previous studies in humans after prolonged exercise and in amphibian muscles with electrical stimulation, have demonstrated that a reduction in Ca^{2+}uptake rate does not necessarily lead to a slowing in the rate of muscle relaxation (Westerblad et al. 1991, Booth et al. 1997). Thus, the marked slowing of muscular relaxation with fatigue most likely reflects other processes, such as slowed detachment of cross bridges. The slowed relaxation with fatigue would tend to preserve torque during repeated contractions. A reduced SR Ca^{2+} uptake would however lead to elevations in resting cytosolic [Ca^{2+}], which might lead to deterioration in both SR RyR and Ca^{2+}ATPase (Chin and Allen 1995, Lamb et al. 1995, Williams 1997).

4.5 Conclusions

This study showed for the first time a depression in the *in-vitro* rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity with intense exercise in human skeletal muscle homogenate preparations. The depressions in SR Ca^{2+} uptake and in SR Ca^{2+} ATPase activity with fatigue were closely correlated. The actual mechanism(s) responsible for these changes cannot be deduced from this study, but most likely involve structural alterations in the SR Ca^{2+} release channel and the Ca^{2+}ATPase enzyme. Marked metabolic perturbations were also demonstrated in fatigued muscle, which have been shown in animal experiments to adversely affect SR Ca^{2+} release and Ca^{2+} uptake. Thus, it is possible that fatigue effects on *in-vivo* SR function could be greater than those *in-vitro* effects measured here, due to the combined effects of the observed structural alterations and the probable metabolic changes. Further work is required to determine the causes of the depressed SR function with fatiguing exercise.

Chapter V INFLUENCES OF MUSCLE FIBER TYPE, TRAINING STATUS AND FATIGUE ON SKELETAL MUSCLE SARCOPLASMIC RETICULUM Ca^{2+} REGULATION.

5.1 Introduction

Muscle SR characteristics vary markedly with different fibre types, with each of SR volume density, RyR density and Ca^{2+} ATPase activity higher in mammalian muscles rich in Type II fibres (Chapter 2.2). Very little is known about SR characteristics in human skeletal muscles, but a relationship between SR function and fibre type would also be expected in human muscles. This is supported by the findings of Madsen et al. (1994), who found a significant correlation between the SR Ca^{2+} ATPase concentration and the proportion of Type II fibres in vastus lateralis muscle. Benders et al. (1994) also estimated that the rates of SR Ca^{2+} uptake and Ca^{2+} ATPase were 4-6-fold higher in fast than slow twitch muscle in humans, based on differences in soleus and vastus lateralis muscles. In the previous chapter, the rate of Ca^{2+} uptake was significantly related to, but no clear relationships were seen with either the Ca^{2+} ATPase activity, or the rate of Ca^{2+} release, to the proportion of Type II fibres in human vastus lateralis muscle. These findings were however, limited by the small sample size investigated. Thus, more extensive analyses are required to determine the influence of muscle fibre type on SR characteristics in human skeletal muscle.

Training status is known to effect skeletal muscle fibre composition in humans, inducing an increase in the proportion of oxidative fibres in muscle (Chapter 2.5). This may be marked for endurance training, with transition from Type IIB to IIA muscle fibres, and in

some circumstances to Type I fibres (see Chapter 2.5). Thus, a reduction in SR function might be expected on the basis of these altered muscle fibre types, consistent with findings in animal muscle (as reviewed in McKenna et al. 1996). Muscle fibre transitions with resistance training are less marked and limited to within the Type II fibre pool, with a reported transition from Type IIB to IIA fibres. Thus, on the basis of changes in fibre composition, less if any changes in SR function would be expected in resistance trained compared with endurance trained muscle. However, additional training-induced factors might affect SR characteristics in resistance trained muscle. Resistance training increases muscle fiber cross sectional area, particularly in Type II fibers (Staron et al. 1990b, Hather et al. 1991), with consequent reductions in mitochondrial and capillary density area (MacDougall et al. 1979, 1986). It is possible that muscle hypertrophy might induce a similar reduction in muscle SR volume relative to cell volume. This possibility has not been investigated.

An increased proportion of IIA compared to IIB fibres might be expected to confer an increased fatigue resistance in both resistance trained (RT) and endurance trained (ET) muscle. Thus, training might be hypothesised to have important protective effects against the depression with fatigue in SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity (Chapter 4). No published studies have investigated whether training induces protective effects against the deterioration in human skeletal muscle SR function with fatigue.

Therefore, this study examined the influence of muscle fibre composition, physical training status and fatigue, on skeletal muscle SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity in humans. Four major hypotheses were tested in this study. First, that clear relationships will be evident between muscle fibre composition and SR function in human vastus lateralis muscle. Second, that an increased proportion of Type I fibres, with a reduction in the SR characteristics, will be evident in resting muscle in ET relative to untrained controls. Third, that resting RT muscle will have lower SR Ca^{2+} release and

Ca^{2+} uptake relative to untrained controls. Finally, that the detrimental effects of fatigue on muscle SR function will be attenuated in both ET and RT, relative to untrained controls.

5.2 Methods

Eight untrained controls (UT, Chapter 4), eight resistance-trained subjects (RT) and eight endurance-trained subjects (ET) participated in the study. All subject details and experimental procedures for exercise tests, muscle biopsy and blood sampling and analyses have been fully described in Chapter III. Muscle biopsies were taken from the vastus lateralis muscle at rest and immediately after completion of 50 maximal exhaustive knee contractions.

5.3 Results

The three groups were first compared with respect to anthropometric characteristics, incremental exercise performance and isokinetic muscle function.

5.3.1 Anthropometric Differences Between Groups

The sum of skin folds at 8 sites and percentage body fat were less in ET than both UT and RT (P <0.05, Table 5.1). The thigh muscle cross-section area was higher in RT than in both UT and ET (P<0.05, Table 5.1).

5.3.2 Exercise Performance Differences Between Groups

5.3.2.1 Incremental Exercise Performance

Higher peak workrate, $\dot{V}O_2$, and duration of exercise time were found in ET than in both RT and UT (P<0.05, Table 5.2).

Table 5.1 Comparison of anthropometric characteristics in UT, RT and ET subjects.

	UT	RT	ET
Sum of 8 skin folds (mm)	74.7 ± 24.4	58.3 ± 13.9	45.4 ± 16.3 †‡
Body Fat (%)	14.5 ± 3.6	11.9 ± 2.3	9.4 ± 2.7 †‡
C.S.A. of Thigh muscle (cm^2)	207.5 ± 6.9	232.4 ± 5.7 †#	219.9 ± 10.7

Values are mean ± SD, n=8 each group. C.S.A. = cross-sectional area. † different from UT; # different from ET $P<0.05$; ‡ different from RT; all $P<0.05$

Table 5.2 Performance, cardiac and metabolic responses to incremental exercise in UT, RT and ET subjects.

	UT	RT	ET
Exercise Duration (min)	12.5±0.4	13.6±0.7	16.1 ±0.8 †‡
Workrate peak (W)	313 ±11	334 ±18	400 ± 18 †‡
HR peak ($b.min^{-1}$)	190±3	185±2	188±4
$\dot{V}O_2$ peak ($l.min^{-1}$)	3.55 ± 0.14	3.53 ± 0.14	5.55 ± 0.28 †‡
$\dot{V}O_2$peak ($ml.kg^{-1}.min^{-1}$)	44.4 ±1.8	43.8±3.6	67.6 ± 1.5 †‡

MEAN ±SEM, n=8 each group; † ET different from UT; ‡ ET different from RT; $P<0.05$

5.3.2 Non-Invasive Isokinetic Muscle Function

Torque-velocity relationships.

No significant differences were found between the three groups in the torque-velocity relationships, when expressed either in absolute units (Nm), or relative to body mass ($Nm.kg^{-1}$, Figure 5.1).

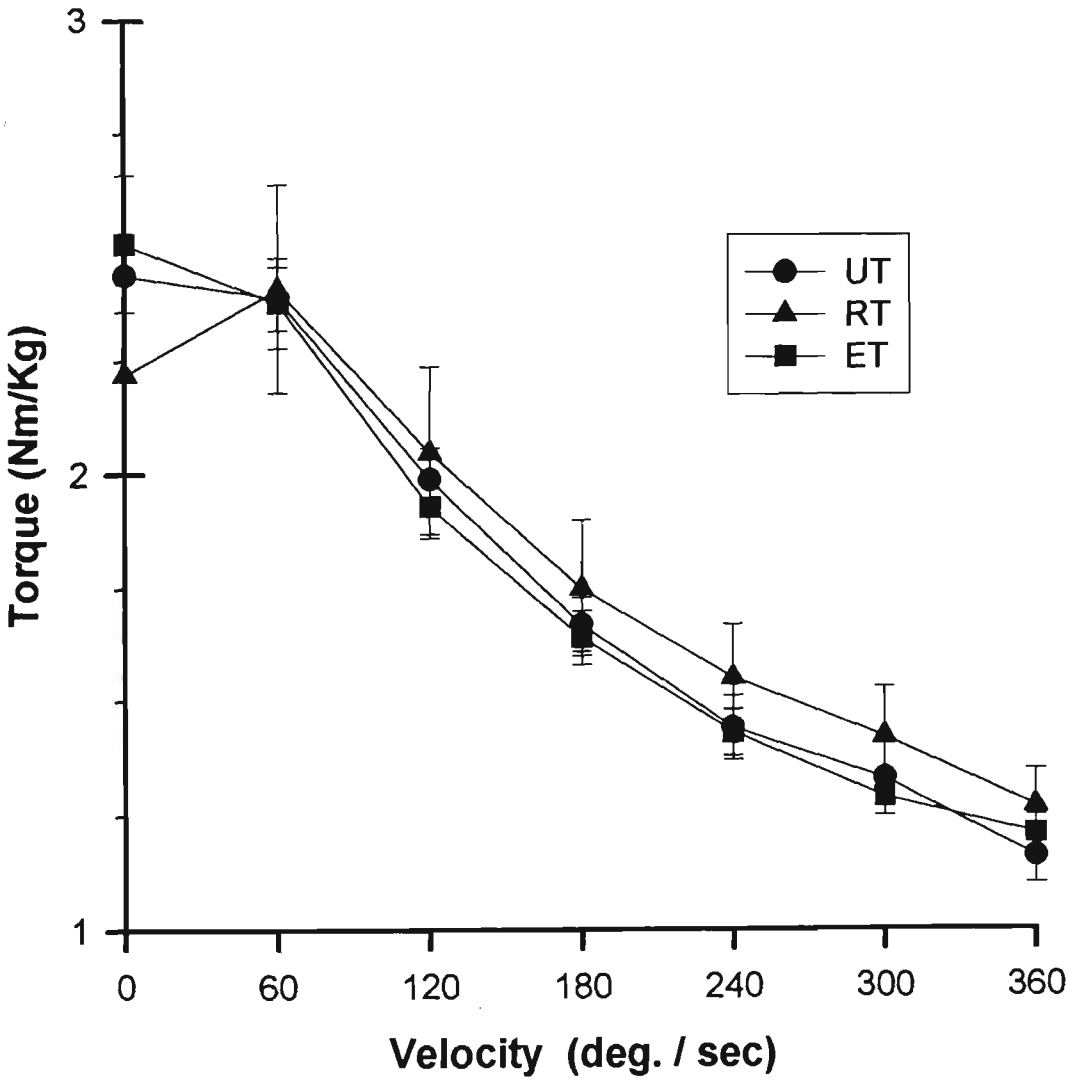


Figure 5.1 The torque-velocity relationship during isometric and isokinetic contractions on a Biodex dynamometer, in UT, RT and ET subjects. n=8, mean ± SEM.

Muscle Fatigue Test

Performance variables measured during the non-invasive fatigue test completed on the Biodex isokinetic dynamometer are shown in Table 5.3. Peak work was less in ET than in UT and RT ($P<0.05$), but no differences were evident between groups in peak torque. When expressed relative to body mass, no differences were found between the groups in either peak torque or peak work. The fatigue index related to the decline in peak torque (FI_{torque}) was lower in ET than in UT and RT, by ~27% and 25%, respectively ($P<0.05$). Similar differences were shown when the fatigue index was related to the decline in maximal work (FI_{work}, $P<0.05$).

Table 5.3 Non-invasive muscle fatigue test results in UT, RT and ET.

	UT	RT	ET
Peak Torque (Nm)	165.7±15.3	**185.5 ± 18.5**	168.3 ±8.32
Peak Torque (Nm/kg)	2.06 ±0.11	2.27 ± 0.23	2.28 ± 0.14
Peak Work (J)	146.5 ± 4.7	153.1 ±4.9	118.9 ±3.3 †‡
Peak Work (J/kg)	1.84± 0.18	1.88 ± 0.06	1.61 ± 0.07
FI_{work} (%)	55.5±3.9	48.1 ±4.0	24.8 ±3 2 †‡
FI_{torque}(%)	52.3±3.5	50.1 ±3.3	25.0 ±5.6 †‡

Mean±SEM, n=8 each group. † $P<0.05$ different from UT; ‡ $P<0.05$ different from RT.

5.3.2.3 Invasive Isokinetic Muscle Function

All groups demonstrated a significant decline in peak muscle torque during the muscle fatigue test with invasive measurements, conducted on the Cybex dynamometer. There were no significant differences in the FI_{torque} between the Biodex and Cybex tests. The FI_{torque} for the UT, RT and ET groups were 43.4 ±3.3, 47.4 ±5.0 and 29.9 ±4.3%,

respectively. Thus, the FI_{torque} was 14% and 18% lower in ET than in UT and RT, respectively ($P<0.05$), indicating reduced fatiguability in the ET group.

In summary, ET had lower body fat levels, higher $\dot{V}O_{2\,peak}$ and a lesser fatigability during the muscle fatigue test, than either the RT or UT. The RT had a higher thigh muscle CsA than UT but did not differ from the other groups in $\dot{V}O_{2\,peak}$, muscle function, or fatigability during the fatigue test.

5.3.3 Muscle Fibre Type

Endurance-trained subjects had a higher proportion of Type I fibres and a lower proportion of Type IIB fibres, compared with both the UT and RT subjects ($P<0.05$, Table 5.4). However, the muscle fibre composition did not differ between RT and UT (Table 5.4).

Table 5.4 Muscle fibre type proportions in UT, RT and ET groups.

	UT	RT	ET
Type I (%)	50.7±2.9	43.6±4.9	67.4 ±3.3 †‡
Type II (%)	49.3±2.9	56.4±5.1	32.6 ±3.3 †‡
Type IIa (%)	38.4±1.6	46.1±2.4	30.8±3.1
Type IIB (%)	10.9±2.9	10.3±2.7	1.8±0.7 †‡

Mean ± SEM, n = 8 each group. UT untrained; RT Resistance trained; ET Endurance trained. † $P<0.05$ different from UT; ‡ $P<0.05$ different from RT.

5.3.4 Muscle SR Characteristics

5.3.4.1 SR Function in Resting Muscle

In resting skeletal muscle, when expressed relative to muscle wet weight, each of the rates of SR Ca^{2+} release (19 and 20%), Ca^{2+} uptake (18 and 7%), and Ca^{2+}ATPase activity (19 and 26%) were lower in ET than in RT and UT, respectively ($P<0.05$, Figures 5.2, 5.3 and 5.4). No significant differences were found in resting skeletal muscle between RT and UT in any of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase rates.

When expressed relative to total homogenate protein, resting SR Ca^{2+} uptake was 13% lower in RT than in UT ($P<0.05$, Table 5.5). Lower resting values were apparent in ET for muscle SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase compared to UT ($P<0.05$). Both Ca^{2+} uptake and Ca^{2+} ATPase were also less in ET compared to RT ($P<0.05$).

5.3.4.2 SR Function in Fatigued Muscle

With fatigue, each of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase declined in all groups, when expressed relative to muscle wet weight ($P<0.05$, Figures 5.2-5.4), or homogenate protein ($P<0.05$, Table 5.5).

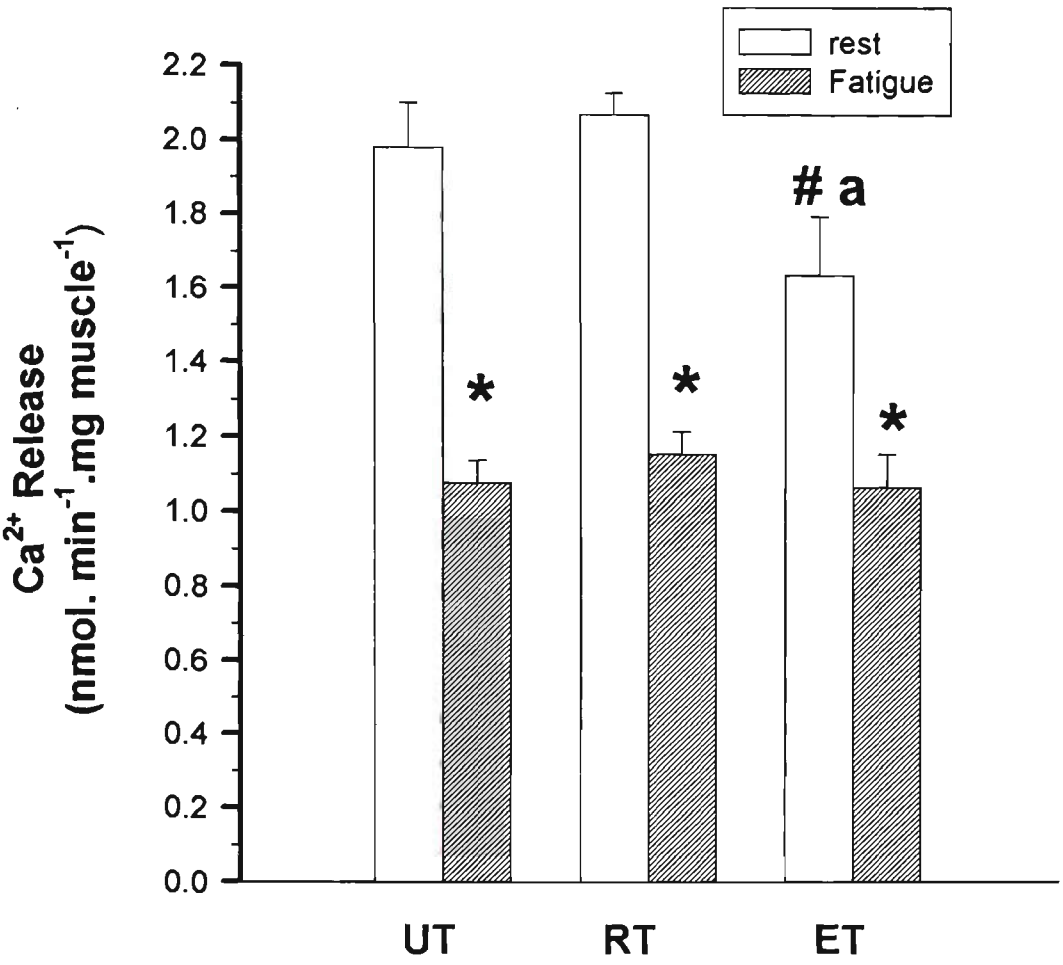


Figure 5.2 Maximal Ca^{2+} release rates in crude muscle homogenate at rest and after fatigue in UT, RT and ET.

n=8, Mean ± SEM. SR units: nmol.min^{-1}.mg muscle^{-1}; *, Fatigue <Rest; #, different from UT; a, different from RT, $P<0.05$.

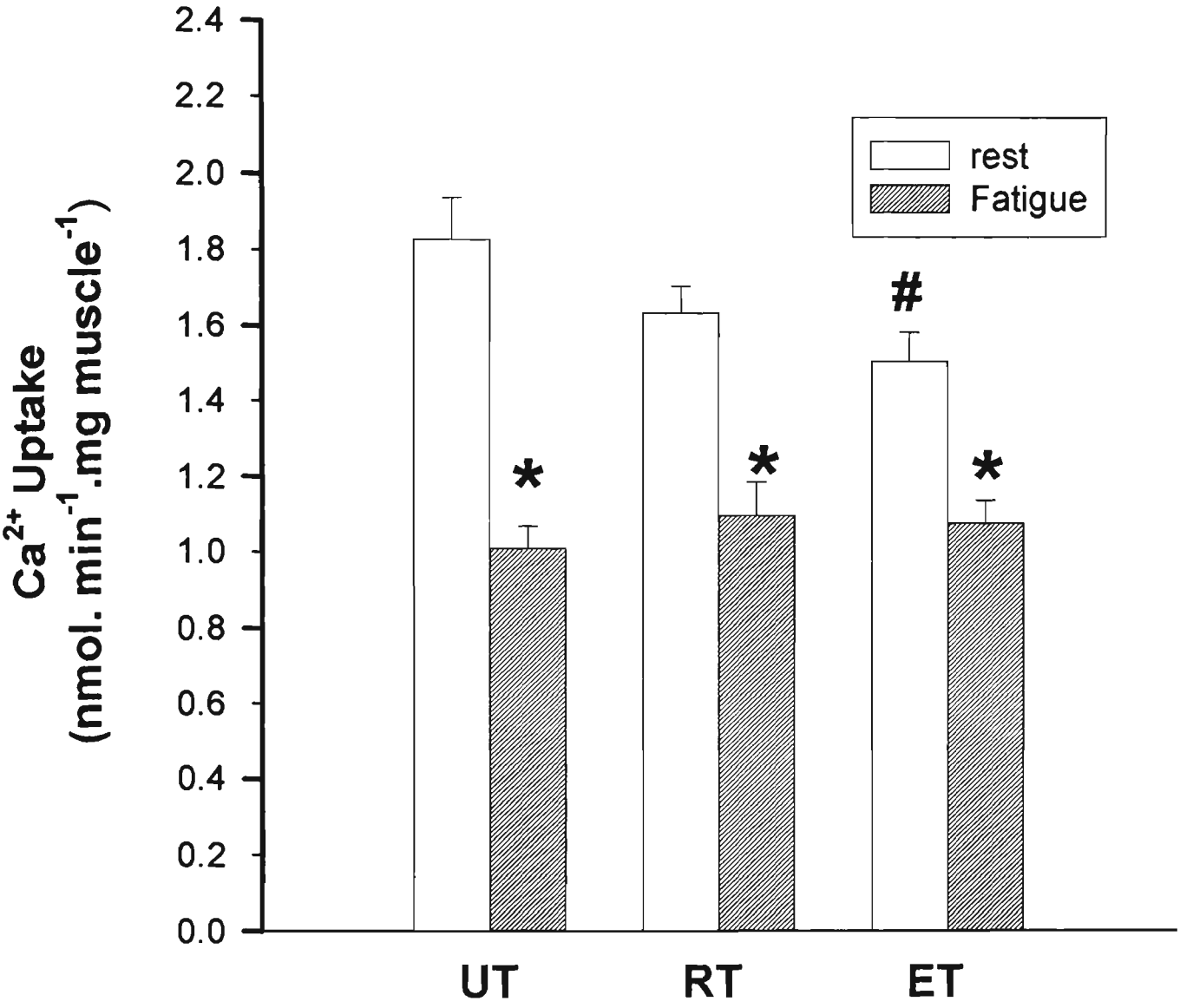


Figure 5.3 Maximal Ca^{2+} uptake rates in crude muscle homogenate at rest and after fatigue in UT, RT and ET.

n=8 in each group: Mean ± SEM. SR units: $nmol.min^{-1}.mg\ muscle^{-1}$; *, Fatigue<Rest; #, different from UT, respectively ($P<0.05$).

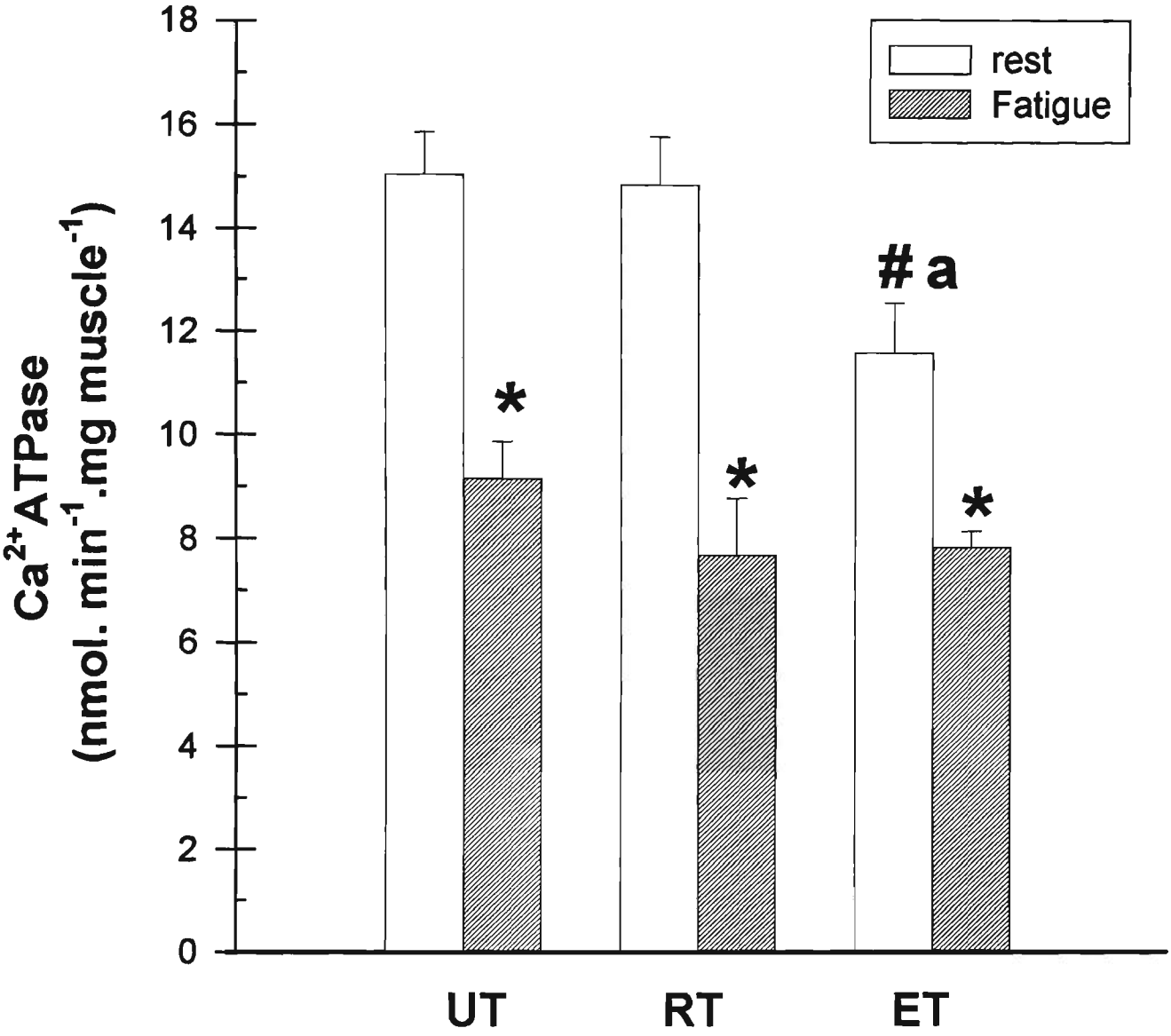


Figure 5.4 Maximal Ca^{2+} ATPase activity in crude homogenate muscle at rest and after fatigue in UT, RT and ET.

n=8 in each group; Mean±SEM. SR units: $nmol.min^{-1}.mg\ muscle^{-1}$; *, Fatigue <Rest; #, different from UT; a, different from RT, respectively ($P<0.05$).

Table 5.5 Effects of training status and fatigue on SR Ca^{2+} release, uptake and Ca^{2+} ATPase activity in human skeletal muscle, expressed relative to muscle protein.

	UT		RT		ET	
SR variables	**Rest**	**Fatigue**	**Rest**	**Fatigue**	**Rest**	**Fatigue**
Ca^{2+} release	12.9±1.0	7.4±0.6 *	12.7±0.8	7.3±0.8 *	10.1±0.6† ‡	6.7± 0.3 *
Ca^{2+} uptake	11.9±0.8	6.2±0.4 *	10.0±0.4 †	6.6±0.5 *	9.4±0.2 †	6.6± 0.2 *
Ca^{2+} ATPase	96.1±2.9	56.0±4.7 *	90.4±4.9	48.1±7.1 *	71.9±2.4 † ‡	50.5± 3.0 *

Mean±SEM. SR units: nmol.min^{-1}.mg protein^{-1}; *Fatigue<Rest; † $P<0.05$ different from UT; ‡ $P<0.05$, different from RT.

5.3.4.3 Absolute and Relative Changes in SR Function in Fatigued Muscle

The absolute decline in SR function in fatigued muscles (per mg muscle) is shown in Table 5.6. The decline in SR Ca^{2+} release did not differ between the three groups. The decline in SR Ca^{2+} uptake and Ca^{2+} ATPase activity with fatigue were less in ET than in UT and RT, respectively ($P<0.05$).

When expressed as a percentage of the values in resting muscle there were no significant differences between the three groups in SR Ca^{2+} release and Ca^{2+} uptake in the decline with fatigue. However, the percentage decline in Ca^{2+} ATPase activity remained less in ET than in RT ($P<0.05$, Table 5.7).

Table 5.6 Decline in SR function after fatiguing exercise in UT, RT and ET groups.

Depression with Fatigue ($nmol.min^{-1}.mg\ muscle^{-1}$)	UT	RT	ET
ΔCa^{2+} release	0.71 ±0.14	0.91 ± 0.10	0.56 ±0.13
ΔCa^{2+} uptake	0.64 ±0.14	0.54 ±0.10	0.43 ±0.05†
ΔCa^{2+} ATPase activity	4.64 ± 0.90	7.15 ± 0.83	3.74 ± 0.88‡

Mean±SEM, n=8 in each group, †, different from UT, ‡ different from RT, $P<0.05$

Table 5.7 Percentage changes in SR function after fatiguing exercise in UT, RT and ET groups.

DEPRESSION WITH FATIGUE (%)	**UT**	**RT**	**ET**
ΔCa^{2+} release (%)	42.1± 5.8	43.4 ±3.9	31.3 ± 6.1
ΔCa^{2+} uptake (%)	43.0 ± 5.2	32.7±4.8	28.4± 2.8
ΔCa^{2+} ATPase activity (%)	38.5± 4.2	48.5±5.1	29.6 ± 5.0‡

Mean±SEM, n=8 in each group, ‡ different from RT, $P<0.05$

The absolute decline in SR function in fatigued muscles expressed relative to homogenate protein is shown in Table 5.8. No significant differences were found between the three groups with respect to the decline in SR Ca^{2+} release. However, the decline in SR Ca^{2+} uptake, as well as in Ca^{2+} ATPase activity with fatigue were less in ET than in both UT and RT ($P<0.05$).

However, when this decline with fatigue was expressed as a percentage of the values in resting muscle, no differences were found between the three groups in SR Ca^{2+} release and Ca^{2+} uptake.

Table 5.8 Decline in SR function after fatiguing exercise in UT, RT and ET groups.

Depression with Fatigue ($nmol.min^{-1}.mg\ protein^{-1}$)	UT	RT	ET
ΔCa^{2+} release	4.76 ±1.23	2.78 ± 0.27	3.37 ±0.57
ΔCa^{2+} uptake	4.69 ±1.16	3.37 ±0.55	2.78 ±0.27† ‡
ΔCa^{2+} ATPase activity	32.49 ± 5.98	42.28 ± 5.48	21.30 ± 4.30† ‡

Mean±SEM, n=8 in each group, †, different from UT, ‡ different from RT, $P<0.05$

Table 5.9 Percentage changes in SR function after fatiguing exercise in UT, RT and ET groups.

DEPRESSION WITH FATIGUE (%)	**UT**	**RT**	**ET**
ΔCa^{2+} release (%)	41.0 ± 6.3	42.2 ± 4.3	31.9 ±5.0
ΔCa^{2+} uptake (%)	45.7 ± 6.6	33.2 ± 4.7	29.3 ± 2.6
ΔCa^{2+} ATPase activity (%)	41.7 ± 4.5	47.6 ± 6.5	28.9 ± 5.1

Mean±SEM, n=8 in each group.

5.3.5 Relationships Between Muscle Fibre Type and Performance

$\dot{V}O_2peak$

The relationships between muscle Type II fibre proportions and $\dot{V}O_2peak$ is shown in Figure 5.5. $\dot{V}O_2peak$ was negatively related to the proportion of Type II fibres ($P<0.005$).

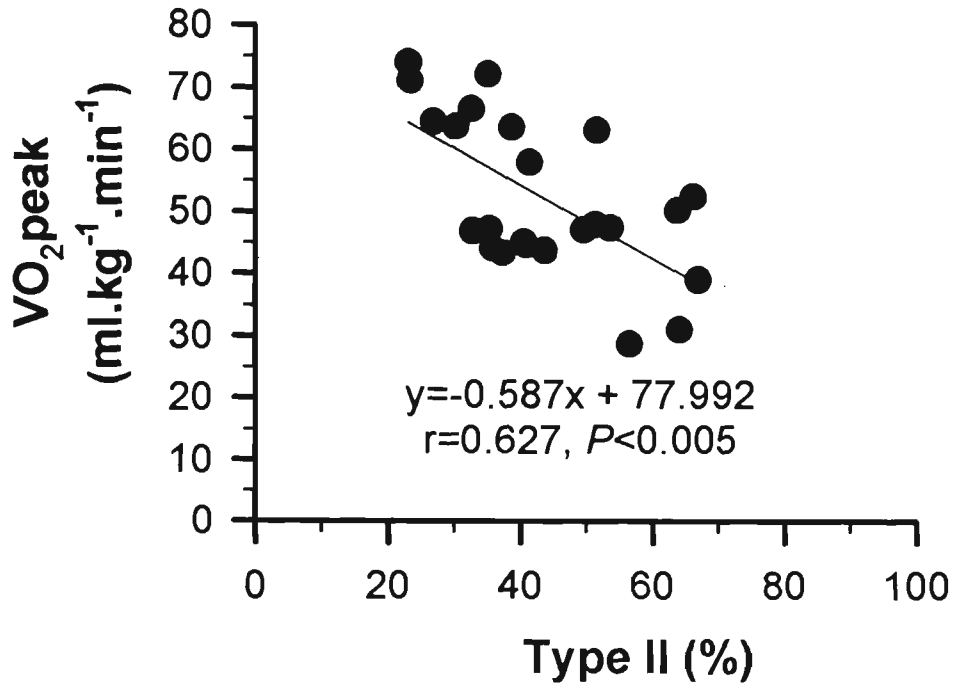


Figure 5.5 Relationship between $\dot{V}O_2peak$ and the proportion of Type II muscle fibres. n=24.

Muscular Fatigue.

Significant relationships were found between the fatigue indices in the two muscle fatigue tests with the proportion of Type II fibres (Table 5.10, $P<0.01$).

Table 5.10 Relationships between muscle fatigue indices (%) and the Type II fibres (%). n=24 for each regression.

Regression equation		r	*P*
Cybex test:	$FI_{torque} = (0.582 \times \text{Type II}) + 14.99$	0.568	$P<0.005$
Biodex test:	$FI_{torque} = (0.584 \times \text{Type II}) + 18.447$	0.530	$P<0.005$
Biodex test:	$FI_{work} = (0.675 \times \text{Type II}) + 13.56$	0.542	$P<0.01$

5.3.6 Relationships between muscle fibre type, SR function and fatigue

The rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase in resting muscle were each positively related to the proportions of Type II fibre ($P<0.001$, Figure 5.6).

Further, both the absolute and the percentage depression in the rates of SR Ca^{2+} release, SR Ca^{2+} uptake and Ca^{2+} ATPase activity in fatigued muscle were also each positively related to the Type II fibre proportion ($P<0.05$, Figure 5.7 and 5.8, respectively).

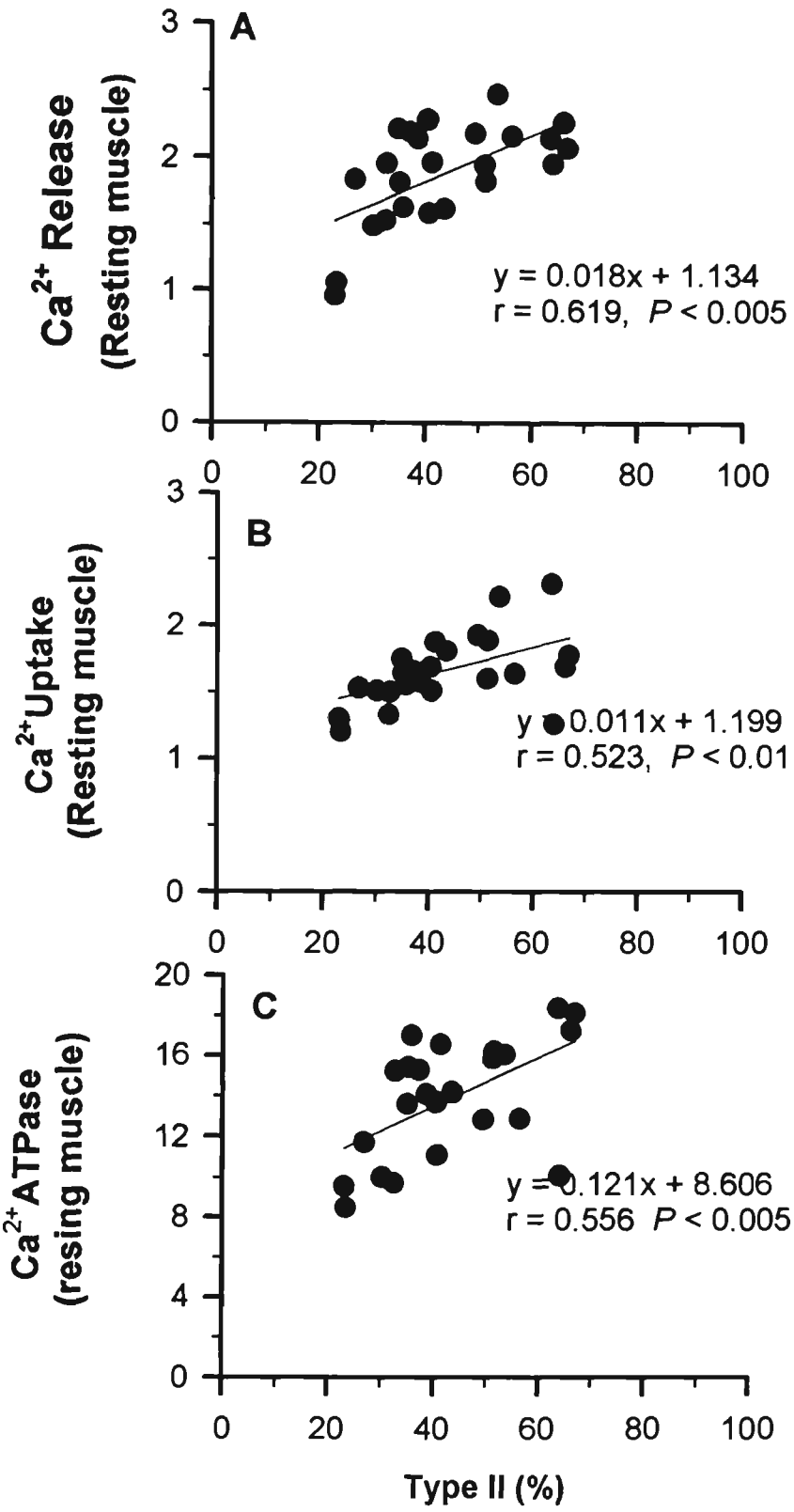


Figure 5.6 Relationships between the rates of Ca^{2+} release (A), Ca^{2+} uptake (B) and Ca^{2+} ATPase activity (C) in resting muscle and the proportion of Type II muscle fibres.

n=24 in each regression.

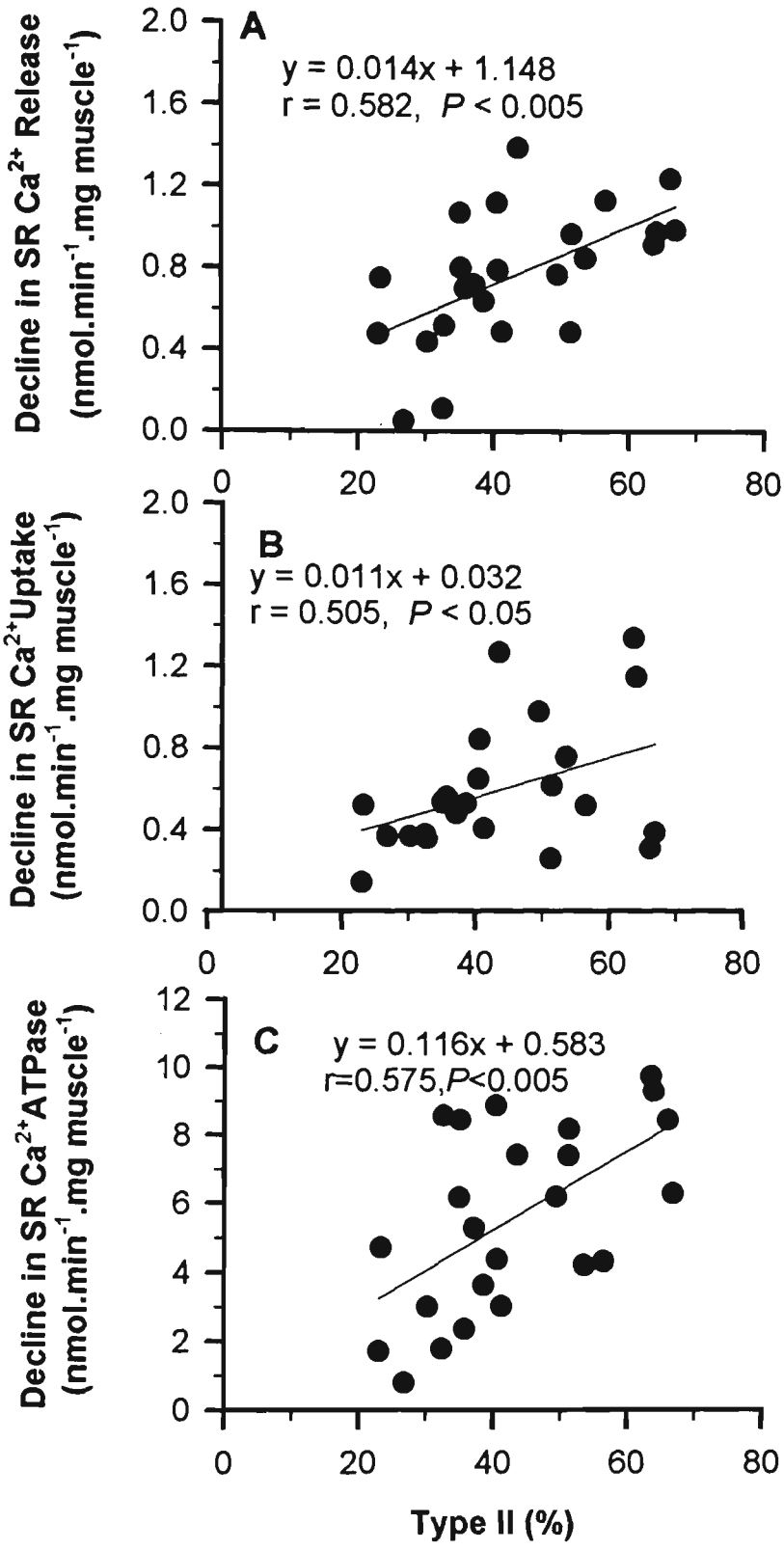


Figure 5.7 Relationships between and the proportion of Type II muscle fibres and the fatigue-induced absolute decline in the rates of Ca^{2+} release (A), Ca^{2+} uptake (B) and Ca^{2+} ATPase activity (C) in muscle. n=24 for each regression.

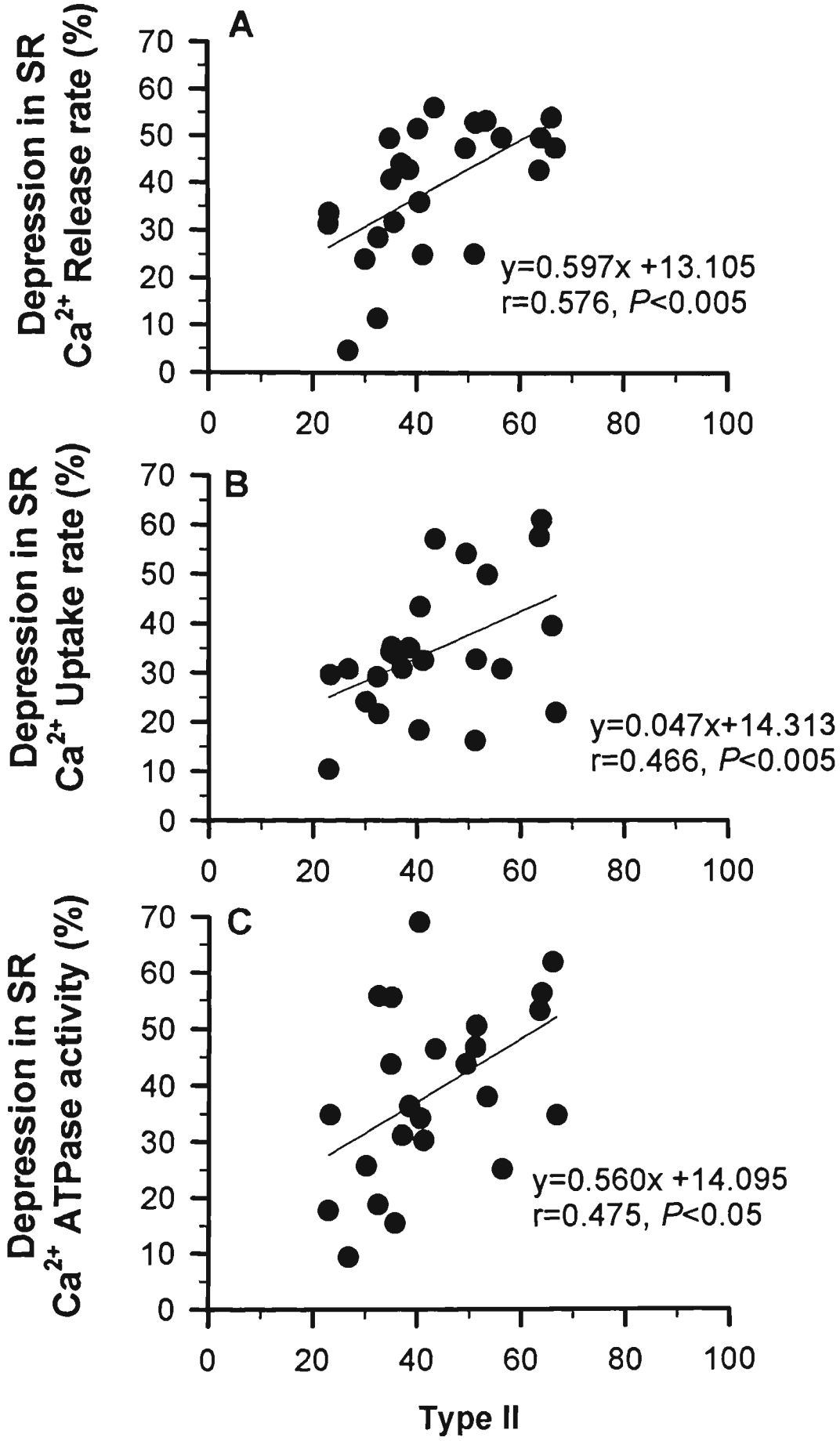


Figure 5.8 Relationships between the proportion of Type II fibres and the fatigue-induced percentage decline in the rates of SR Ca^{2+} release (A), Ca^{2+} uptake (B) and Ca^{2+} ATPase activity (C). n=24 for each regression.

5.3.7 Muscle Metabolites

Resting muscle. The only significant difference between the three groups in resting muscle metabolites was a higher PCr content found in RT than in UT and ET ($P<0.05$, Table 5.11).

Fatigued muscle. Muscle ATP content was decreased with fatigue in UT and RT, when analysed enzymatically ($P<0.05$), but this was not significant in ET. Muscle PCr and glycogen contents and pH were decreased, whilst both lactate and creatine contents were increased with fatigue in all groups ($P<0.05$). At fatigue, muscle lactate and Cr were less, whilst muscle CrP was greater, in ET compared to UT ($P<0.05$). Muscle IMP content was elevated in fatigue in all groups ($P<0.05$), with IMP at fatigue less in both RT and ET compared to UT ($P<0.05$).

5.3.8 Relationships between SR Function and Muscle Metabolites

The relationships between the absolute and the percentage decline in SR function versus changes in muscle metabolites, with data pooled for all subjects, are shown in Table 5.12. No significant correlations were found for any regression pair.

Table 5.11 Skeletal muscle metabolites at rest and after fatigue exercise in UT, RT and ET.

	UT *(n=8)*		RT *(n=8)*		ET *(n=6)*	
	Resting	**Fatigue**	**Resting**	**Fatigue**	**Resting**	**Fatigue**
ATP (ENZ.)	25.67 ± 0.85	19.51 ± 1.71*	26.28 ± 1.33	21.21 ± 1.29 *	24.13 ± 1.37	20.80 ± 0.94
ATP (HPLC)	24.71±0.94	21.41±1.43 *	27.03±0.98	24.32 ± 0.87	28.93 ± 2.46	23.64 ± 2.80
ADP	2.77 ± 0.18	2.90 ± 0.47	3.40 ± 0.24	3.52 ± 0.38	3.14 ± 0.26	3.83 ± 0.33
IMP	0.08 ± 0.01	3.26 ± 0.84*	0.09 ± 0.03	1.39 ± 0.63 * #	0.08 ± 0.01	2.75 ± 0.45 * †
PCr	93.3 ± 2.3	35.8 ±3.5*	104.8 ± 2.9 # a	41.8 ± 3.4 *	82.3 ± 2.3	54.6 ± 3.3 * † ‡
Cr	42.0 ± 2.6	99.5 ± 4.6*	44.0 ± 3.5	105.7± 2.6 *	49.0 ± 4.3	76.7 ± 3.6 * † ‡
Lac-	8.0 ± 0.8	91.6 ± 10.0*	9.6 ± 1.0	84.8 ± 7.5 *	6.7 ± 0.9	66.1± 7.2 * † ‡
pH	7.17 ± 0.02	6.83 ± 0.03 *	7.14 ± 0.02	6.79± 0.03 *	7.19 ± 0.02	6.88 ± 0.03 *
Glycogen	469 ± 34	356 ± 30 *	494 ± 34	376 ± 19 *	510± 32	349 ± 29 *

Values are expressed by mmol.kg^{-1} dry mass, except glycogen mmol glucosyl units.kg^{-1} dry mass, and pH. Mean ± SEM;. * Rest different from fatigue; † ET different from UT; ET different from ‡RT; # RT different from UT; *a*, RT different from ET; $P<0.05$.

ENZ- determined by enzymatic method; HPLC- determined by high performance liquid chromatography.

Table 5.12 Relationships between Fatigue decline, muscle SR Ca^{2+} release, uptake and Ca^{2+} ATPase (%) and the absolute change in ATP, lactate and IMP.

n=24 for each regression.

Regression equation	**r**	***P***
ΔSR Ca^{2+} release = (-0.954 x ΔATP) +34.054	0.257	*ns*
ΔSR Ca^{2+} release = (0.145 x ΔLac-) +28.43	0.259	*ns*
ΔSR Ca^{2+} release = (0.044 x ΔIMP) +38.72	0.006	*ns*
ΔSR Ca^{2+} uptake = (-0.390 x ΔATP) +33.815	0.115	*ns*
ΔSR Ca^{2+} uptake = (0.217 x ΔLac-) +18.893	0.399	*ns*
ΔSR Ca^{2+} uptake = (1.604 x ΔIMP) +31.945	0.241	*ns*
ΔSR Ca^{2+} ATPase = (-0.572 x ΔATP) +36.763	0.140	*ns*
ΔSR Ca^{2+} ATPase = (0.557 x ΔLac-) +34.838	0.09	*ns*
ΔSR Ca^{2+} ATPase = (1.367 x ΔIMP) +42.878	0.172	*ns*

5.5 Discussion

This study demonstrates a clear dependence in human muscle of each of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity on muscle fibre type, with these rates in Type II estimated to be 3, 2.4 and 4-fold higher, respectively, than in Type I muscle fibres. Muscle SR characteristics also varied with training status, with lower SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity in endurance trained muscle and lower SR Ca^{2+} uptake in resistance trained muscle, compared to untrained muscle. The less SR characteristics in ET muscle most likely reflected their less proportion of Type II muscle fibres, whilst that of RT might indicate additional training effects on muscle SR. Finally, chronic endurance and resistance training did not prevent the decline in SR function with fatigue. Whilst lesser absolute depressions in muscle Ca^{2+} uptake and Ca^{2+} ATPase activity were noted in ET compared with UT, when expressed as a percentage of their resting levels, these did not differ between training groups.

5.5.1 Fibre Type and SR function

This study reports a strong relationship between SR characteristics and muscle fibre composition in resting human skeletal muscle. Significant relationships were found between each of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity in resting muscle and the proportion of Type II fibres. On the basis of regression analyses, the rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase in Type II muscle fibres were estimated to be ~3, 2.4 and 4 fold higher, respectively, than in Type I muscle fibres (Table 5.13). These relative differences in human muscle fibre types are comparable with those of other species. In rat muscle, SR Ca^{2+} uptake and Ca^{2+} ATPase were 2 to 4-fold higher, in fast- compared to slow- twitch muscles (Kim et al. 1982, Feher et al. 1988). Similarly, the density of RyR was 2 to 4-fold higher in fast compared to slow-twitch muscles (Appelt et al. 1989). The current results are also consistent with the 4-6-

fold higher rates of Ca^{2+} uptake and Ca^{2+} ATPase estimated in human muscle (Benders et al. 1994) and with the positive relationship between muscle SR Ca^{2+} ATPase concentration and Type II muscle fibres (Madsen et al. 1994). Thus, it is apparent that any measurements of SR function in human muscle must also be accompanied by measurements of muscle fibre type composition.

Table 5.13 The estimated relationships between SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase and proportion of Type II fibres in resting muscle, n=24.

Muscle Fibre Type	Ca^{2+} release	Ca^{2+} uptake	Ca^{2+} ATPase activity
100 %Type I	1.11	1.03	6.46
100 %Type II	2.92	2.43	23.5

Units: $nmol.min^{-1}.mg\ muscle^{-1}$. All relationships based on the following regression analyses. Regression equations Ca^{2+} release =(0.018 x Type II) +1.115; Ca^{2+} uptake =(0.014 x Type II) + 1.034; Ca^{2+} ATPase =(0.171x Type II) +6.459.

The regression analyses suggest that the proportion of muscle Type II fibres can explain ~40% of the variation in Ca^{2+} release, 50% of the variation in Ca^{2+} uptake and 61% of the variation in Ca^{2+} ATPase activity. Therefore, although fibre type is an important determinant of SR characteristics, other factors must also be considered. Methodological factors are important and the specificity of these measurements was discussed in Chapter 4. The variability in these SR measurements was small, being less than 8% (see Appendix Table A10), but this would contribute to a variable result for a given muscle fibre type. Second, small errors in fibre type determination would also be expected. Part of the variation in SR characteristics for a given fibre type, was the

classification into only Type I and II fibres, since higher SR characteristics would be expected for Type IIB than for IIA fibres. Another possible contributory factor was that the training status of two of the three groups of individuals analysed in this study might exert an influence on SR function independent to that based solely on muscle fibre type.

5.5.2 Training status and SR characteristics in resting muscle

It was hypothesised that a higher proportions of Type I fibres would be observed in ET muscle, with consequent lesser SR function, when compared to UT. This study clearly demonstrated that the ET subjects firstly had a higher proportion of Type I muscle fibres, and secondly, also had lower rates of muscle SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity than untrained subjects. This finding is consistent with the close association between fibre type and SR function. Unfortunately, with this experimental model, it is not possible to discern whether any additional endurance training effects on muscle SR characteristics may be superimposed upon differences due to muscle fibre type. However, such training effects are likely to be small in human muscle. Previous short duration endurance training studies in humans (4-11 weeks) have shown no change in SR function, or muscle fibre type (Madsen et al. 1994, Green et al. 1995). But after long duration running training (14 years), increased Type I fibre proportions and both decreased IIB fibres and Ca^{2+} ATPase content were found in the old group compared with young control group (Klitgaard et al. 1989). However, this was not seen in the old swim- trained group (Klitgaard et al. 1989). The training model may therefore influence both SR function and fibre type proportion. Large changes in SR Ca^{2+} uptake and Ca^{2+} ATPase activity have been shown with endurance training in animals, although these changes are somewhat conflicting (see Table 2.9).

Long-term resistance training can induce muscle fibre hypertrophy, especially in type II fibres, and can also improve muscle strength (MacDougall et al. 1980, Hather et al. 1991, Staron et al. 1990). Therefore, it was hypothesised that a resistance training effect might be observed on SR function independent of fibre type, due to a "dilution" of muscle SR. In rat fast-twitch skeletal muscle, active muscle loading decreased the total SERCA protein (SERCA1+ SERCA2), from 51.4 to 45.9 (by 5.5 AU/10ug) (Kandarian et al. 1994). Therefore, decreases in SR characteristics with chronic RT were hypothesised.

No differences were found between RT and UT in the muscle fibre proportions, although the mean Type IIa fibre percentage was 8% higher (ns) in RT muscle. Thus, any differences in SR function on the basis of the observed fibre type differences would be expected to be slightly greater in RT muscle. No clear differences in SR function were found between the two groups. SR Ca^{2+} release did not differ between groups (-2%, nmol.min^{-1}.mg protein $^{-1}$, ns; +4%, nmol.min^{-1}.mg^{-1}, ns). In contrast, resting muscle SR Ca^{2+} uptake was lower in RT compared to UT (-16%, nmol.min^{-1}.mg protein $^{-1}$, $P<0.05$; -10%, nmol.min^{-1}.mg^{-1}, ns), whilst SR Ca^{2+} ATPase activity did not differ between the two groups (-6%, nmol.min^{-1}.mg protein $^{-1}$, ns; -7%, nmol.min^{-1}.mg^{-1}, ns). These data are equivocal, with a tendency for lesser SR Ca^{2+} uptake, but not for the other two SR variables. This suggests a training effect on SR Ca^{2+} uptake that is independent of fibre type in RT, which may reflect a volume dilution effect on SR, or a reduced expression of SR Ca^{2+} ATPase (Kandarian et al. 1994).

An unexpected limitation in this study was that the RT group did not differ markedly from the untrained subjects in muscle mass, or peak muscle torque, with the only clear difference being a greater muscle thigh cross-sectional area. No measurements were made to confirm any muscle fibre hypertrophy. A functional criterion was used to differentiate this RT group from recreational gymnasium users, but this may not have

been stringent enough. Further, the expected greater proportion of Type IIA fibres in RT compared with UT proportion (Staron et al. 1990, Klitgaard et al 1990, Kraemer et al 1995) was not evident. Although the proportion of Type IIA fibres in RT tended to be higher (8%), this was not significant, which might be due to the small sample size in each group. Thus, the lack of consistent effect of RT on SR function could simply be due to the first that this group was insufficiently trained to show this effect. To investigate this question further, a more highly trained group is required and a longitudinal study design is preferably.

5.5.3 Interaction between training status and fatigue effects on SR

The third hypothesis tested in this chapter was that fatigue effects on SR function would be attenuated in the endurance and resistance trained groups. However, an important finding was that fatigue significantly depressed *in vitro* SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity in all three groups. This confirms the findings from the previous Chapter in a larger subject population and suggests that this down-regulation in SR function may be obligatory with fatigue. To test the hypothesis that training status attenuates the depressive fatigue effects on SR, the absolute decline in SR function was compared between the three groups. A lesser absolute decline in SR Ca^{2+} uptake and Ca^{2+} ATPase activity was found in ET, consistent with their higher aerobic performance and reduced muscular fatiguability. However, a lesser absolute decline would be expected in ET due to their low proportion of Type II fibres. When expressed as a percentage decline, these changes were not significant, although the tendency was to be less with ET (-15%, -9%, respectively). There were no clear effects of training status on the depression in SR Ca^{2+} release expressed in either absolute or relative terms.

The increased subject numbers in this Chapter allow further investigation into the susceptibility of different fibre types on the depression in SR function with fatigue. Significant relationships were found between depressed SR function and the proportions of Type II fibres, strongly suggesting that in human muscles, the Type II fibres were more susceptible to impaired SR function with fatigue. This might be a major underlying mechanism linking the greater fatiguability observed in those subjects with a high proportion of Type II fibres. Byrd et al. (1989b) reported greater depressions in SR Ca^{2+} uptake in red gastrocnemius, than in white vastus muscles in the rat during prolonged exercise (see Fig 2, Byrd 1992). One possible reason for this difference might be that prolonged exercise would primarily involve slow-twitch motor unit recruitment, whereas both slow and fast units would be recruited during brief maximal contractions, as in the present study.

In the previous Chapter, significant relationships were found between numerous muscle metabolites and SR function at rest and with fatigue. Differences in these metabolic changes in the different training groups also allow further light to be shed on the importance of these changes to SR function with fatigue. Lesser changes were found with fatigue in each of ATP, PCr, lactate and IMP in the ET group, and the absolute decline in SR function was also less in this group compared to UT. However, none of the SR function-metabolite relationships were significant. This strongly suggests that the structural impairments in SR RyR and Ca^{2+}ATPase with fatigue are not linked with metabolic changes in muscle, at least bulk changes. Thus, the apparent linkage found in the previous chapter between changes in muscle metabolites and SR function must reflect simple parallel exercise effects, rather than causally related processes.

5.5.4 Conclusions

In human skeletal muscle, each of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity were clearly dependent upon muscle fibre type. Training status also effected SR function. SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity were all lower in endurance trained athletes than in untrained controls, but this was entirely consistent with their higher proportion of Type I fibres. In resistance trained subjects, SR Ca^{2+} uptake was less than in controls, suggesting training effects that were independent of fibre composition, although these effects were not striking. Finally, the depressive effects of fatiguing exercise on muscle SR function were not prevented by chronic training, although lesser absolute changes were found in the endurance trained group.

CHAPTER VI REDUCED SKELETAL MUSCLE SARCOPLASMIC RETICULUM FUNCTION DESPITE A HIGH PROPORTION OF TYPE II MUSCLE FIBRES IN LUNG TRANSPLANT RECIPIENTS

6.1 Introduction

Both acute and chronic exercises have marked effects on skeletal muscle SR function. An acute bout of intense fatiguing muscular contractions caused marked reductions in SR Ca^{2+} release and uptake rates (Chapter 4). Chronic training as well as muscle fibre compositions were suggested to effect SR function (Chapter 5). Lower rates of SR Ca^{2+} release and uptake were found in endurance trained subjects, who had a high proportion of Type I fibres. This finding was consistent with another report indicating a positive relationship between SR Ca^{2+} ATPase content and the proportion of type II muscle fibres in humans (Madsen et al. 1994). However, training may exert an effect independent of changes in muscle fibre composition, with a lower rate of SR Ca^{2+} uptake found in the resistance trained compared to the control group (Chapter 5).

In animal models, muscle disuse may change muscle fibre composition from-slow to fast-twitch and thus, would also be expected to alter skeletal muscle SR function. Following hindlimb suspension and immobilisation in rats and guinea pigs, the SR Ca^{2+} ATPase activity and the rates of SR Ca^{2+} uptake and release were initially increased, in association with slow-to-fast fibre transition (Kim et al. 1982, Leivseth et al. 1992, Arkhipenko et al. 1993, Stevens and Mounier 1992). The magnitude of these changes were detailed in Chapter 2.

Several studies show that muscle fibre type proportions can also be affected by muscular disuse in humans. A decrease of 5-7% in the percentage of human Type I fibres was found in vastus lateralis muscle after 4-6 weeks knee immobilization for acute ligamentous injuries (Young et al. 1982, Ingemann-Hansen and Halkjaer-Kristensen 1983, Halkjaer-Kristensen and Ingemann-Hansen 1985). The proportion of Type IIB fibres was increased in the soleus muscle after a similar period of disuse (Haggmark and Eriksson 1979). However, a recent study reported no significant changes in fibre type proportions after 6 weeks of bed-rest (Berg et al. 1997). There do not appear to be any studies that have investigated the effects of long-term disuse *per se* in healthy humans. However, the combined effects of disuse and disease have been investigated in several patient groups who suffer from inactivity over many years, revealing a reduced proportion of Type I fibres, and a corresponding increased proportion of Type II fibres. Lower-limb amputees show an increased proportion of Type IIB and IIC (transitional) fibres in the quadriceps of the amputated limb versus the non-amputated limb (Renstrom et al. 1983). In patients with congestive heart failure, the proportion of Type IIB fibres in vastus lateralis muscle was more than 2- fold higher than in controls (Massie et al. 1996, Vescovo et al. 1996, Martin 1989). In heart transplantation patients, high proportions of Type II fibres in vastus lateralis muscle were found before, and at 3 and 12 months after transplantation (Type II ~ 66, 67 and 70 % respectively, Bussières et al. 1997). Patients with chronic obstructive pulmonary disease (COPD) also demonstrated a high (78-83%) proportion of Type II fibres (Jakobsson et al. 1990). This suggests that long term inactivity may play an important role in a slow-to-fast twitch muscle fibre transformation in humans, consistent with the large body of evidence in animal studies (see review by Pette and Staron 1997). Unfortunately none of those studies also investigated whether SR function was correspondingly changed with fibre type.

Lung transplant recipients (LTx) have usually been chronically inactive for many years and this may therefore have a major adverse impact on their skeletal muscle characteristics, and may also be a major cause of reduced exercise tolerance. The $\dot{V}O_2$ peak in heart-lung (HLTx), bilateral lung (BLTx) and single lung (SLTx) transplant recipients remains only 45%-70% of that seen in normal subjects (Miyoshi 1990, Theodore 1987, Williams et al. 1992, Banner et al. 1992 Levy et al. 1993). This exercise limitation may be due to abnormalities in pulmonary (especially SLTx) or cardiovascular function (SLTx and/or HLTx) during exercise (Miyoshi 1990, Levine et al. 1994, Ambrosino et al. 1996, Williams and Snell 1997). Alternatively, peripheral defect(s), or abnormalities in skeletal muscle, may the primary site of exercise limitation post-lung transplantation.

LTx recipients are an interesting model to investigate the effect of immobilization or disuse on skeletal muscle characteristic. Post-operative rehabilitation should remove the effects of "acute" disuse and allow us to study the more long-term adaptive changes. These may include the persistence of alterations in fibre type proportion reported in patients with chronic obstructive pulmonary disease, and/or abnormal SR function. Impaired SR function is most likely an important mechanism of muscle fatigue and thus, these changes may contribute to the premature exercise termination in these patients. However, there is little evidence to confirm this hypothesis. Further, little is known about the morphological changes or SR Ca^{2+} regulation in skeletal muscle in LTx.

The aim of this study was, therefore to investigate the possibility that skeletal muscle SR function, muscle fibre types and metabolism may be abnormal in lung transplant recipients. We hypothesised that lung transplant recipients will exhibit a high proportion of Type II fibres and correspondingly high rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity in the vastus lateralis muscle. In addition, metabolites

in resting muscle and incremental exercise performance were also measured in these patients to determine the extent of any abnormalities in the rates SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity .

6.2 Methods

Seven lung transplant recipients (LTx) and seven age- and sex-matched healthy controls (CON) participated in this study. All subject details, and experimental procedures for respiratory function and exercise tests, muscle biopsy and blood sampling and analyses have been fully described in Chapter III.

6.3 Results

6.3.1 Pulmonary Function.

The individual subject pulmonary function data are shown in the Table 6.1. Both the vital capacity (VC) and forced expiratory volume in the first second (FEV_1) in the LTx showed a mild mixed obstructive and restrictive ventilation defect compared to CON (Table 6.2, $P < 0.005$), but the FEV_1/VC ratio did not differ between groups. The arterialised venous oxygen saturation remained above 97% in both groups during exercise.

Table 6. 1 Clinical characteristics and pulmonary function in LTx.

LTx Patient	Operation	Duration Post-operation (mo)	FEV_1 (L)	VC (L)	FEV_1/VC (%)
1	SLTx	12	2.08	4.51	46
2	SLTx	9	1.36	2.22	61
3	HLTx	24	2.36	2.46	96
4	DLTx	8	3.48	4.23	82
5	DLTx	21	3.24	3.82	85
6	DLTx	3	3.40	3.6	94
7	DLTx	4	2.56	3.38	76

Table 6.2 Summary of Lung Function Measurements

Variable	LTx	Controls
FEV_1 (L)	2.64 ± 0.38 †	3.87 ± 0.15
Predicted (%)	75 ± 8**	109 ± 7
VC (L)	3.46 ± 0.32 †	4.78 ± 0.18
Predicted (%)	78 ± 7*	111 ± 9
FEV_1/VC (%)	77.2 ± 6.8	81.1 ± 2.0
Predicted (%)	96 ± 8	98 ± 3

Mean ± SEM, n = 7, * $P<0.05$, ** $P<0.01$, † $P<0.005$, LTx compared with CON values.

6.3.2 Exercise Performance

The peak incremental exercise workrate, $\dot{V}O_2$, $\dot{V}E$ and HR were markedly lower in LTx compared with controls ($P < 0.05$, Table 6.3).

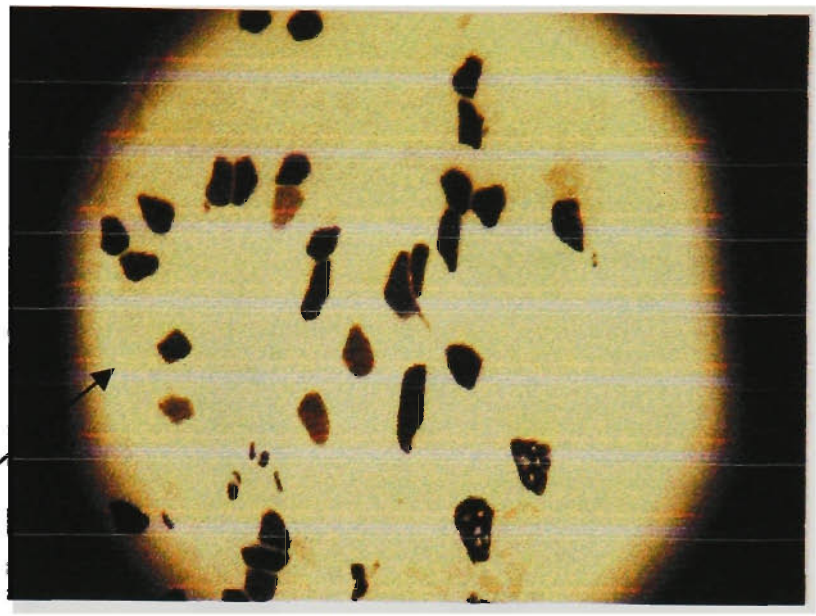
Table. 6.3 Incremental Exercise Results

Variable	LTx	Controls
Peak workrate (W)	88 ± 10 †	218 ± 30
Exercise time (min)	5.43 ± 0.57 *	8.71 ± 1.14
HR peak (bpm)	137 ± 6 ‡	180 ± 4
Predicted (%)	74 ± 2 ‡	95 ± 2
$\dot{V}O_2$ peak ($ml.min^{-1}.kg^{-1}$)	18.7 ± 1.5 ‡	36.9 ± 2.4
Predicted (%)	56 ± 3‡	111 ± 3
$\dot{V}_E$ peak ($L.min^{-1}$)	64.1 ± 7.1*	118.6 ± 12.9
MVV ($L.min^{-1}$)	92.4 ± 10.4	135.5 ± 5.3
$\dot{V}_E$ peak /MVV (%)	72.0 ± 8.0	86.57 ± 6.87

Abbreviations: Predicted $\dot{V}O_2$ peak and HR peak for cycle ergometry from Wasserman et al. (1994); $\dot{V}_E$ peak = minute ventilation at peak exercise; MVV (Predicted maximum voluntary ventilation) = FEV_1 X 35. Values are mean ± SEM, n = 7, * $P <$ 0.05, † $P < 0.005$, ‡ $P< 0.001$, LTx compared with controls.

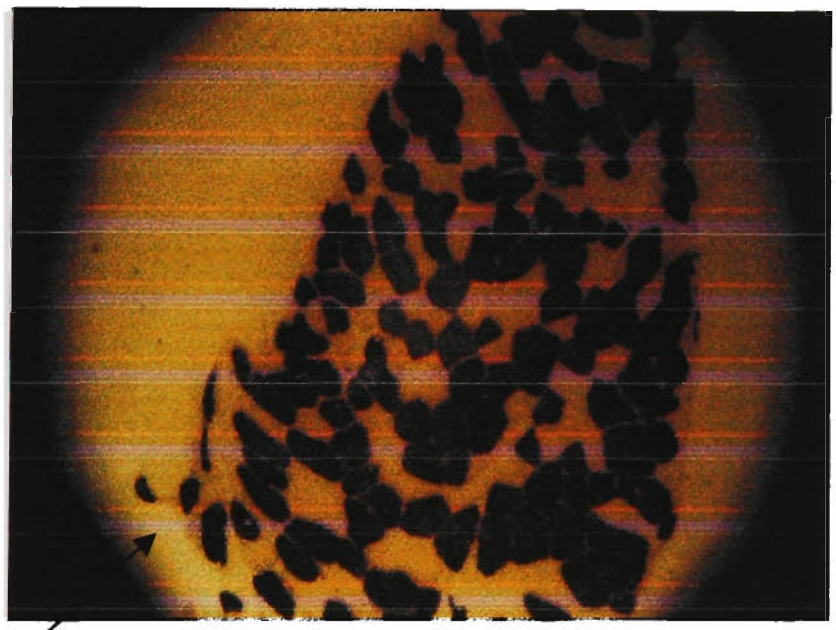
6.3.3 Skeletal Muscle Fibre Types.

A typical histochemical myosin ATPase stain for Type I fibres for one control and one LTx is shown in Figure 6.1. The LTx patients exhibited a lower proportion of Type I and a higher proportion of Type II fibres, compared with the controls ($P < 0.01$, Figure 6.2).



LTx

Type I Fibres (dark color)



Control

Figure 6.1 Myosin ATPase stain for Type I fibres for one control and one LTx .

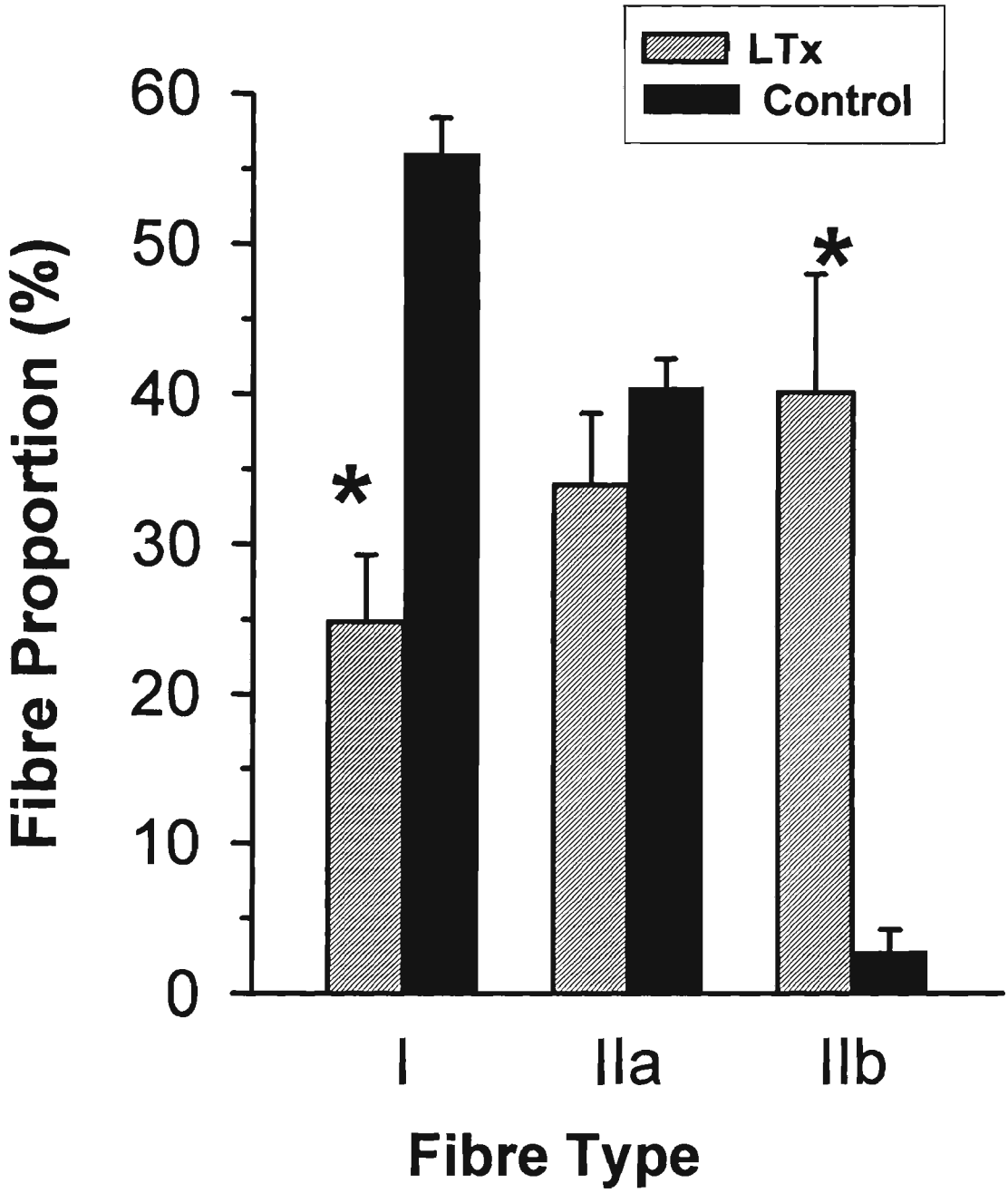


Figure 6.2 The Quadriceps muscle fibre type proportions in LTx patients and controls.

Mean ± SEM, n = 7, $*P < 0.01$.

6.3.4 Skeletal Muscle Metabolites

Resting skeletal muscle ATP content was lower ($P < 0.005$), whilst both lactate and IMP contents were raised in LTx when compared with controls ($P < 0.05$, Table 6.4).

Table 6.4 Resting skeletal muscle metabolites

	LTx	Controls
ATP (HPLC)	21.41 ± 1.16 †	25.97 ± 1.3
ADP	2.81 ± 0.19	2.41 ± 0.14
AMP	0.12 ± 0.02 †	0.24 ± 0.06
Creatine	55.02 ± 4.48	46.88 ± 4.01
PCr	96.81 ± 4.96	93.18 ± 2.67
TCr	151.84 ± 7.52	140.60 ± 5.34
IMP	0.26 ± 0.04 *	0.05 ± 0.01
Lactate	16.26 ± 0.96 †	8.40 ± 0.86
pH	7.06 ± 0.02*	7.11 ± 0.01
ATP/ADP ratio	7.67 ± 0.27 †	11.44 ± 0.45

Values are mean ± SEM, n = 7, expressed in mmol.kg^{-1} dry mass, * P< 0.05. † P < 0.005.

6.3.5 Muscle SR Function

The maximal rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity when expressed relative to muscle weight (nmol.min^{-1}.mg muscle^{-1}) were 34%, 29.8% and 23.8% respectively, lower, in LTx compared to controls ($P < 0.05$, Figures 6.3, 6.4 and 6.5). Similarly, when expressed relative to muscle total protein content (nmol.min^{-1}.mg

protein^{-1}), the maximal rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity were 28% ($P < 0.05$), 26% ($P < 0.05$), and 16% ($P < 0.06$), respectively, lower in LTx compared to controls (Table 6.5).

Table 6.5 SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity in LTx *vs.* controls. expressed relative to muscle total protein.

SR Characteristics	LTx	Controls
Ca^{2+}release	8.96 ± 0.97*	12.69 ± 1.25
Ca^{2+}uptake	8.92 ± 0.62*	12.02 ± 1.27
Ca^{2+}ATPase activity	76.73 ± 4.55 *f*	91.39 ± 4.06

Data are mean±SEM, n = 7; units: nmol.min^{-1}.mg protein^{-1}; *P<0.05, *f* < 0.06.

There were positive relationships between the rate of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity versus the type II muscle fibre proportions in quadriceps muscle 29 healthy controls (included other 5 subjects). However, that didn't have any relationships with seven lung transplant recipients (Figure 6.6 and 6.7). Controls include seven age- and sex-matched healthy controls; as well as eight untrained, eight resistance-trained and eight endurance-trained subjects (from chapter 5).

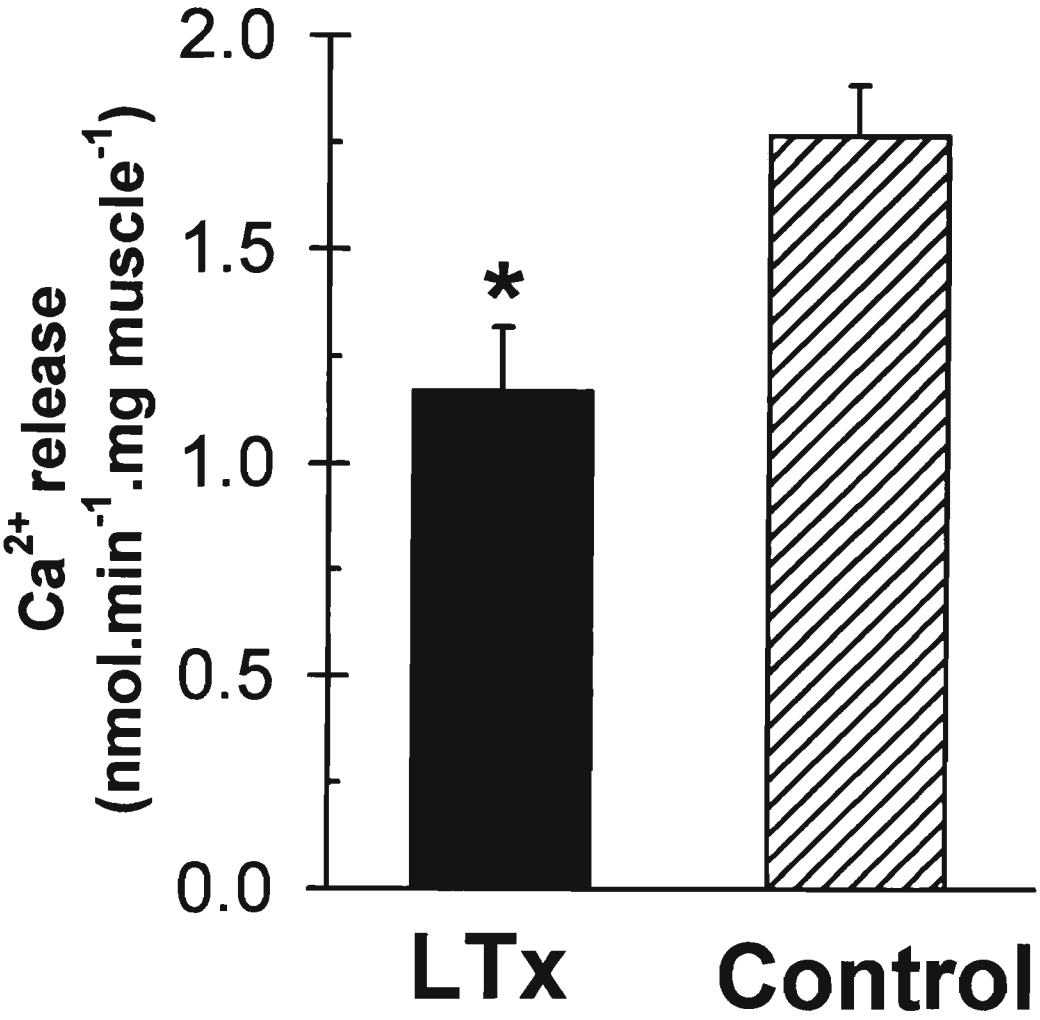


Figure 6.3 Maximal rate of muscle SR Ca^{2+}release in LTx vs Controls. Data are mean±SEM; n=7; *P<0.05; Units: $nmol.min^{-1}.mg\ muscle^{-1}$.

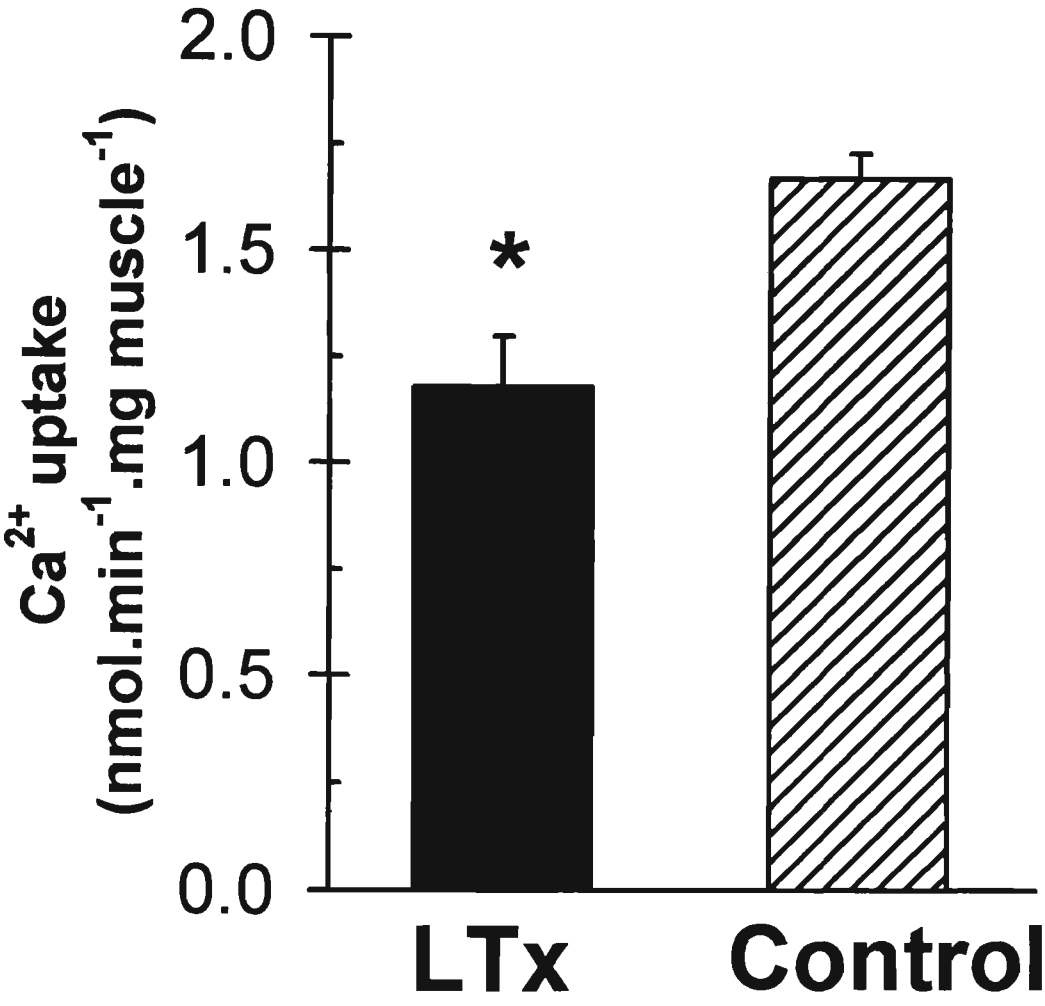


Figure 6.4 Maximal rate of muscle SR Ca^{2+}uptake in LTx vs Controls. Data are mean±SEM; n=7; *P<0.05; Units: $nmol.min^{-1}.mg\ muscle^{-1}$.

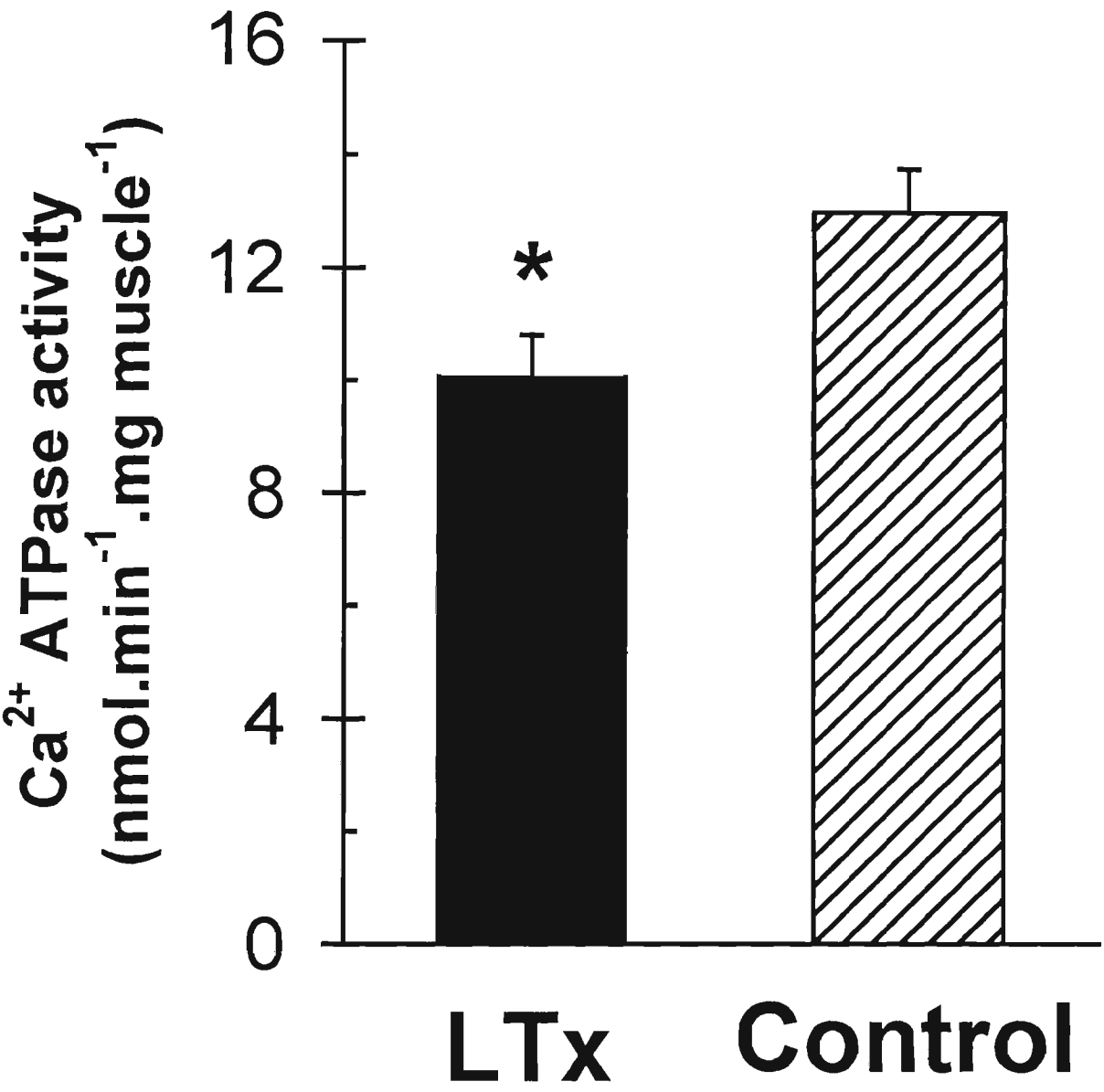


Figure 6.5 Maximal rate of muscle SR Ca^{2+}ATPase activity in LTx vs Controls.

Data are mean±SEM; n=7; *P<0.05; Units: nmol.min^{-1}.mg muscle^{-1}.

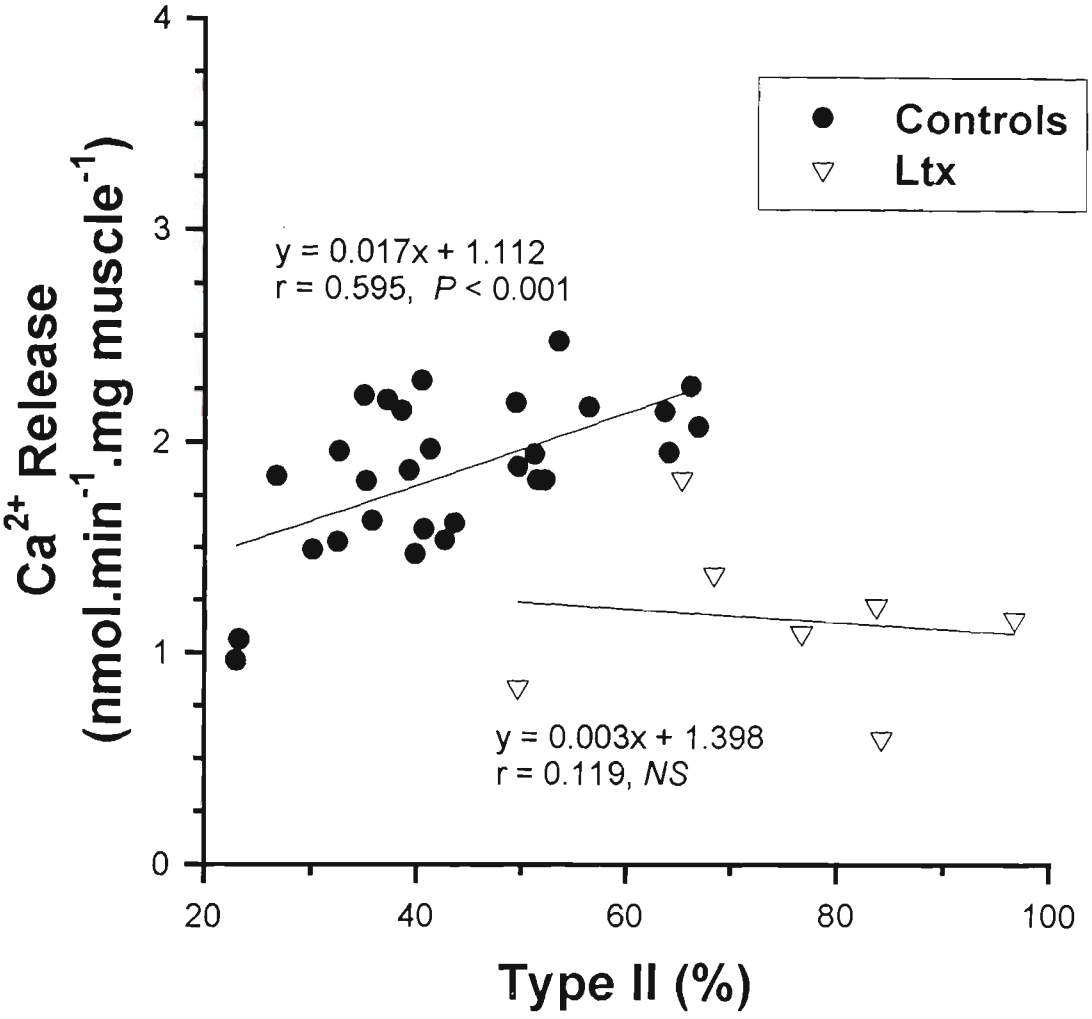


Figure. 6.6 Relationships between the rate of SR Ca^{2+} release versus the type II muscle fibre proportions in quadriceps muscle of seven lung transplant recipients (LTx) and 29 healthy controls. Controls include seven age-and sex-,matched healthy controls; as well as eight untrained, eight resistance-trained and eight endurance-trained subjects (from chapter 5).

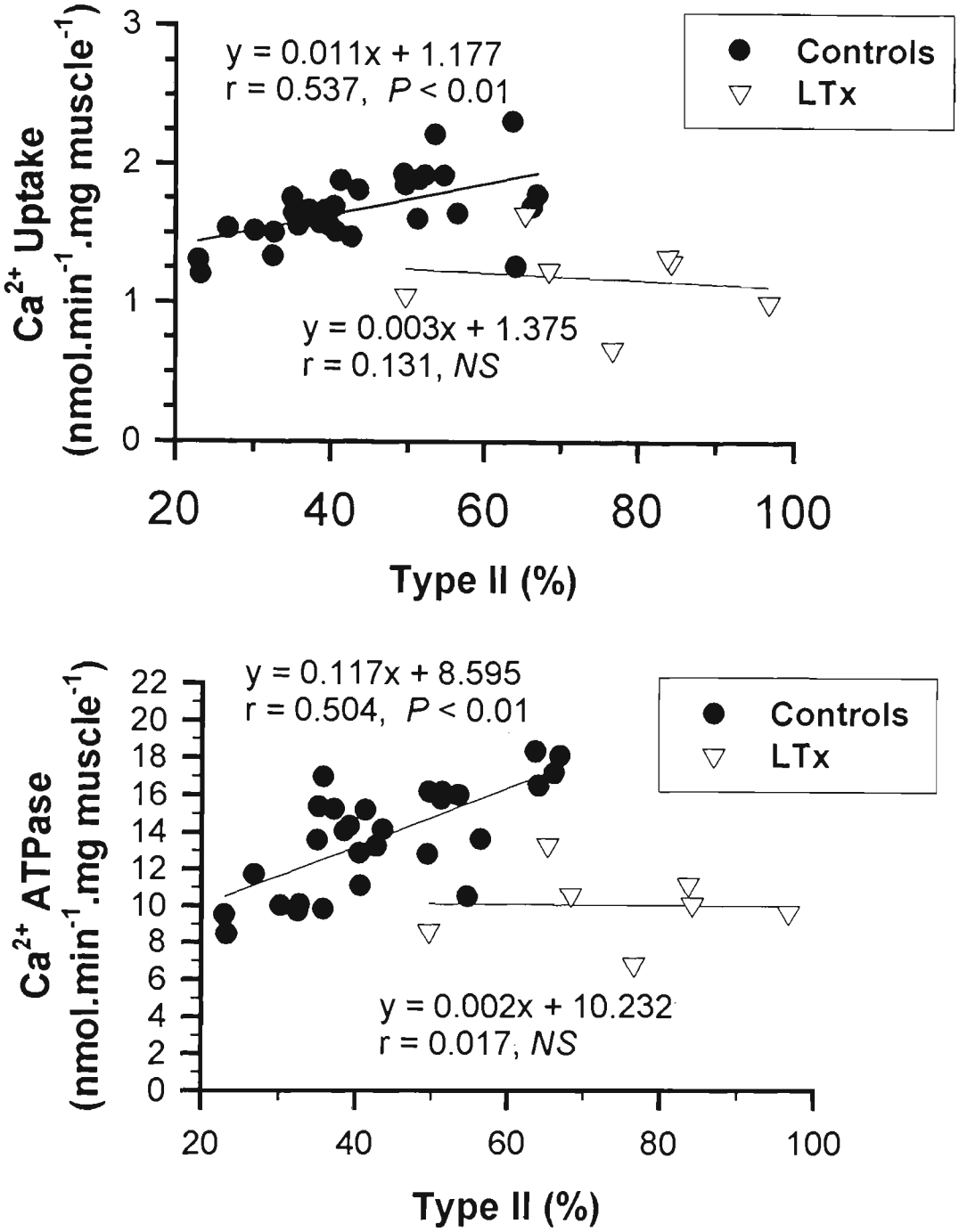


Figure. 6.7 Relationships between SR Ca^{2+} uptake and Ca^{2+} ATPase activity versus the type II muscle fibre proportions in quadriceps muscle of seven lung transplant recipients (LTx) and 29 healthy controls. Controls include seven age- and sex-matched healthy controls; as well as eight untrained, eight resistance-trained and eight endurance-trained subjects (from chapter 5).

6.4 Discussion

The major finding in this study was that each of the sarcoplasmic reticulum (SR) Ca^{2+} release and Ca^{2+} uptake rates, as well as Ca^{2+} ATPase activities, were considerably depressed in the vastus lateralis muscle obtained from lung transplant recipients, when compared to healthy controls. Further, these patients exhibited a higher proportion of Type II muscle fibres than controls, which would normally be expected to result in higher rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity. Thus, these results suggest severe abnormalities in muscle SR function in these patients and/or a dissociation between the expression of myosin ATPase and SR proteins in fast-twitch muscle. it is also possible that these abnormalities in SR characteristics may contribute to reduce muscular performance, consistent with the low VO_2 peak observed in these patients.

6.4.1 Altered Muscle Fibre Types in LTx

The histochemical method used in the present study identifies Types I, IIA and IIB fibres, on the basis of their myosin ATPase staining patterns, representing fibres with increasing myosin ATPase activity (Brooke and Kaiser 1970a, Salmons 1994). This fibre classification system corresponds closely with molecular techniques documenting the presence of the different myosin heavy chain (MHC) isoforms, namely the slow, fast IIA and fast IIB isoforms, respectively (Billeter et al. 1981). Only two fast MHC isoforms have been identified in adult human limb musculature, initially identified as MHCIIA and MHCIIB, with the human MHCIIB isoform being homologous to the rat MHCIID/X isoform (Smerdu et al. 1994, Ennion et al. 1995).

Human vastus lateralis muscle typically contains approximately 50% Type I fibres (Saltin and Gollnick 1983). In LTx muscle, a (~ 38 %) greater proportion of Type IIB

fibres and a (~ 31 %) lesser proportion of Type I fibres was found, compared to healthy controls. The mechanisms underlying these altered fibre proportions observed in LTx in the present study are not clear. Several possible contributory factors include chronic disuse, iatrogenic effects, and/or secondary effects of diseases such as COPD or hypertension. It is seems most likely that the major factor causing this alteration in skeletal muscle fibre type is chronic disuse.

Chronic Disuse. Skeletal muscle is a remarkably adaptable tissue and it is well established that altered physical activity may result in transitions between Type I and II fibres (see Chapter 2, Section 2.5). There is considerable evidence in animal models which supports a slow-to-fast fibre transition with muscular disuse, but there are relatively few reports in humans. However, these few studies suggest that humans demonstrate qualitatively similar muscle fibre transitions with muscular disuse to those observed in small mammals (Young et al. 1980, Ingemann-Hansen and Halkjaer-Kristensen 1983, Halkjaer-Kristensen and Ingemann-Hansen 1985, Saltin et al. 1977, Sargeant et al. 1977, Häggmark and Eriksson 1979, Renström et al. 1982). Therefore, the altered fibre composition found in LTx muscle is consistent with a slow-to-fast fibre conversion, and with the MHCI → MHCIIA→ MHCCIID/X→MHCIIB transition demonstrated with disuse in other mammalian muscle (Pette and Staron 1997). Only a small percentage change in skeletal muscle fibre composition has been reported with training in humans (Chapter 2). However, the time course of most training studies is quite short, typically being 8-12 weeks in duration. However, LTx suffer from disuse for many years and therefore this is likely contributes to the large differences observed between muscles in LTx and in healthy controls.

Iatrogenic Effect. The immunosuppressive drugs taken by LTx patients may induce myopathy. Prior to transplantation, all patients were optimally treated with corticosteroids, whilst after transplantation, the immunosuppressive drug cyclosporine

A (CsA) was also administered. The usual pathology of a corticosteroid myopathy is one of selective atrophy of Type IIB muscle fibres, but a Type IIa fibre atrophy has also been reported (Gardiner et al. 1978, Touno et al. 1996, Wilcox et al. 1989, Nava et al. 1996). However, there is no data indicating that either corticosteroid therapy or CsA alter skeletal muscle fibre type proportions.

Disease Effects. High proportions of Type II fibres have been reported in the quadriceps muscle in a number of different types of patients. These include COPD patients, either with (83% Type II) and without (78%) respiratory failure (Jakobsson et al. 1990); heart failure patients (64%, Sullivan et al. 1990, Mancini et al. 1989); heart transplant recipients (66%, Bussières et al. 1997) and hypertensive patients without peripheral vascular disease (Juhlin-Danfelt et al. 1979, Duey et al. 1993). Each of these patient groups would also suffer from chronic disuse, suggesting that inactivity this may be an important factor underlying their skeletal muscle fibre type abnormalities. However, it is also possible that each disease state directly and/or indirectly effects skeletal muscle fibre type.

6.4.2 SR Function-Fibre Type Dependence

Clear differences exist in SR characteristics between different muscle fibre types, with 2- to 6-fold higher rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2-} ATPase activity reported in Type II compared with Type I fibres in animals (see Table 2.4, Chapter 2). Further, muscles rich in Type IIB fibres (e.g. superficial vastus lateralis 100% Type IIB) have higher rates of SR Ca^{2+} uptake and Ca^{2+} ATPase activity than muscles rich in Type I fibres (eg. soleus muscle, 80% Type I) (Kim et al. 1982). There appears to be limited data confirming this SR function- fibre relationships in human skeletal muscle. Madsen et al. (1994) showed a high correlation between SR Ca^{2+} ATPase content and the

proportion of Type II fibres. Benders et al. (1994) reported a higher SR Ca^{2-} uptake in quadriceps than in soleus muscle and on the basis of fibre types reported a 6-fold higher Ca^{2+} uptake in Type II fibres. This was similar to the difference reported in animal muscles (Kim et al. 1982). This suggests that healthy human muscle also exhibits large differences in SR function between different fibre types. This is consistent with the findings in the previous Chapter, of a significant positive relationship between the proportion of Type II fibres and the maximal rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activities in human muscle (Figure 6.6 and 6.7). Therefore, with the high proportion of Type II fibres found in LTx muscle, high rates of SR Ca^{2+} release, Ca^{2+}uptake and Ca^{2+} ATPase activity would also be expected. To the contrary, these measures of SR function were reduced by between 22- 33% in the LTx muscle relative to the controls. This discrepancy is clearly shown in Figure 6.3, 6.4 and 6.5.

6.4.3 Altered SR Function in LTx

The cause of the reduced SR function in LTx observed in the present study is not clear. Chronic electrical stimulation induces fast-to-slow fibre transition in mammalian muscle, but with different rates of decrease in muscle proteins; among the earliest decrease are in SR proteins, which precede reductions in myosin ATPase (Sarzala et al. 1982, Salmons 1994). Chronic electrical stimulation induces an early decrease in the Ca^{2+}ATPase activity in fast- twitch muscle, but this is largely due to a partial inactivation of the Ca^{2+}ATPase enzyme, rather than a reduction in enzyme numbers (Leberer et al. 1987). Since mysosin ATPase was altered in LTx, then one would expect to also see altered SR function. Normal skeletal muscle adaptation involves changes in contractile function, Ca^{2+} transport kinetics and metabolism (Salmons 1994). Since this was not observed in LTx, the changes observed may reflect dysfunction of the SR in

skeletal muscle in LTx.

6.4.3.1 Chronic disuse effects on SR function

Studies investigating the effects of disuse on SR function have yielded conflicting results, which may be related to different models and durations of disuse, as well as species differences. In rats, increases in SR Ca^{2+}uptake and Ca^{2+} ATPase activity were reported after 1-3 weeks of immobilisation or hindlimb suspension, in the soleus, deep vastus lateralis and gastrocnemius muscles (Kim et al. 1982, Arkhipenko et al. 1993, Stevens and Mounier 1992). However, the time course and stability of these changes were inconsistent. A subsequent reduction was reported in Ca^{2+}ATPase activity after 4 and 6 weeks immobilisation in rat soleus muscle (84% Type I), but not in vastus lateralis (70% Type IIa) or superficial vastus lateralis (100% Type IIB) muscles (Kim et al. 1982). There appear to be no studies investigating long-term disuse on skeletal muscle SR characteristics. Few studies have investigated the effects of muscle disuse on SR function in human muscle. Ten days of cast immobilisation of one leg in women reduced the SR vastus lateralis muscle Ca^{2+} uptake by 21%, but did not significantly change Ca^{2+} ATPase activity or the rate of Ca^{2+} release (Thom et al. 1997). It is possible that the period of immobilisation was too brief to induce a more severe deterioration in SR function. Madsen et al. (1994) found no change in the muscle Ca^{2+} ATPase concentration after 6 weeks detraining, following an endurance training program. Further studies on the effects of inactivity on skeletal muscle SR function in humans are clearly required.

Several factors may contribute to disuse-induced deterioration of SR function, including structural alterations and an increase in muscle oxidative damage. Disuse-induced SR structural changes were recently reported in rat slow and fast fibres (Takekura et al. 1996). They observed increases in the T tubular network, with more triads than in

normal fibres after immobilization. It is possible that this may represent a protective mechanism against the deterioration in SR function with severe disuse.

It is also possible that some of the SR abnormalities seen in LTx result from muscle damage consequent to increased free radical accumulation within muscle. Endogenous scavenging enzymes in muscle prevent excessive accumulation of free radical species, including the enzyme catalase, which removes H_2O_2 (Chance et al. 1979). In rat muscle, catalase activity was increased by 574% after seven days of hindlimb casting, and by 252% seven days after tenotomy (Jenkins et al. 1982). These findings suggest that free radical production within muscle must be increased with muscular disuse. Free radicals depress the rate of Ca^{2+} release from isolated skeletal muscle SR and homogenates in rabbits, via inhibition of the RyR (Kobzik et al. 1994, Mészáros et al. 1995). In addition, the SR Ca^{2+} ATPase activity is depressed by oxidative modification and Ca^{2+}uptake may be slowed by oxidative stress (Scherer and Deamer 1986 a, b; Favero et al. 1998). However, no one has investigated whether free radical compounds accumulate with disuse in human muscle.

6.4.3.2 Disease effects on SR function.

It is not known whether a pre-existing impairment in SR function in LTx muscle is due to disease. It is well known that patients with COPD exhibit abnormal skeletal muscle function (Jacobson et al. 1990, Maltais et al,1996, Clark et al. 1996), but there are no investigations of abnormal SR function in COPD. Two of our LTx patients were diagnosed with cystic fibrosis. Cystic fibrosis, an inherited autosomal recessive condition, is characterised by abnormal chloride channels in the cell of the airway at its luminal surface (Aitken and Fiel 1993). Cystic fibrosis is the most common cause of chronic suppurative lung disease in children. Patients with cystic fibrosis have reduced leg strength, but this is more likely due to malnutrition (Lands et al. 1990).

Five of the LTx patients were hypertensive and this condition may be associated with SR dysfunction. Spontaneously hypertensive rats exhibit reduced Ca^{2+} release channel opening (Po) and sensitivity of DHP binding to Ca^{2+} calcium channels, which may protect against any increase in cytosolic [Ca^{2+}] (Ebata et al. 1990). To date, no study has investigated these possibilities in human muscle.

6.4.3.3 Iatrogenic effects on skeletal muscle SR in LTx

Both corticosteroids and CsA can cause systemic hypertension and myopathy, and these may be linked with SR dysfunction in skeletal muscle. CsA and corticosteroids also can cause deleterious effects by oxygen free radical production, leading to functional and structural cellular changes (Kuo 1994, Kehrer 1993).

6.4.3.3.1 Corticosteroids

Corticosteroids induce a significant loss of muscle protein due to an inhibition of protein synthesis in fast-twitch glycolytic fibres, and a reduction in the speed of relaxation is also shown (Rannels et al. 1978, Wilcox et al. 1989, Paul et al. 1979). Studies investigating SR function in corticosteroid myopathy have given conflicting results, which may be related to dosage, duration, and species differences. In corticosteroid (Triamcinolone) -treated rats, no significant changes were found in rat skeletal muscle SR Ca^{2+} uptake or release (Peter et al. 1972). In contrast, in Triamcinolone - treated rabbits, decreased SR Ca^{2+} uptake and Ca^{2+} release, as well as reduction in Ca^{2+}ATPase activity were all found in EDL muscle (Shoji et al. 1969). Administration of corticosteroids reduced SR and myofibrillar protein concentration in rat diaphragm (Lieu et al. 1993). It would therefore seem likely that corticosteriods may have an adverse effect on skeletal muscle SR function in human muscle. However, this remains unknown.

6.4.3.3.2 Antihypertensive medication

In the present study, five of the seven LTx patients were treated for hypertension with calcium channel blockers such as Diltiazem or Verapamil, or other antihypertensive drugs. The antihypertensive effect of Diltiazem is less than that of Verapamil. Diltiazem inhibited frog skeletal muscle contractures induced by Ag^{+}, which opens the RyR and leads to SR Ca^{2+} release (Oba and Hotta 1987). Therefore, the antihypertensive drugs used might potentially adversely affect SR function in LTx.

6.4.3.3.2 Cyclosporine A

Sarcoplasmic Reticulum function. There are no studies that have investigated the effects of CsA on skeletal muscle SR characteristics. However, several studies have demonstrated detrimental effects of CsA on cardiac muscle. High concentrations of CsA inhibited Ca^{2+} ATPase activity in rat cardiac muscle (Hutcheson et al. 1995) and had an acute cardiotoxic effect, which in part appeared to be calcium related (Olbrich et al. 1990). Kingma et al. (1991) investigated the cardiotoxic effect of CsA in the isolated perfused heart model in rats. They found significantly lower peak systolic pressures genrated in hearts from CsA treated rats. A reduced peak stress by cardiac trabeculae was found with CsA and this could be explained by reduced Ca^{2+} release from the SR with each action potential (Banijamali et al. 1993). Abnormalities in the diastolic properties of the heart have been described following human cardiac transplantation and may reflect, at least in part, decreased SR Ca^{2+} uptake, which tended to decline 4-5 months postoperatively (Limas et al. 1987). The decrease Ca^{2+} uptake in SR following cardiac transplantation may in part be the biochemical basis for the reported impairment in diastolic relaxation.

Lamb and Stephenson (1996) reported that the immunosuppressive agent FK506 resulted in an irreversible loss of coupling between the FK506- binding protein and the

RyR. The biological properties of CsA and FK506 are remarkably similar (Johansson and Moller 1990; Lin et al. 1991) and both are used as immunosuppressive agents in organ transplants. It is therefore possible that CsA may similarly affect the Ca^{2+} release channels in skeletal muscle and thereby diminish Ca^{2+} release in LTx. The vesicle preparation used in this thesis to measure *in-vitro* SR function does not rely on the DHPR to activate the Ca^{2+} release channels. However, a possible *in-vivo* structural alteration would persist and could be involved in the *in-vitro* impairment in SR function seen in LTx muscle.

Other common side effects if CsA treatment include nephrotoxicity, and magnesium loss (Mervaala et al. 1997). Withdrawal of CsA induced a rise in serum magnesium in all kidney transplant patients, as well as a marked decrease in the serum level of urate within one week (Hilbrands et al. 1996). Hypomagnesaemia may lead to skeletal muscle SR dysfunction and myopathy (Larner, 1994, Astier et al. 1996). Dietary magnesium deficiency in rats enhances free radical production and affects the structural and the functional properties of skeletal muscle SR membranes. The SR Ca^{2+}ATPase activity was reduced in hypomagnesaemia (Rock et al. 1995, Astier et al. 1996). The LTx patients in this study were regularly tested and aggressively treated for hypomagnesaemia, thus their plasma [Mg^{2+}] was kept at the low end of the normal clinical range. It is therefore not clear whether this state may have contributed to their reduced rate of SR uptake and Ca^{2+}ATPase activity. Hypomagnesaemia is expected to enhance SR Ca^{2+} release and therefore may not be the cause of the depressed rate of SR Ca^{2+} release in LTx.

CsA causes oxidative stress in rat hepatocytes (Wolf et al. 1997). Further, lipid peroxidation and/or modifications in lipid metabolism may provide a potential explanation for the structural and functional alterations in Mg-deficient SR membranes

(Astier et al. 1996). Oxidative stress in skeletal muscle has been shown to impair SR membrane integrity, Ca^{2+} release and Ca^{2+}ATPase activity (Davies et al. 1982, Scherer and Deamon 1986a, Favero et al. 1998, Reid 1996).

6.4.3.3.3 Other influences of CsA on skeletal muscle.

A further potential adverse effect of CsA is an inhibition of skeletal muscle mitochondrial respiration *in vitro* (Hokanson et al. 1995, Mercier et al. 1995). In a study on the same patients, the rates of mitochondrial ATP production and mitochondrial enzyme activities were found to be markedly reduced compared to controls (Wang et al. 1997). The numbers of capillaries adjacent to individual fibers was reduced by CsA in rat EDL muscle (Biring et al. 1998). Finally, CsA can interact with other medications. For example, CsA alters the disposition of HMG-CoA reductase inhibitor, which caused marked myofibre injury (Smith et al. 1991). Rhabdomyolysis, myolysis and myoglobinuric acute renal failure due to combinations of CsA and Lovastatin have been reported in heart transplant patients (Corpier et al. 1988, Tober 1988, Alejandro, 1994).

Thus, a variety of iatrogenic effects and in particular corticosteriods and CsA may have a marked influence on skeletal muscle SR in LTx.

6.4.4 Do SR Abnormalities Contribute to the Impaired Peak Exercise Performance in LTx?

The LTx had a markedly lower VO_2 peak compared to the control subjects, consistent with previous studies (Theodore et al. 1987, Gibbons et al. 1991, Williams, and Snell. 1997). Although the low VC and FEV_1 demonstrated mild obstructive and restrictive ventilatory defects in LTx, only one of the patients reached a ventilatory limitation during exercise and none significantly desaturated. This suggests that peripheral factors were primarily responsible for the severe exercise limitation observed in these patients,

consistent with other reports (Theodore et al. 1987, Gibbons et al. 1991).

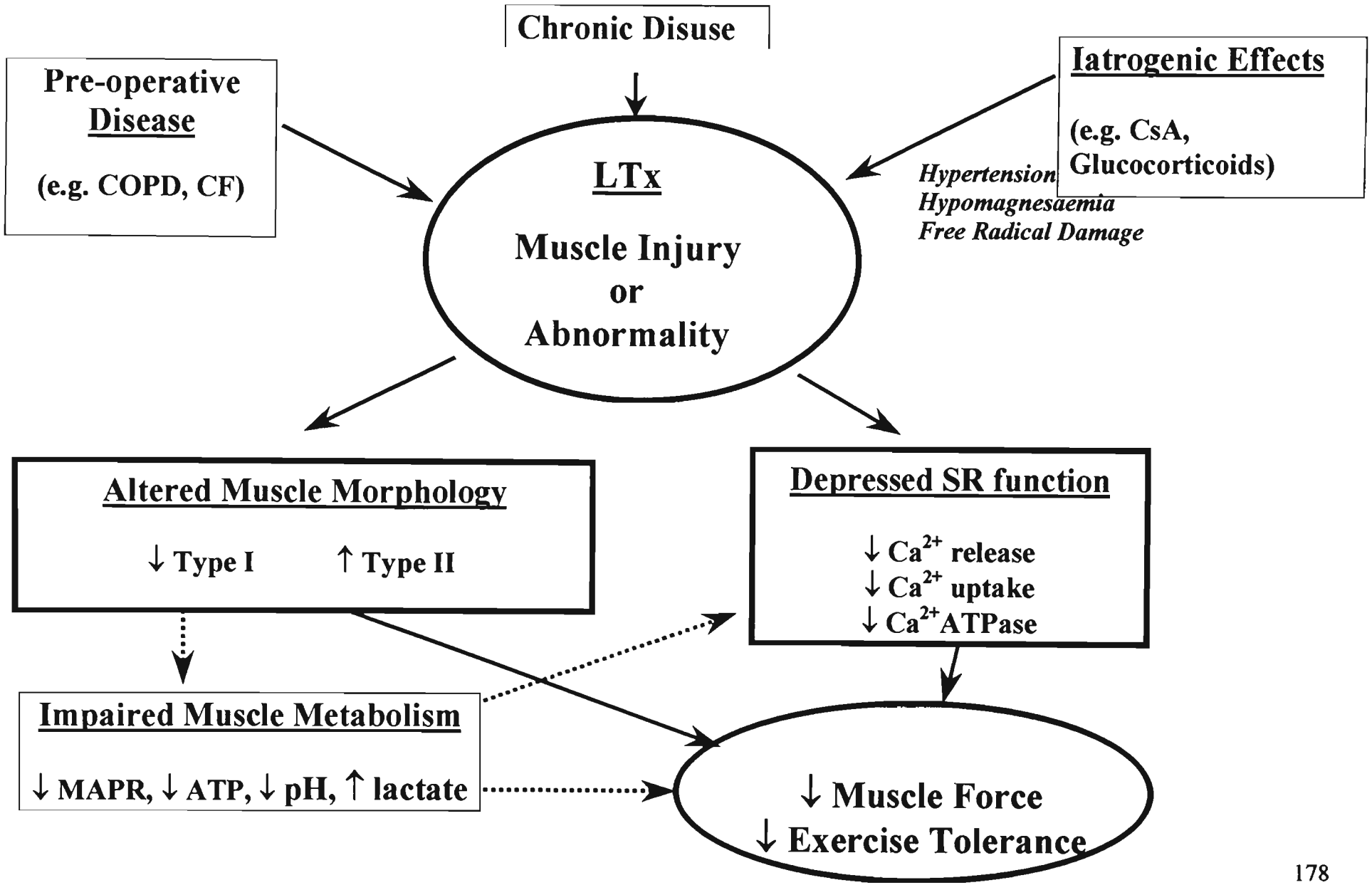
It is well known that the SR regulates the cytosolic [Ca^{2+}], and thereby regulates force development and relaxation in skeletal muscle (see Chapter 2). The LTx exhibited impaired *in-vitro* SR function with decreased rates of Ca^{2+} release and Ca^{2+} uptake, when compared to both their own fibre composition and to the rates observed in healthy controls. It is important to note that the SR measurements in this study were conducted *in-vitro* and therefore indicate structural abnormalities in the RyR or associated proteins, and in the Ca^{2+}ATPase enzyme in resting muscle in these patients. A reduced SR Ca^{2+} release may lead to lower cytosolic [Ca^{2+}] and a consequent reduction in force output in LTx. However, it is likely that this deficiency in SR function may be exacerbated *in-vivo* as evidenced by several metabolic abnormalities, which were present in resting muscle. These include elevations in muscle Lac^- and IMP, as well as reductions in muscle ATP content. If these metabolic abnormalities present in resting muscle persist during exercise, these adverse effects may be even more marked and contribute to muscular fatigue. Consistent with this premise, these patients demonstrated an early rise in plasma [Lac^-] during the incremental exercise test, which strongly suggests an earlier intramuscular Lac^- accumulation. Further, LTx demonstrate impaired skeletal muscle mitochondrial function (Wang et al. 1997, Evans et al. 1997), which suggests that muscle ATP may be compromised to a greater extent in these patients than in controls. Both elevations in lactate and local reductions in ATP would be expected inhibit opening of the SR RyR (Meissner et al. 1987, Favero et al. 1995). These may then explain the poor exercise capacity in these patients. A further abnormality in these patients was the low muscle pH at rest, consistent with a ^{31}P-MRS study in which the quadriceps muscle intracellular pH in LTx was found to be less at rest and declined earlier during incremental exercise compared to controls (Evans et al. 1997). A reduced pH can inhibit SR Ca^{2+} ATPase activity and thus Ca^{2+} uptake (Lamb et al. 1992). Thus,

the depression in SR Ca^{2+} uptake in resting muscle in LTx might also be exacerbated during exercise compared with in healthy muscle. The initial effect of reduced SR Ca^{2+} uptake in LTx would be to elevate cytosolic [Ca^{2+}] and therefore help preserve force output. However, SR Ca^{2+} uptake may lead to reduced loading of SR and therefore limit the Ca^{2+} available for release. Thus, decreased SR function could conceivably contribute to the marked exercise limitation in these patients. This is shown schematically in Figure 6.8.

6.5 Conclusions

A higher proportion of type II fibres, together with a significantly reduced SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity were found in skeletal muscle in LTx compared to controls. Thus, the depression in SR function was even worse when considered in the context of the existing muscle fibre type in these patients. This impaired SR function in skeletal muscle in LTx may result from chronic disuse, disease and/or iatrogenic effects, of which CsA and corticosteriods may be the most important. The results of this study cannot directly determine which factors limit muscular performance in patients post lung transplantation. However, the demonstrated impairment in muscle SR function is likely to be a important factor to their limited exercise capacity and exercise tolerance.

Figure 6.8.SR Function and Skeletal Muscle Morphological Alteration in Lung transplant recipients (LTx). MAPR mitochondria ATP production rate; CsA Cyclosporine A; COPD chronic obstructive pulmonary disease; CF cystic fibrosis.



Chapter VII Exposure to Cyclosporine A Reduces in vitro Sarcoplasmic Reticulum Calcium Release and Uptake in Rat Skeletal Muscle

7.1 Introduction

Cyclosporine A (CsA) has become a principal immunosuppressive agent used in post organ transplantation. Numerous adverse side-effects of CsA have been reported including common nephrotoxicity, hyperuricemia (Bennett et al. 1994), systematic hypertension, myocardial toxicity, hepatotoxicity, hyperkalemia and gout (Jung and Pergande 1985, Broekmeirer et al. 1989, Paul et al 1989). Further, many studies have shown that CsA significantly depressed mitochondrial function, in each of rat renal cortical cells (Simonl et al. 1997), liver (Broekmeier et al. 1989, Samuta 1993), cardiac and skeletal muscle (Altschuld 1992, Mercier 1995, Hokanson 1995), as well as in human renal tissue (Jung and Reinholdt 1987). Skeletal muscle toxicity has also previously been postulated on clinical grounds (Goy et al. 1989, Fernandez-Sola et al. 1991, Biring et al. 1998).

An alteration of the expression of the SERCA calcium pump isoforms with CsA was shown in rat lymphocytes (Launay et al. 1997). Enhanced generation of hydrogen peroxide *in vitro* (Wolf et al. 1994, 1997) and lipid peroxidation *in vivo* (Inselmann et al. 1988) has also been demonstrated.

At high concentrations CsA has an acute cardiotoxic effect, which in part appears depresses SR Ca^{2+}ATPase activity in cardiac muscle in rats (Hutcheson et al 1995). Further, chronic exposure to CsA inhibited maximal force development by cardiac

trabeculae which was suggested to results from increased SR Ca^2 release (Banijamali et al. 1993). CsA can also influence Ca^{2+} regulation in myocyte (Salducci et al. 1996, Zamzami et al. 1995, Broekmeirer et al. 1989).

In the previous chapter, abnormal SR function was found in lung transplant recipients, with the maximal rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity 37%, 31% and 24%, respectively, lower in lung transplant recipients compared to controls. It is possible that part of this effect may be due to toxic effects of CsA on skeletal muscle SR. However, no studies to date have examined the acute effects of CsA on sarcoplasmic reticulum (SR) function in skeletal muscle. This study therefore determined the effects of CsA on *in vitro* SR function in rat skeletal muscle homogenate, using CsA at concentrations close to clinical levels in skeletal muscle (about 1ug/ml).

7.2 Materials And Methods

7.2.1 General Protocol

The extensor digitorum longus (EDL) and soleus (SOL) muscles of eight female Sprague-Dawley rats were used in this study, as fully described earlier (Chapter 3). Details of muscle homogenisation and of the muscle homogenate Ca^{2+} uptake, Ca^{2+} release and Ca^{2+} ATPase assays are described fully in Chapter 3.

7.2.2 Cyclosporine A Preparation

Pure drug Cyclosporine A, which was kindly provided by Sandoz Australia, was dissolved in 50% ethanol at room temperature (~25°5) and 0.02 mg/ml, 0.5 mg/ml, and 1 mg/ml stock solutions of CsA were prepared. The homogenate from a single muscle sample was divided into four 100 µl aliquots, which were then placed into separate cryule tubes, each containing 5µl of either 0 (50% ethanol), 1, 25 or 50 µg/ml CsA. The cryule tubes containing homogenate plus CsA were then rapidly frozen and stored in liquid nitrogen

for later analyses. A second set of CsA stock solutions were then made up, at [CsA] of 0.2 $mg.ml^{-1}$, 5.0 $mg.ml^{-1}$ and 10.0 $mg.ml^{-1}$. Then 11 µl from the 0.2 $mg.ml^{-1}$, 5 $mg.ml^{-1}$ and 10 $mg.ml^{-1}$ CsA stock solutions were then added to the 2200 µl working buffer for the SR assay, to give a final cuvette [CsA] of 1, 25 and 50 $\mu g.ml^{-1}$, respectively. To initiate the SR assay, the previously frozen muscle homogenate plus CsA solution was then added to the working buffer with the appropriate [CsA].

Each of the four samples was analysed in the same measurement session for Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity (Chapter 6). All homogenates were analysed over a period of 2 months.

7.2.3 Statistical Analyses.

Statistical analyses were using a one-way analysis of variance, with a Newman-Kuels post-hoc analysis. A level of significance of $P<0.05$ was accepted for all analyses. All data are presented as mean ± standard error of the mean (SEM).

7.3 Results

All the concentrations of CsA in the range (1-50 µg /ml) inhibited the rates of SR Ca^{2+} release (Figure 7.1), Ca^{2+}uptake (Figure7. 2), and Ca^{2+}ATPase activity (Figure 7. 3), in both EDL and SOL muscles in the rat. Which were significant depressions in 1, 25 50 µg /ml CsA treated muscle ($P<0.05$). SR Ca^{2+}ATPase activity decreased association in a dose-dependent manner in EDL muscle.

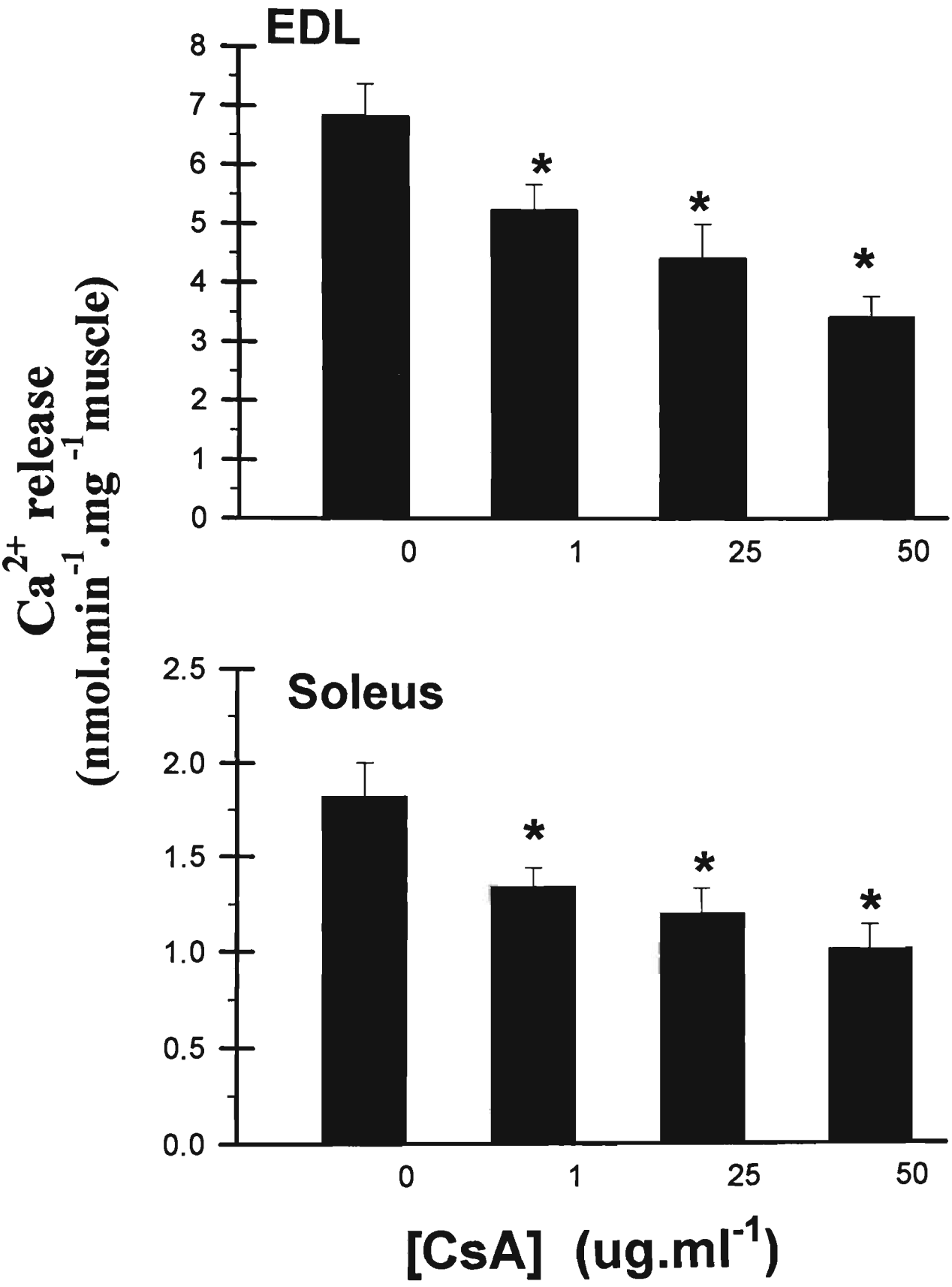


Figure 7.1 CsA inhibited SR Ca^{2+} release in both EDL and SOL muscle. Mean ± SEM.

N=8; * P <0.05.

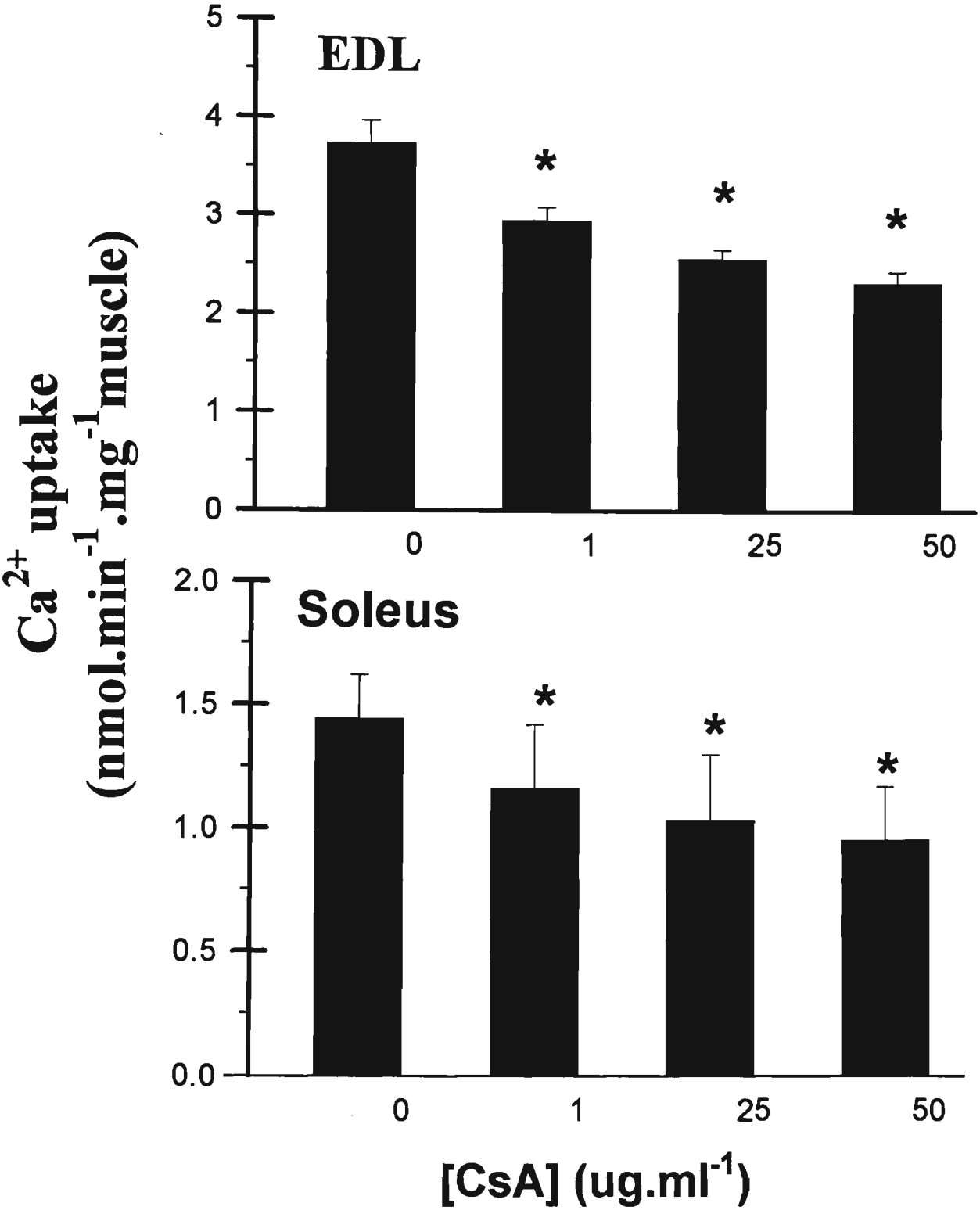


Figure 7. 2 CsA inhibited SR Ca^{2+} uptake in both EDL and SOL muscle. mean ± SEM. n=8; * P <0.05.

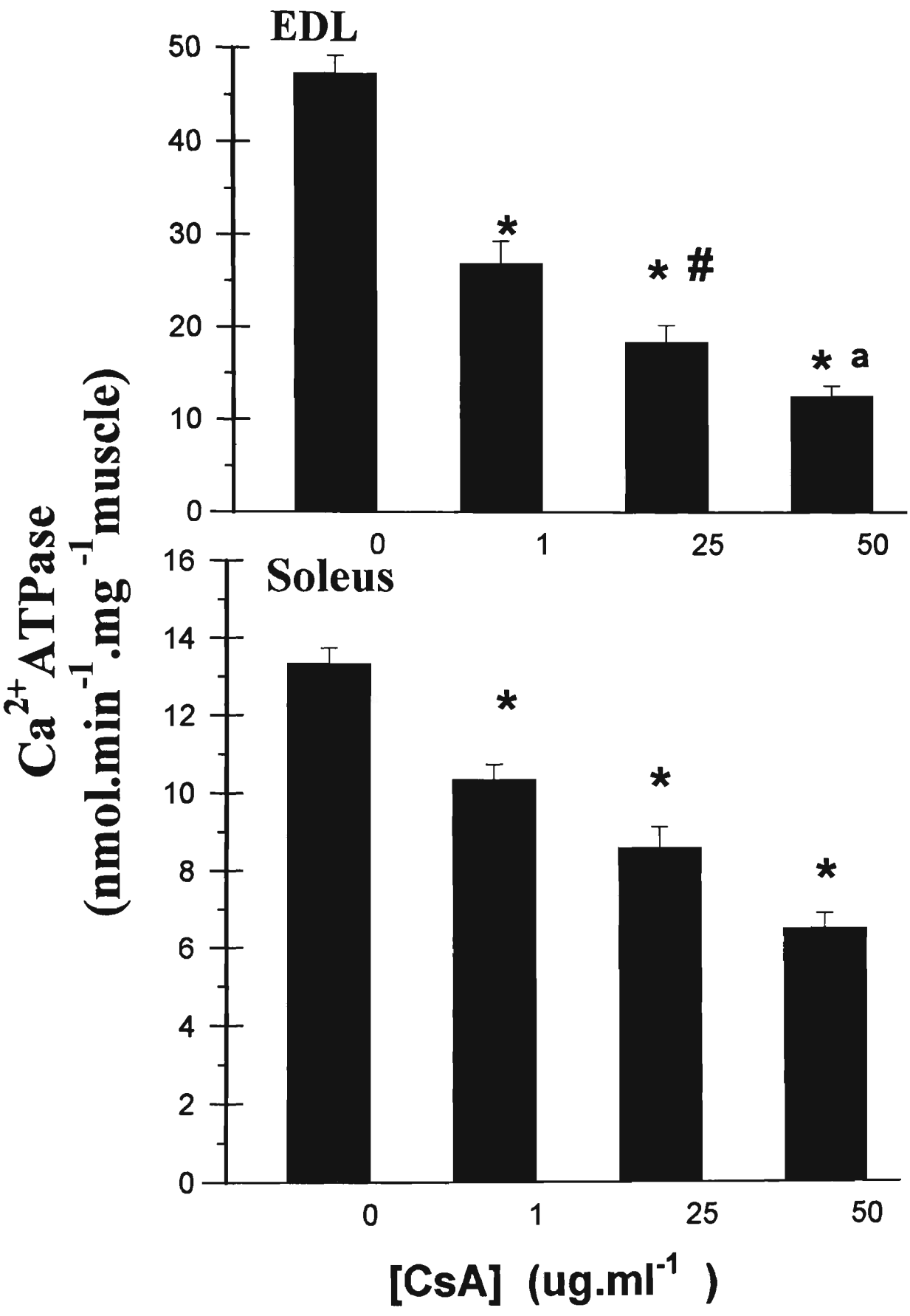


Figure 7.3 CsA inhibited SR Ca^{2+} ATPase activity in both EDL and SOL muscle. mean±SEM; N=8; * 0 vs 1μg, 25μg, 50 μg /ml; #1μg vs 25μg /ml; a, 25μg vs 50 μg /ml (P<0.05).

7.4. Discussion

The major finding in this Chapter was that acute exposure to CsA at concentrations tested (1-50 μg /ml) inhibited the rates of SR Ca^{2+} release, Ca^{2+}uptake and Ca^{2+}ATPase activity in both EDL and SOL muscles in the rat. There are no published studies that have investigated the effects of CsA on skeletal muscle SR characteristics.

A difficulty in designing the present study is that the CsA concentration in skeletal muscle in patients receiving CsA is not exactly known. The lowest CsA concentration of 1μg/ml was chosen based on an estimate of muscle CsA concentration from the limited number of clinical observations available. Patients receiving organ transplantation usually receive long term CsA treatment, with their blood CsA concentration maintained at 150-500 ng /ml. There is no data currently available detailing the skeletal muscle CsA concentration in these patients. Lensmeyer et al. (1988) reported the deposition of the metabolites of CsA in skeletal muscle in three deceased patients who had received CsA. The patients were a heart transplant recipient who had been treated with CsA for 3 days, a bone marrow transplant recipient and a liver transplantation recipient who had each been maintained on CsA for 3 months. The results for CsA distribution were inconclusive, with higher CsA present in muscle in one patient, in blood in another, and with similar levels in muscle and blood in the third patient. However, the CsA metabolite M17, hydroxycyclosporine was ~2-3 fold higher in the muscle than in the blood in all three patients. Further, Wager et al. (1987) measured the ^{3}H-cyclosporine deposition in several tissues in rats, finding a 5 to 8-fold higher CsA concentration in muscle than in blood. Therefore, it seems likely, that at a least in patients undertaking CsA therapy the deposition of CsA in the muscle may be approximately 2 to 3-fold higher than in the blood. Since blood CsA is maintained at a concentration of 150-500 ng/ml, we estimated muscle CsA concentration to be least 1μg/ml. In the present study, marked depressive effects of CsA were found with acute exposure a concentration of 1μg/ml, the rates of SR Ca^{2+} release, Ca^{2+}uptake and

Ca^{2+}ATPase activity 23.2, 20.2 and 43.1% were reduction in EDL and 24.7, 19.7 and 34.3 % SOL muscles. This strongly suggests that similar changes to SR function would occur in muscle of lung transplant recipients as a result of CsA. Thus these findings suggest that the 37 %, 31 % and 24 % depression in SR function reported in the previous Chapter could in part be due to the effects of CsA.

In the present study, depressions were 1.5 to 4 times higher in EDL muscle, which suggest the influence may be dominant in fast-twitch muscle. Since lung transplant recipients have an abnormally high (75%) type II fibre proportion, it is possible that their SR is inhibited by CsA more severely than other population.

7.4.1 Possible Mechanisms of CsA on Muscle SR

The current study does not demonstrate the mechanism(s) by which CsA either acutely, or chronically inhibits SR function in skeletal muscle. Several possibilities for the *in-vivo* CsA effects include oxidative stress and hypomagnesemia. CsA causes oxidative stress, and can induce acute organ failure in hepatocytes and renal cortical cells in rats (Wolf et al. 1997, Baliga et al. 1997). CsA induced Mg^{2+}-deficiency has been demonstrated in rats and in humans (Rock et al. 1995, Astier et al. 1996). Further, lipid peroxidation and/or modifications in lipid metabolism may provide a potential explanation for the structural and functional alterations in Mg^{2+}-deficient SR membranes (Astier et al. 1996) and the inhibited SR Ca^{2+} uptake and Ca^{2+} ATPase activity in rat (Rock et al. 1995). Oxidative stress in skeletal muscle has been shown to impair SR membrane integrity, Ca^{2+} release and Ca^{2+}ATPase activity (Davies et al. 1982, Scherer and Deamon 1986a, Favero et al. 1998, Reid 1996).

Low ATP can reduce the rate of Ca^{2+} release, Ca^{2+} uptake and ATPase activity of SR (Meissner et al. 1987). Also CsA can damage mitochondria morphologically and functionally (Stone et al. 1989, Lochner et al. 1987, Darly-Usmar et al. 1990), leading to subsequent loss of energy supply (Fourier et al 1987, Crompton et al. 1992).

The structurally and functionally similar immunosuppressive agent, FK506 (Johansson and Moller 1990, Lin et al. 1991) induced an irreversible loss of coupling between the FK506- binding protein and the RyR in the rat (Lamb and Stephenson 1996). It is therefore interesting to speculate that CsA may similarly effect the Ca^{2+} release channels in skeletal muscle. However, it was speculated that chronic CsA increased opening of the RyR in cardiac muscle (Barjimali et al. 1993).

7.4.2 Other Effects of CsA

A variety of toxic side effects of CsA treatment have been reported, such as neprotoxicity, hepatotoxicity, myocardiotoxicity, hypertension, gout, hyperuricemia, hypomagnesaemia, hyperkalemia and hypertrichosis (Paul et al 1989, Hans et al. 1990, Rock et al. 1995, Astier et al. 1996).

Several studies have demonstrated detrimental effects of CsA on cardiac muscle. High concentrations of CsA (~100 ug/ml) inhibited Ca^{2+} ATPase activity in rat cardiac muscle (Hutcheson et al. 1995) and had an acute cardiotoxic effect. Kingma et al (1991) investigated the cardiotoxic effect of Cyclosporine A in the isolated perfused heart in rats. They found low peak systolic pressures in hearts obtained from Cyclosporine A- treated rats that had received 15 or 7.5mg /kg /24h subcutaneously for three weeks ($p<0.05$), and indicated a decrease in myocardial contractile force that was apparently unrelated to decreased to renal function or changes in mean arterial pressure. CsA A reduced peak stress in cardiac muscle and this could be explained by reduced Ca^{2+} release from the SR (Banijamali et al. 1993).

Several groups of organ transplant recipients demonstrated markedly impaired exercise performance. Post lung transplant (LTx) patients have an exercise intolerance, which persists in spite of restoration of near normal lung function (Williams and Snell 1997). Low $\dot{V}O_2max$ values were also shown in cardiac-transplant and in kidney-transplant

recipients (Theodore et al. 1987, Painter et al. 1986). This suggests that the exercise deficit may be related to skeletal muscle dysfunction. Since acute CsA exposure exerted multiple influences on *in-vitro* SR function and presumably then also on excitation - contraction coupling in intact skeletal muscle, it is possible that CsA may also directly influence the exercise capacity of skeletal muscle. Further studies investigating contractile performance of muscle cells after acute and chronic CsA exposure are warranted. Also, the experiments may necessary measure possible differences with longer incubation times and comparisons of adding CsA to fresh *vs.* frozen homogenates.

7.5 Conclusion

In conclusion, this study has confirmed that CsA concentrations at 50μg/ml, 25μg/ml and 1μg/ml significantly inhibit the rates of SR Ca^{2+} release, Ca^{2+} uptake and ATPase activity in rat skeletal muscle. These results indicate that CsA could then inhibit SR function in skeletal muscle post lung transplantation.

Chapter VIII General Discussion And Recommendations For Further Research

8.1 Introduction

This thesis demonstrated the important effects of five factors on SR function measured in a crude homogenate preparation from human skeletal muscle biopsies. The first four comprised muscle fibre type, training status, chronic inactivity and acute exercise. The final study demonstrated that the immunosuppressive drug cyclosporin A also affected SR function in rat skeletal muscle.

Major Findings

8.2.1 Fibre Type Influences SR Ca^{2+} Release, Ca^{2+} Uptake and Ca^{2+}ATPase Activity in Human Skeletal Muscle

Highly significant relationships were found in human skeletal muscle between the proportion of Type II muscle fibres and the rates of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity. These rates were estimated from regression analyses to be respectively ~2, ~3 and ~4-fold higher in Type II than Type I fibres in vastus lateralis muscle. This has important implications for studies in which SR function is measured in human muscle.

The importance of muscle fibre composition is clearly evidenced in the interpretation of SR function measurements in chronic training and chronic disuse/disease. In endurance trained athletes, significantly lower SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+}ATPase activity were measured in resting muscle, relative to untrained healthy controls. However, this

was heavily dependent upon muscle fibre type, as these athletes also had a significantly lower proportion of Type II fibres. In subjects who had undertaken resistance training for at least two years, a lower rate of SR Ca^{2+} uptake (per protein) was found at rest. However, this could not be attributed to differences in muscle fibre composition. No significant differences in muscle fibre type were found between resistance trained and untrained muscle, but the proportion of Type II fibre was 7% higher (not significant) in resistance trained subjects. Thus the expected direction for SR function differences on the basis of muscle fibre type II proportion would be slightly higher values of SR function in resistance trained than in control muscle. However, lower SR Ca^{2+} uptake was found in the resistance trained muscle compared with control group. Therefore, the lower SR Ca^{2+} uptake in resistance trained muscle group might reflect muscular hypertrophy and/or a reduced expression of the SR calcium pump. It is also possible that differences in expression of Ca^{2+} ATPase isoforms might also be important (Kandarian et al. 1994).

The importance of fibre type in evaluating SR function was particularly pronounced in lung transplant recipients. These patients had a much higher proportion of Type II muscle fibres (more than 32%) than their age- and sex-matched healthy controls, consistent with a possible long-term effect of disuse altering muscle phenotype towards IIB fibres. Despite the higher proportion of type II fibres, which should result in higher SR characteristics, each of SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity in skeletal muscle were significantly reduced in LTx compared to controls, by 33%, 29% and 22%, respectively. Thus, the depression in SR function was even worse when considered in the context of the existing muscle fibre type in these patients. This impairment in muscle SR function is likely to be an important factor to their limited exercise capacity and exercise tolerance.

8.2.2 Fatiguing Exercise Depresses SR Ca^{2+} Release, Ca^{2+} Uptake and Ca^{2+}ATPase Activity In Human Skeletal Muscle

Acute, fatiguing exercise of short duration was shown to depress each of the rates of SR Ca^{2+} release (42%), Ca^{2+} uptake (43%) and Ca^{2+}ATPase activity (39%) in human skeletal muscle. This is the first report of a depressed rate of SR Ca^{2+} release in human muscle with exercise. This finding supports previous direct and indirect measurements of reductions in SR Ca^{2+} release in other mammalian and amphibian muscles (Westerblad et al. 1991, 1993, Favero et al. 1993, Williams et al. 1997). SR Ca^{2+} uptake was also depressed after fatiguing exercise in human skeletal muscle, confirming other earlier reports (Gollnick et al. 1991, Booth et al. 1997, Warmington 1997, Hargreaves et al. 1998). However, this thesis demonstrated for the first time with high intensity exercise, that this depression in SR Ca^{2+} uptake was also highly related to, and thus, most likely directly caused by, a depression in SR Ca^{2+}ATPase activity.

Positive relationships were found between the proportion of Type II fibres and the absolute and percentage depressions in each of muscle SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity with fatigue. This strongly suggests that the SR in Type II fibres in human muscles is more susceptible to fatigue than the SR in Type I fibres. This is consistent with the conclusions reached in a recent review examining numerous factors affecting SR in different muscle fibre types (Stephenson et al. 1998).

Significant relationships were found between muscle SR function and muscle metabolites, when resting and fatigued muscle data were pooled. However, this most likely reflected parallel processes as a result of exercise, rather than causal relationships. This was more clearly shown when the changes in SR function were compared with the changes in muscle metabolites as a result of fatiguing exercise. Both the absolute and percentage depression in each of muscle SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity had no significant relationships with any of muscle ATP, PCr, glycogen, IMP, lactate and pH.

This strongly argues against alterations in muscle metabolites inducing the impairments observed in SR function. Due to the *in-vitro* nature of the analyses undertaken, the most likely mechanisms for these fatigue effects involve structural alterations in the SR Ca^{2+} release channel and the Ca^{2+}ATPase enzyme. The actual mechanisms responsible for these changes cannot be deduced from this study. However, it is likely that fatigue effects on *in-vivo* SR function are even greater than the *in-vitro* effects measured here, due to the combined effects of the observed structural alterations and the probable *in-vivo* metabolic effects on SR.

8.2.3 Different Trained States Express Different Fatiguability

A clear depression in SR function was seen in each of the untrained, resitance trained and endurance trained groups. Thus, chronic training did not prevent the deterioration in SR function. However, some differences in the effects of fatigue on SR were found in the two trained groups compared to controls. An attenuated absolute reduction in SR Ca^{2+} uptake and Ca^{2+} ATPase activity was found with fatigue in ET compared to untrained and/or resistance trained muscle. This could be interpreted as a protective effect of training on SR function, and/or may reflect either a lesser fatigue effect on SR in Type I fibres. To control for the lower resting values for SR function due to a higher proportion of Type I fibres in the endurance trained group, the percentage reduction in these variables was also calculated. This did not differ significantly between the three groups, which then argues against a protective training effect on SR function. However, the small subject numbers studied in each group and the 9-15% lower (n.s.) values in SR function in endurance trained muscle raise the possibility of a Type II error and therefore limit the interpretations possible from this study.

8.2.4 Chronic Inactivity, Disease and/or Iatrogenic Effects Impair Skeletal Muscle SR Function In Lung Transplant Recipients

Abnormally high proportions of Type II fibres, coupled with significantly reduced SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity were found in skeletal muscle in LTx compared to controls. This impaired skeletal muscle SR function may result from chronic inactivity, disease and/or iatrogenic effects. The alterations in muscle fibre type are consistent with changes seen in animal muscle models with chronic disuse and also with reports in muscle of chronic heart failure patients. This thesis confirmed that at the concentrations investigated (1 – 50 μg/ml), the immunosuppressive agent, CsA significantly inhibited the rates of SR Ca^{2+} release, Ca^{2+} uptake and ATPase activity in rat skeletal muscle. Thus, acute exposure to CsA depressed SR function in both EDL and SOL muscles in the rat, which suggests that CsA could also inhibit skeletal muscle SR function in lung transplant recipients. The CsA concentration in skeletal muscle in humans is unknown, but a concentration of 1 μg/ml is similar to the blood CsA concentration in these patients. Therefore these results suggest that CsA could inhibit skeletal muscle SR function post lung transplantation.

8.3 Recommendations for Further Research.

Studies conducted in this thesis have raised many further questions regarding the effects of acute exercise, training status, chronic inactivity and disease on SR function in human skeletal muscle. These questions lie within the realms of physiology, pharmacology and pathology and require further investigation. Use of a longitudinal experimental design would be of benefit in studies investigating the time course of training effects on SR function in both healthy and diseased individuals, as well as the time course of progressive disease effects on SR function. No studies have examined long-term alterations in SR function and fibre type in human skeletal muscle, in training or disuse.

Relatively little is known about DHPR and RyR function in human skeletal muscle and the effects of training, fatigue and disuse. Such studies would clearly be of importance.

Fatiguing exercise induces marked declines in SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase activity. However the underlying mechanisms for these changes are still unclear. Reactive oxygen species are likely to be important factors, which complex influences on SR function. Studies examining the effects of antioxidants on muscular fatigeability would be valuable.

The use of histocytochemical and immunocytochemical methods co-operatively would be of great benefit to future work in human muscle. Such work might focus on the expression of SR calcium pumps (eg. SERCA1 and SERCA2 DNA, RNA), as well as subunits of the DHPR and RyRs.

The effects of acute exercise, training and chronic inactivity on SR function in human skeletal muscle will continue to be an interesting area for research in the coming decade. Research in this area can be expected to have considerable impact on understanding the mechanisms of fatigue and of exercise limitation in sporting and clinical domains, in the prevention or attenuation of intracellular substructural changes and therefore ultimately, to result in enhanced exercise capacity in both healthy and patient.

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APPENDIX A
VICTORIA UNIVERSITY OF TECHNOLOGY

STANDARD CONSENT FORM FOR SUBJECTS INVOLVED IN EXPERIMENTS

CERTIFICATION BY SUBJECT

I, ...
of.....
certify that I have the legal ability to give valid consent and that I am voluntarily giving my consent to participate in the experiment entitled :

"Effects of Resistance Training on Human Skeletal Muscle Ionic Regulation and Mitochondrial Function."

being conducted at Victoria University of Technology by :

Dr Michael J. McKenna, Associate Professor Michael F. Carey

I certify that the objectives of the experiment, together with any risks to me associated with the procedures listed hereunder to be carried out in the experiment, have been fully explained to me by :

.....

and that I freely consent to participation involving the use on me of these procedures.

Procedures:

1. Anthropometric measurements, including underwater weighing
2. Lung function measurements
3. Maximal incremental exercise test on a cycle ergometer
4. Venous catheterisation and blood sampling during incremental exercise test
5. Biodex muscle function tests
6. Muscle biopsies at rest and following fatiguing knee extensor exercise on Cybex.
7. Venous catheterisation and blood sampling during Cybex fatigue test

I certify that I have had the opportunity to have my questions answered and that I understand that I can withdraw from the experiment at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the confidentiality of the information I provide will be safeguarded.

Signed : .....)
)
Witness other than the experimenter :) Date : .....
)
.....)

VICTORIA UNIVERSITY OF TECHNOLOGY

Subject Information Sheet

"Effects of Resistance Training on Human Skeletal Muscle Ionic Regulation and Mitochondrial Function."

INVESTIGATORS:
Dr Michael J. McKenna, Assoc. Prof. Michael F. Carey,
Department of Physical Education & Recreation, Department of Chemistry & Biology, Victoria University of Technology, Footscray, Vic.

Aim of the study:
This study will compare muscle size, biochemistry, maximal strength and the development of fatigue during repeated contractions, between strength trained an untrained individuals.

Subject participation:
As a volunteer to participate as a control subject, you are free to withdraw from the study at any time, without any adverse effects, reactions or discrimination.

Exercise Testing and Lung Function Testing Procedures:
You will be required to attend the Exercise Physiology Laboratory (Room L329, Building L), at the Footscray Campus of Victoria University of Technology on two occasions. On the first visit to the laboratory you will be required to perform a maximal incremental exercise test on a cycle ergometer to evaluate your maximal aerobic exercise characteristics. Blood samples will be taken from a hand vein at rest, during and following exercise to indicate your metabolic response to exercise. During this visit we will also measure the capacity of your lungs and the rate at which you can breath air in and out, by getting you to blow air in/out of a special instrument. We will also determine the size of your leg muscles by measuring the girth, length and volume of your leg and your lean .

Muscle Function Testing
Your maximal thigh muscle strength will be determined using a Biodex isokinetic dynamometer located in the C.R.E.S.S. biomechanics laboratory at 300 Flinders Street, City Campus. The test will require you to perform several maximal contractions at different speeds of contraction. This will be followed by a test to fatigue your thigh muscles, involving 50 repeated contractions of your leg muscles.

Blood Samples:
Blood samples (each 5 ml) will be drawn from a catheter inserted into a vein on the back of your hand. A catheter is a thin, flexible, sterile piece of tubing which sits inside your vein. With the use of a small tap, blood samples can be drawn from the catheter at specific times. A total of around fifty (50) ml of blood will be taken during the test. (Normally 400 ml is taken when you donate at the Blood Bank). Blood samples will be analysed for haemoglobin, haematocrit, plasma pH, gases, ions, lactate and other metabolites.

The catheters used for drawing blood are routinely used for clinical purposes and exercise testing; however, slight bruising may occur at the site of catheter insertion. On very rare occasions, blood clots may form. Emergency equipment will be present at all tests. To

avoid any risk of blood borne diseases all catheters, syringes and disposable items will be sterile, not previously used and discarded immediately following use.

Muscle Biopsies and Muscle Fatigue Testing:
On the second visit to the Human Performance Laboratory, Footscray Campus, a muscle biopsy will be taken from your thigh muscle whilst you are lying on a couch. You will then be asked to perform the muscle fatigue test involving 50 repeated contractions of your thigh muscle, on the Cybex dynamometer. A muscle biopsy will be taken immediately after exercise finishes. Muscle biopsies are routinely carried out in our laboratory, with no adverse effects. The three possible complications with a muscle biopsy are pain, infection and internal bleeding. To prevent you from suffering pain, a local anaesthetic (needle) will be given in the skin overlying your thigh muscle. To prevent infection, only sterilised instruments will be used. To prevent excessive bleeding, pressure will be manually applied after biopsies have been taken and maintained through use of a pressure bandage. In addition, no major nerves or blood vessels are located in the vastus lateralis muscle that we biopsy.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your co-operation.

CARDIOVASCULAR RISK FACTOR QUESTIONNAIRE

In order to be eligible to participate in the experiment investigating:
"Effect of Resistance Training on Human Skeletal Muscle Ionic Regulation and Mitochondrial Function"
you are required to complete the following questionnaire which is designed to assess the risk of you having a cardiovascular event occurring during an exhaustive exercise bout.

Name: _____ **Date:** _____

Age: _____ **years Weight:** _____ **kg Height:** _____ **cms**

Give a brief description of your average activity pattern in the past 2 months:

_____

_____

_____

Circle the appropriate response to the following questions.

1.	Are you overweight?	Yes	No	Don't know
2.	Do you smoke?	Yes	No	Social
3.	Does your family have a history of premature cardiovascular problems (eg. heart attack, stroke)?	Yes	No	Don't Know
4.	Are you an asthmatic	Yes	No	Don't Know
5.	Are you a diabetic?	Yes	No	Don't Know
6.	Do you have a high blood cholesterol level?	Yes	No	Don't Know
7.	Do you have high blood pressure?	Yes	No	Don't Know
8.	Are you on any medication?	Yes	No	

If so, what is the medication? _____

9. Do you think you have any medical complaint or any other reason which you know of which you think may prevent you from participating in strenuous exercise? No Yes, please elaborate _____

I, _____, believe that the answers to these questions are true and correct.

Signed: _____ Date: _____

MUSCLE BIOPSY QUESTIONNAIRE

NAME: _____

ADDRESS: _____
DATE: _____ **AGE:** _____ **years**

1. Have you or your family suffered from any tendency to bleed excessively ? (eg. haemophilia) or bruise very easily ?

   Yes No Don't Know

   If yes, please elaborate...____
   _____

2. Are you allergic to local anaesthetic?

   Yes No Don't Know

   If yes, please elaborate...____
   _____

3. Do you have any skin allergies?

   Yes No Don't Know

   If yes, please elaborate...____
   _____

4. Have you any allergies that should be made known?

   Yes No Don't Know

   If yes, please elaborate... _____
   _____

5. Are you currently on any medication?

   Yes No Don't Know

   If yes, what is the medication? _____
   _____

6. Do you have any other medical problem that should be made known?

   Yes No

   If yes, please elaborate... _____
   _____

To the best of my knowledge, the above questionnaire has been completely accurately and truthfully.

Signature: _____ Date: _____

MUSCLE BIOPSY PROCEDURE

The muscle biopsy is a relatively painless procedure that is used to obtain small samples of skeletal muscle tissue for metabolic analysis. A small incision is made in the skin overlying the muscle, under local anaesthetic. The biopsy needle is then inserted into the muscle and a small piece of tissue removed from the muscle. During this part of the procedure you may feel some pressure and a tendency for the muscle to cramp, however, this only persists for a few seconds. Following the biopsy the incision will be closed using a steri-strip and a pressure bandage will be applied which should be maintained for 24-48 hours. Steri-strip closure should be maintained for a few days. You should not exercise for 24-48 hours after biopsies and you should avoid heavy knocks. It is common for subjects to experience some mild soreness in the muscle over the next 2-3 days, however this passes and does not restrict movement. In some rare cases mild haematomas have been reported, but these symptoms disappear within a week. The whole procedure will be preformed under sterile conditions by a qualified medical practitioner. Although the possibility of infection and significant bruising is quite small, if by chance it does eventuate, inform us immediately and then consult your doctor.

CATHETERISATION

At specific intervals throughout the exercise test a small blood sample will be taken via a catheter placed into a hand vein. The catheter consists of a needle and teflon tubing. The tubing is fed over the top of the needle (which has punctured the vein) and into the vein. The needle is then withdrawn, leaving only the teflon tubing in your vein for the remainder of the experiment. A tap (stopcock) is placed into the tubing so the flow of blood along the tubing can be altered at will. This procedure allows the taking of multiple blood samples without the need for multiple venepunctures (puncturing of the vein). Each time a blood sample is taken, a small volume of sterile heparinised saline will be injected to clear the catheter and keep it patent. Catheterisation of subjects is slightly discomforting and can lead to the possibility of bruising and infection. The use of sterile, disposable catheters, syringes, swabs etc. will markedly reduce the possibility of infection caused by the catheterisation procedure. The use of qualified and experienced staff will reduce the likelihood of bruising as this is primarily caused by poor venepuncture techniques. Although the possibility of infection and significant bruising is quite small, if by chance it does eventuate, inform us immediately and then consult your doctor.

VICTORIA UNIVERSITY OF TECHNOLOGY

STANDARD CONSENT FORM FOR SUBJECTS INVOLVED IN EXPERIMENTS

CERTIFICATION BY SUBJECT

I, ...
of.....
certify that I have the legal ability to give valid consent and that I am voluntarily giving my consent to participate in the experiment entitled :

"Skeletal muscle analysis post thoracic transplantation: control subjects"

being conducted at Victoria University of Technology by :

Dr Michael J. McKenna, Assoc. Prof. Michael F. Carey, Dr Trevor Williams, Dr Michael Hall

I certify that the objectives of the experiment, together with any risks to me associated with the procedures listed hereunder to be carried out in the experiment, have been fully explained to me by :

.....

and that I freely consent to participation involving the use on me of these procedures.

Procedures

.....
.....
.....
.....
.....

I certify that I have had the opportunity to have my questions answered and that I understand that I can withdraw from the experiment at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the confidentiality of the information I provide will be safeguarded.

Signed : .....)
)
Witness other than the experimenter :) Date : .....
)
.....)

VICTORIA UNIVERSITY OF TECHNOLOGY

Subject Information Sheet

"Skeletal muscle analysis post thoracic transplantation: control subjects"

INVESTIGATORS:
Dr Michael J. McKenna, Assoc. Prof. Michael F. Carey,
Department of Physical Education & Recreation, Department of Chemistry & Biology,
Victoria University of Technology, Footscray, Vic.

Dr Trevor Williams, Dr Michael Hall
Department of Respiratory Medicine, The Alfred Hospital, Prahran, Vic.

Aim of the study:
This study will compare your muscle composition and metabolism, as well as maximal exercise performance, with that of patients that have undergone heart, lung or heart and lung transplants. The study is designed to determine why these transplant patients have a reduced exercise performance.

Subject participation:
As a volunteer to participate as a control subject, you are free to withdraw from the study at any time, without any adverse effects, reactions or discrimination.

Exercise Testing Lung Function Testing Procedures:
On two occasions, separated by a four week time period, you will be required to attend the Exercise Physiology Laboratory (Room L329, Building L), at the Footscray Campus of Victoria University of Technology. On the first visit to the laboratory you will be required to perform a maximal incremental exercise test on a cycle ergometer to evaluate your maximal aerobic exercise characteristics. Blood samples will be taken from a hand vein at rest, during and following exercise to indicate your metabolic response to exercise.
We will measure the capacity of your lungs and the rate at which you can breath air in and out, by getting you to blow air in/out of a special instrument. This carries no additional risk.

Blood samples:
Blood samples (each 5ml) will be drawn from a catheter inserted into a distal forearm vein. A catheter is a thin, flexible, sterile piece of tubing which sits inside your vein. With the use of a small tap, blood samples can be drawn from the catheter at specific times. A total of around fifty (50) ml of blood will be taken during the test. Blood samples will be analysed for haemoglobin, haematocrit, plasma pH, gases, ions, lactate and other metabolites.

The catheters used for drawing blood are routinely used for clinical purposes and exercise testing; however, slight bruising may occur at the site of catheter insertion. On very rare occasions, blood clots may form. Emergency equipment will be present at all tests. To avoid any risk of blood borne diseases all catheters, syringes and disposable items will be sterile, not previously used and discarded immediately following use.

Muscle Biopsies:
On the second visit to the laboratory, a muscle biopsy will be taken from your thigh muscle whilst you are lying on a couch. Muscle biopsies are routinely carried out in our laboratory, with no adverse effects. The three possible complications with a muscle

biopsy are pain, infection and internal bleeding. To prevent you from suffering pain, a local anaesthetic (needle) will be given in the skin overlying your thigh muscle. To prevent infection, only sterilised instruments will be used. To prevent excessive bleeding, pressure will be manually applied after biopsies have been taken and maintained through use of a pressure bandage. In addition, no major nerves or blood vessels are located in the vastus lateralis muscle that we biopsy.

By signing the informed consent form you are indicating that the tests and procedures have been explained to you and are understood by you. Also, it is accepted by the investigators and by yourself that you are participating voluntarily in the study and that you are free to withdraw from the investigation at any time. Thank you for your co-operation.

APPENDIX B TABLES

Table A 1a. SUBJECT CHARACTERISTICS IN UT

Untrained	Age (year)	Height (cm)	Body Mass (kg)	VO2peak ($ml.min^{-1}.kg^{-1}$)	HRmax (bpm)	AT %VO2peak (%)	Sum of 6 (mm)	Body Fat (%)	VC (L)	FeV1.0 (L)
SF	24	186.0	83.1	44.82	179	63%	67.4	13.88	5.00	4.70
BK	20	172.0	64.6	50.51	200	65%	37.9	8.30	5.10	4.40
SM	26	182.2	84.6	44.30	183	65%	88.1	16.06	5.20	4.00
CS	27	185.0	82.9	48.20	186	67%	65.3	14.00	5.80	4.10
DM	34	181.4	79.4	43.58	182	60%	53.4	12.12	5.20	4.30
PT	27	190.5	87.2	47.32	195	74%	84.0	15.10	5.00	4.35
DA	27	188.5	80.6	47.61	192	65%	83.3	15.40	7.09	5.32
PA	26	181.0	80.9	43.99	200	75%	118.1	21.20	4.37	3.60
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	26.4	183.3	80.4	46.0	189.6	0.67	74.7	14.5	5.35	4.35
SD	3.9	5.7	6.8	2.5	8.3	0.1	24.4	3.6	0.8	0.5
SEM	1.4	2.0	2.4	0.9	2.9	0.0	8.6	1.3	0.3	0.2

Table A1b. SUBJECT CHARACTERISTICS

Resistance trained	Age (year)	Height (cm)	Body Mass (kg)	VO2peak ($ml.min^{-1}.kg^{-1}$)	HRmax (bpm)	AT %VO2peak (%)	Sum of 6 (mm)	% BF (%)	VC (L)	FeV1.0 (L)
CG	20	171	81.62	39.26	189	63%	81.9	14.4	5.2	4.00
CN	37	173.5	88.09	28.95	176	54%	48.2	11.2	3.7	3.00
CH	25	175.8	76.66	45.27	189	58%	48.7	10.1	5.5	4.50
DF	20	182.7	79.32	52.74	189	81%	40.4	8.4	4.81	3.97
KG	26	183.6	82.86	47.47	188	76%	66.6	12.7	7.66	6.32
DK	18	172.9	82.39	58.2	186	76%	53.9	10.8	6.06	5.07
SZ	39	173	81.39	47.04	171	77%	54.9	11.6	5.24	4.32
AK	29	176.5	80.31	31.24	189	86%	72.0	15.7	5.66	4.80
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	26.8	176.1	81.6	43.8	184.6	0.7	58.3	11.9	5.5	4.5
SD	7.9	4.7	3.3	10.1	7.1	0.1	13.9	2.3	1.1	1.0
SEM	2.8	1.7	1.2	3.6	2.5	0.0	4.9	0.8	0.4	0.3

Table A1c. SUBJECT CHARACTERISTICS IN ET

Endurance trained	Age (year)	Height (cm)	Body Mass (kg)	VO2peak ($ml.min^{-1}.kg^{-1}$)	HRmax (bpm)	AT %VO2peak (%)	Sum of 6 (mm)	% BF (%)	VC (L)	FeV1.0 (L)
PH	25	189.4	89.0	74.25	197	84%	51.6	9.27	6.2	4.7
AW	25	184	81.0	71.27	192	73%	45.2	9.09	5.41	4.27
FS	29	177	74.4	72.41	185	74%	34.3	7.6	6.68	5.47
PL	25	174.8	66.2	66.8	171	85%	27.1	6.5	5.24	4
JM	22	168	67.1	63.84	201	82%	69.6	13.5	4.6	3.8
RA	32	169	59.4	63.92	185	78%	23.6	6.1	6.15	4.62
GP	25	179	76.2	64.73	180	84%	50	10.4	4.38	3.9
PB	28	176	83.4	63.53	195	76%	62.1	12.8	6.68	5.47
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Mean	26.4	177.2	74.6	67.59	188	0.80	45.44	9.41	5.59	4.50
SD	3.1	7.1	9.9	4.4	9.9	0.0	16.3	2.7	0.9	0.7
SEM	1.1	2.5	3.5	1.5	3.5	0.0	5.8	1.0	0.3	0.2

Table A 2. Muscle Fiber Type Results

Untrained subject	Total Fibers No.	Type I %	Type II %	Type IIb %
BK	212	36.3	63.7	24.5
CS	193	48.7	51.3	23.3
SM	376	62.8	37.2	2.4
DM	399	64.2	35.8	3.3
PT	404	56.4	43.6	0
DA	226	59.3	40.7	4.9
PA	112	46.4	53.6	13.4
SF	281	50.5	49.5	0
n	**8**	**8**	**8**	**8**
mean	**275.38**	**53.08**	**46.93**	**8.98**
SD	**108.18**	**9.41**	**9.41**	**10.13**
SEM	**38.25**	**3.33**	**3.33**	**3.58**

Resistance Trained				
subject	**Total Fibers No.**	**Type I %**	**Type II %**	**Type IIb %**
CG	133	33.1	66.9	13.5
DF	161	43.5	56.5	11.8
CN	231	33.8	66.2	16.9
CH	222	59.5	40.5	1.4
KG	128	35.9	64.1	10.9
DK	214	67.3	32.7	0
SZ	165	64.8	35.2	3
AK	412	58.7	41.3	1.2
n	**8**	**8**	**8**	**8**
mean	**210**	**43.6**	**56.4**	**10.3**
SD	**85.6**	**11**	**11**	**5.9**
SEM	**28.5**	**4.9**	**4.9**	**2.7**

Endurance Trained				
Subject	**Total Fibers No.**	**Type I %**	**Type II %**	**Type IIb %**
PH	80	67.5	32.5	0
AW	120	65	35	2.5
FS	137	61.3	38.6	0
PL	122	77	23	2.5
JM	96	69.8	30.2	3.1
RA	231	73.2	26.8	1.7
GP	202	76.7	23.3	0
PB	130	48.5	51.5	4.6
n	**8**	**8**	**8**	**8**
mean	**139.8**	**67.4**	**32.6**	**2**
SD	**51.4**	**9.4**	**9.4**	**1.4**
SEM	**18.2**	**3.3**	**3.3**	**0.7**

Table A 3a. Metabolites In Untrained

expressed in $mmol.kg^{-1}$ dry mass

	ATP (HPLC)		ADP		AMP		IMP		ATP/ADP		Total N	
	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue
SF	24.22	27.03	2.06	2.44	0.11	0.09	0.10	2.54	10.24	7.02	26.39	29.55
BK	27.71	20.08	3.16	3.95	0.20	0.20	0.04	4.15	8.98	8.09	31.07	24.24
SM	24.74	21.81	3.16	5.47	0.16	0.22	0.09	1.29	9.74	9.98	28.06	27.50
CS	29.16	27.42	2.30	2.30	0.07	0.08	0.07	2.30	6.09	10.91	31.52	29.81
PT	23.63	22.08	2.74	2.92	0.11	0.11	0.11	2.92	7.49	4.10	26.48	25.10
DA	22.80	17.15	2.22	2.14	0.09	0.11	0.09	2.14	7.01	5.93	25.11	19.40
PA	22.31	18.42	3.34	2.87	0.11	0.14	0.03	8.79	10.75	9.21	25.76	21.43
DM	23.12	17.31	3.19	1.07	0.22	0.22	0.09	1.92	5.63	5.03	26.53	18.61
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
MEAN	24.71	21.41	2.77	2.90	0.13	0.15	0.08	3.26	8.24	7.53	27.62	24.46
SD	2.45	4.04	0.51	1.32	0.05	0.06	0.03	2.39	1.95	2.43	2.42	4.36
SEM	0.87	1.43	0.18	0.47	0.02	0.02	0.01	0.84	0.69	0.86	0.86	1.54

	Total N + IMP		Lactate		Glycogen		PCr		Cr		ATPEZ	
	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue
SF	26.50	32.09	10.99	82.74	446	309	91.72	22.31	32.51	101.92	26.31	27.12
BK	31.10	28.39	5.02	92.60	451	336	105.41	37.18	43.97	112.2	29.42	19.49
SM	28.15	28.79	10.82	72.53	579	550	93.94	46.39	34.54	82.09	27.21	21.72
CS	31.59	32.11	7.13	76.13	445	351	99.23	41.29	53	110.94	25.35	21.99
PT	26.58	28.02	7.93	71.25	344	251	90.76	47.61	34.59	77.74	25.87	20.52
DA	25.20	21.54	9.82	128.04	433	371	83.22	24.61	44.35	102.96	22.31	12.83
PA	25.79	30.23	6.53	142.44	645	359	90.14	27.5	45.43	108.07	22.29	12.5
DM	26.62	20.53	6.07	66.74	409	321	91.84	39.33	47.86	100.37	26.6	19.9
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
MEAN	27.69	27.71	8.04	91.56	469	356	93.28	35.78	42.03	99.54	25.67	19.51
SD	2.41	4.41	2.26	28.35	96	87	6.60	9.80	7.34	12.88	2.41	4.84
SEM	0.85	1.56	0.80	10.02	34	31	2.33	3.46	2.60	4.55	0.85	1.71

Table A 3b. Metabolites In Resistance Trained expressed in $mmol.kg^{-1}$ dry mass

	ATP (HPLC)		ADP		AMP		IMP		ATP/ADP		Total N	
	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue
CG	31.20	23.16	3.05	3.30	0.07	0.11	0.15	5.70	10.23	7.02	34.32	26.57
CH	23.08	23.56	2.57	2.91	0.06	0.20	0.15	0.75	8.98	8.10	25.71	26.67
DF	28.12	25.27	2.89	2.53	0.07	0.16	0.21	1.07	9.73	9.99	31.08	27.96
SZ	24.32	26.81	3.99	2.46	0.08	0.07	0.04	0.90	6.10	10.90	28.39	29.34
AK	27.43	20.48	3.66	5.00	0.18	0.11	0.01	0.21	7.49	4.10	31.27	25.59
DK	26.30	28.31	3.75	4.77	0.19	0.09	0.05	0.79	7.01	5.94	30.24	33.17
CN	30.10	24.21	2.80	2.63	0.09	0.11	0.03	0.86	10.75	9.21	32.99	26.95
KG	25.71	22.79	4.50	4.53	0.29	0.22	0.10	1.45	5.71	5.03	30.50	27.54
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
MEAN	27.03	24.32	3.40	3.52	0.13	0.13	0.09	1.47	8.25	7.53	30.56	27.97
SD	2.76	2.45	0.67	1.07	0.08	0.05	0.07	1.75	1.93	2.43	2.65	2.37
SEM	0.98	0.87	0.24	0.38	0.03	0.02	0.03	0.62	0.68	0.86	0.94	0.84

	Total N + IMP		Lactate		Glycogen		PCr		Cr		ATPEZ	
	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue
CG	34.47	32.27	10.6	103.1	555	483	108.5	36.3	49.7	121.9	28.51	20.90
CH	25.86	27.42	12.2	59.1	513	371	101.3	30.7	34.6	105.2	26.77	23.96
DF	31.29	29.03	11.3	67.9	420	351	104.4	54.7	42.0	91.7	27.16	23.30
SZ	28.43	30.24	9.6	115.6	404	370	101.2	53.4	63.5	111.3	24.64	13.20
AK	31.28	25.80	5.3	101.1	625	408	89.3	29.5	49.9	109.6	27.01	26.65
DK	30.29	33.96	8.0	64.4	337	315	118.7	48.2	43.3	113.8	30.07	29.19
CN	33.02	27.81	6.6	95.2	513	324	109.4	43.0	38.7	105.1	26.88	20.69
KG	30.60	28.99	13.2	72.2	585	387	105.6	38.4	36.2	103.5	26.69	19.09
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
MEAN	30.66	29.44	9.6	84.8	494	376	104.8	41.8	44.7	107.8	27.22	22.12
SD	2.65	2.66	2.8	21.3	99	53	8.4	9.7	9.4	8.8	1.56	4.89
SEM	0.94	0.94	1.0	7.5	35	19	3.0	3.4	3.3	3.1	0.55	1.73

Table A 3c. Metabolites in Endurance Trained expressed in mmol.kg^{-1} dry mass

	ATP		ADP		AMP		IMP		ATP/ADP		Total N	
	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue
AW	27.34	27.1	2.67	3.86	0.19	0.40	0.10	2.54	10.24	7.02	26.39	29.55
PL	23.62	24.55	2.63	4.23	0.37	0.28	0.04	4.15	8.98	8.09	31.07	24.24
JM	39.15	34.22	4.02	2.46	0.35	0.41	0.09	1.29	9.74	9.98	28.06	27.50
RA	33.06	16.26	2.67	3.03	0.63	0.08	0.07	2.30	6.09	10.91	31.52	29.81
GP	24.42	16.2	3.26	3.95	0.26	0.43	0.11	2.92	7.49	4.10	26.48	25.10
PB	26.01	23.54	3.71	3.97	0.08	0.55	0.09	2.14	7.01	5.93	25.11	19.40
n	6	6	6	6	6	6	6	6	6	6	6	6
MEAN	28.93	23.65	3.16	3.58	0.31	0.36	0.08	2.56	8.26	7.67	28.11	25.93
SD	6.02	6.85	0.60	0.69	0.19	0.16	0.03	0.95	1.64	2.54	2.65	3.92
SE	2.46	2.80	0.25	0.28	0.08	0.07	0.01	0.39	0.67	1.04	1.08	1.60

	Total N + IMP		LACTATE		Glycogen		PCr		Cr		ATP	
	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue	Rest	Fatigue
AW	26.50	32.69	2.9	37.5	572	366	76.6	61.2	48.2	63.5	22.11	23.14
PL	31.10	26.74	4.9	44.9	517	484	76.8	55.8	55.8	76.9	23.16	18.05
JM	28.15	26.89	7.3	61.9	579	381	89.2	49.1	31.1	71.2	30.78	20.93
RA	27.51	23.64	4.8	91.3	370	244	78.3	42.0	50.9	87.3	23.41	18.23
GP	26.58	36.47	9.0	85.3	642	431	87.0	63.9	62.4	85.5	23.67	23.40
PB	25.20	30.00	5.8	50.7	527	278	85.9	55.8	45.8	76.0	21.65	21.05
PH			8.8	72.6	457	316						
FS			9.7	84.4	416	290						
n	6	6	8	8	8	8	6	6	6	6	6	6
MEAN	27.51	29.40	6.7	66.1	510	349	82.3	54.6	49.0	76.7	24.13	20.80
SD	2.03	4.64	2.4	20.4	91	82	5.7	8.0	10.6	8.9	3.35	2.30
SE	0.83	1.90	0.9	7.2	32	29	2.3	3.3	4.3	3.6	1.37	0.94

Table A 4a Biodex testing peak torque *vs* velocity

Torque Unit: Nm/Kg						
Velocity:Degree/sec.	**UT**	**SEM**	**RT**	**SEM**	**ET**	**SEM**
0	11.97	1.35	11.39	2.37	15.50	1.69
60	2.91	0.08	3.09	0.14	2.73	0.13
120	0.77	0.03	0.82	0.03	0.75	0.06
180	0.69	0.03	0.74	0.03	0.68	0.06
240	0.60	0.02	0.62	0.01	0.61	0.01
300	0.67	0.02	0.67	0.02	0.67	0.01
360	0.69	0.02	0.70	0.02	0.74	0.03

Table A 4b Cybex testing peak torque *vs* velocity

Subjects	**UT**	**Subjects**	**RT**	**Subjects**	**ET**
SF	-38.57	CG	-51.14	PH	-12.04
BK	-53.40	CN	-53.131	AW	-15.17
SM	-38.81	CH	-41.66	PB	-40.17
CS	-29.33	DF	-54.06	PL	-20.79
DM	-53.03	KG	-23.19	GP	-37.65
PT	-36.37	DK	-59.07	RA	-43.14
PA	-42.65	SZ	-32.29	JM	-35.66
DA	-54.72	AK	-64.72	FS	-34.41
N	**8**		**8**		**8**
Mean	**-43.36**		**-47.41**		**-29.88**
SD	**9.36**		**14.01**		**12.03**
SEM	**3.31**		**4.95**		**4.25**

Table A5a SUBJECT CHARACTERISTICS 1 (LTx and Controls)

						VO2peak/pred w.		
Subj.No	**SEX**	**Age**	**H(CM)**	**Wt**	**VO2**	**Pred.**	**re-pred**	**%**
P2	M	53.0	176.0	86.5	16.0	31.0	31.6	50.7
P3	F	49.0	167.0	56.4	14.3	29.9	28.0	51.0
P4	F	43.0	160.5	64.4	16.1	32.1	24.9	64.5
P5	F	34.0	171.5	62.6	21.6	35.4	32.3	66.8
P6	F	26.0	158.5	73.6	15.9	38.4	26.6	59.9
P7	M	29.0	178.0	59.4	23.8	44.1	48.4	49.2
P8	M	25.0	175.0	61.7	23.1	46.8	48.1	48.0
n		**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**
mean		**37.0**	**169.5**	**66.4**	**18.7**	**36.8**	**34.3**	**55.7**
SD		**11.4**	**7.7**	**10.4**	**4.0**	**6.6**	**9.9**	**7.8**
SE		**4.3**	**2.9**	**3.9**	**1.5**	**2.5**	**3.7**	**3.0**

Age Matched						VO2peak/pred w.		
Subj.No	**SEX**	**Age**	**Hight(CM)**	**Wt**	**VO2**	**Pred.**	**re-pred**	**%**
1	M	53.0	165.0	65.7	32.1	28.8	44.9	111.4
2	F	46.0	167.0	64.0	33.4	26.7	37.6	125.1
3	F	43.0	171.1	65.5	31.8	28.7	43.2	110.7
4	F	35.0	166.5	62.9	36.4	30.2	46.3	120.7
5	F	28.0	159.5	56.2	33.1	31.7	36.9	104.4
6	M	26.0	182.2	84.6	44.3	43.2	52.5	102.7
7	M	27.0	190.5	87.2	47.4	45.4	51.8	104.4
n		**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**
mean		**36.9**	**171.7**	**69.4**	**36.9**	**33.5**	**44.7**	**111.3**
SD		**10.6**	**10.9**	**11.7**	**6.3**	**7.5**	**6.2**	**8.7**
SE		**4.0**	**4.1**	**4.4**	**2.4**	**2.8**	**2.3**	**3.3**

Table A5b SUBJECT CHARACTERISTICS 2 (LTx and Controls)

Subj.No	HRmax	HR Pred.	%	VEmax(L/min.)	VC	VC Pred.	%	FEV1	Pred.	%
P2	118.0	176.0	67.0	81.0	4.5	5.0	90.2	2.1	3.6	57.8
P3	121.0	178.0	68.0	39.0	2.2	3.6	61.5	1.4	2.8	49.5
P4	132.0	182.0	72.5	39.0	2.5	3.5	70.9	2.4	2.8	85.8
P5	159.0	188.0	84.6	80.0	4.2	4.2	101.4	3.5	3.4	103.9
P6	137.0	193.0	71.0	81.0	3.8	3.8	100.8	3.2	3.3	97.6
P7	139.0	191.0	72.8	64.0	3.6	5.6	63.9	3.4	4.5	74.9
P8	153.0	194.0	78.9	65.0	3.4	5.6	60.6	2.6	4.6	55.5
n	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**
mean	**137.0**	**186.0**	**73.5**	**64.1**	**3.5**	**4.5**	**78.5**	**2.6**	**3.6**	**75.0**
SD	**15.2**	**7.3**	**6.2**	**18.6**	**0.9**	**0.9**	**18.4**	**0.8**	**0.8**	**21.6**
SE	**5.7**	**2.8**	**2.3**	**7.0**	**0.3**	**0.4**	**7.0**	**0.3**	**0.3**	**8.1**

Age Matched

Subj.No	HRmax	HR Pred.	%	VEmax(L/min.)	VC	VC Pred.	%	FEV1	Pred.	%
1	166.0	175.6	94.6	105.0	4.2	4.1	102.5	3.8	3.3	113.2
2	189.0	180.1	104.9	89.6	4.7	3.6	129.7	3.6	3.0	119.9
3	159.0	182.1	87.3	94.7	4.2	3.9	108.8	3.3	3.2	103.0
4	164.0	187.3	87.6	129.1	5.5	3.9	143.2	4.4	3.2	134.8
5	185.0	191.8	96.5	86.4	4.6	3.7	125.9	3.8	3.2	118.6
6	183.0	193.1	94.8	148.1	5.2	5.7	91.0	4.0	4.7	84.9
7	195.0	192.5	101.3	177.1	5.0	6.2	80.2	4.4	5.1	86.0
n	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**
mean	**177.3**	**186.0**	**95.3**	**118.6**	**4.8**	**86.6**	**135.5**	**4.8**	**4.4**	**111.6**
SD	**14.0**	**6.9**	**6.5**	**34.2**	**0.5**	**18.2**	**14.1**	**0.5**	**1.1**	**22.5**
SE	**5.3**	**2.6**	**2.5**	**12.9**	**0.2**	**6.9**	**5.3**	**0.2**	**0.4**	**8.5**

Table A 6 SR Results (LTx and Age-controls)

rest

	(m) nmol.min^{-1}.mg muscle^{-1}			(P) nmol.min^{-1}.mg protein^{-1}		
controls	uptake(m)	uptake(p)	release(m)	release(p)	ATPase(m)	ATPase(p)
P1	1.34	10.12	1.38	10.44	10.62	80.32
P2	1.52	9.08	1.84	11.04	13.35	79.94
P3	1.30	7.88	0.60	3.64	10.11	61.27
P4	1.34	10.34	1.23	9.49	11.20	86.39
P5	1.01	9.25	1.17	10.71	9.67	88.55
P6	0.67	5.75	1.10	9.44	6.82	58.25
P7	1.05	10.04	0.84	7.98	8.64	82.40
N	7	7	7	7	7	7
MEAN	1.18	8.92	1.17	8.96	10.06	76.73
SD	0.28	1.63	0.40	2.56	2.04	12.03
SEM	0.11	0.62	0.15	0.97	0.77	4.55

	(m) nmol.min^{-1}.mg muscle^{-1}			(P) nmol.min^{-1}.mg protein^{-1}		
LTx	uptake(m)	uptake(p)	release(m)	release(p)	ATPase(m)	ATPase(p)
JH	1.47	10.17	1.54	10.65	13.24	91.56
WM	1.66	10.32	1.87	11.62	14.32	89.00
SM	1.66	11.02	2.20	14.57	15.24	100.93
RM	1.85	10.32	1.89	10.56	16.18	90.39
FW	1.55	11.48	1.47	10.89	9.82	72.74
MS	1.92	19.53	1.83	18.62	10.50	106.82
PT	1.81	11.31	1.62	10.12	14.14	88.32
N	7	7	7	7	7	7
MEAN	1.70	12.02	1.77	12.43	13.35	91.39
SD	0.16	3.35	0.25	3.11	2.37	10.75
SEM	0.06	1.27	0.09	1.17	0.90	4.06

Table A 7 Muscle Fiber Type (LTx and Age-controls)

LTx

Name	Total Fibers	Type I	Type II	Type IIa	Type IIb
	No.	%	%	%	%
P2	288	31.6	68.4	54.5	13.9
P3	245	34.7	65.3	23.7	41.6
P4	274	15.7	84.3	27.4	56.9
P6	328	16.2	83.8	29.6	54.3
P7	250	3.2	96.8	22.4	74.4
P8	120	23.3	76.7	44.2	32.5
P5	177	50.3	49.7	38.4	11.3
N	7	7	7	7	7
MEAN	240.3	25	75	34.3	40.7
SD	70.7	11.6	11.6	12.9	21.1
SEM	26.7	4.4	4.4	4.9	8

Age matched controls

Name	Total Fibers No.	Type I %	Type II %	Type IIa %	Type IIb %
JH	225	57.3	42.7	42.7	0
WM	178	60.7	39.3	39.3	0
SM	376	62.8	37.2	34.8	2.4
RM	304	50.3	49.7	47	2.6
FW	188	60.1	39.9	31.4	8.5
MS	258	47.7	52.3	46.1	6.2
PT	404	56.4	43.6	43.6	0
N	7	7	7	7	7
MEAN	276.1	56.5	43.5	40.7	3
SD	88.9	5.6	5.6	5.8	3.1
SEM	33.6	2.1	2.1	2.2	1.4

Table A8. Plasma Lactate (LTx and Controls)

LTx

Average	VO2(L)	lac-	workload	Log [VO2]	Log [La-]	Log[workload]	Log [La-]
R	0.48	1.10	0.00	-0.32	0.04	0.00	0.04
1	0.61	1.33	16.34	-0.22	0.13	1.21	0.13
2	0.70	1.71	32.68	-0.16	0.23	1.51	0.23
3	0.86	2.41	49.02	-0.06	0.38	1.69	0.38
4	1.11	3.14	67.60	0.05	0.50	1.83	0.50
5	1.35	3.45	84.40	0.13	0.54	1.93	0.54
	1.40	2.61	103.41	0.14	0.42	2.01	0.42

Controls

Average	VO2(L)	Lac-	workrate	Log VO2	Log[La-]	Log Work	Log La-
R	0.28	0.97	0.00	-0.55	-0.01	0.00	-0.01
E1	0.62	1.09	21.43	-0.21	0.04	1.33	0.04
E2	0.79	1.26	46.43	-0.10	0.10	1.67	0.10
E3	0.97	1.57	71.43	-0.01	0.19	1.85	0.19
E4	1.18	2.12	96.43	0.07	0.33	1.98	0.33
E5	1.38	2.97	121.43	0.14	0.47	2.08	0.47
E6	1.57	4.18	146.43	0.20	0.62	2.17	0.62
E7	1.75	6.00	171.43	0.24	0.78	2.23	0.78
E8	1.86	5.60	200.00	0.27	0.75	2.30	0.75
E9	1.92	5.60	225.00	0.28	0.75	2.35	0.75
E10	2.04	5.69	250.00	0.31	0.76	2.40	0.76
E11	2.19	7.39	275.00	0.34	0.87	2.44	0.87
E12	1.90	8.58	300.00	0.28	0.93	2.48	0.93
E13	1.51	0.83	0.00	0.18	-0.08	#NUM!	-0.08

Table A9. CsA effect on SR Ca^{2+} release, Ca^{2+} uptake and Ca^{2+} ATPase

CsA concentration ug/ml

SOL	Ca^{2+} release nmol.min^{-1}.mg muscle^{-1}				Ca^{2+}uptake nmol.min^{-1}.mg muscle^{-1}				Ca^{2+}ATPase nmol.min^{-1}.mg muscle^{-1}			
Rat NO.	0 [CsA]	1 [CsA]	25 [CsA]	50 [CsA]	0 [CsA]	1 [CsA]	25 [CsA]	50 [CsA]	0 [CsA]	1 [CsA]	25 [CsA]	50 [CsA]
1	1.54	1.39	1.25	0.66	1.31	1.10	1.26	0.99	13.75	11.25	11	8.39
2	1.10	0.80	0.59	0.46	1.15	0.74	0.66	0.61	12.23	10.9	9.6	7.98
3	1.42	1.07	0.91	0.74	1.39	1.04	0.65	0.74	12.4	10.62	9.5	5.95
4	1.68	1.56	1.47	1.25	1.51	1.07	0.93	1.03	14.58	11.15	9.32	5.47
5	2.20	1.47	1.43	1.43	1.44	1.41	1.07	0.94	15.22	11.58	8.25	5.1
6	2.75	1.58	1.64	1.25	1.75	1.04	1.29	1.18	13.3	9.96	6.43	6.32
7	2.05	1.61	1.39	1.37	1.54	1.54	1.05	0.87	12.11	8.47	7.82	6.43
8	1.83	1.25	0.93	0.90	1.47	1.34	1.32	1.26	13.18	8.89	6.75	6.3
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**1.82**	**1.34**	**1.20**	**1.01**	**1.44**	**1.16**	**1.03**	**0.95**	**13.35**	**10.35**	**8.58**	**6.49**
sd	**0.51**	**0.29**	**0.36**	**0.36**	**0.18**	**0.26**	**0.27**	**0.22**	**1.13**	**1.14**	**1.56**	**1.14**
SE	**0.18**	**0.10**	**0.13**	**0.13**	**0.06**	**0.09**	**0.09**	**0.08**	**0.40**	**0.40**	**0.55**	**0.40**

EDL	Ca^{2+} release nmol.min^{-1}.mg muscle^{-1}				Ca^{2+}uptake nmol.min^{-1}.mg muscle^{-1}				Ca^{2+}ATPase nmol.min^{-1}.mg muscle^{-1}			
Rat NO.	0 [CsA]	1 [CsA]	25 [CsA]	50 [CsA]	0 [CsA]	1 [CsA]	25 [CsA]	50 [CsA]	0 [CsA]	1 [CsA]	25 [CsA]	50 [CsA]
1	5.68	4.64	2.58	1.59	2.77	2.62	2.65	2.23	45.30	35.65	25.30	16.70
2	4.99	2.77	2.29	2.22	3.14	2.78	2.08	1.74	46.00	30.90	27.65	17.15
3	6.06	5.61	4.47	3.10	4.76	3.23	2.38	2.12	46.10	22.60	18.20	9.65
4	5.36	4.65	3.40	3.42	3.61	2.23	2.20	2.46	52.00	36.55	18.60	15.20
5	6.91	4.73	4.10	4.04	3.95	3.27	2.64	2.23	54.80	25.50	13.75	9.75
6	9.12	6.61	6.60	4.25	4.33	3.27	2.82	2.36	40.50	19.50	15.30	10.45
7	8.78	6.17	6.83	4.25	3.95	3.56	3.19	2.93	52.90	26.20	13.30	9.60
8	7.70	6.69	4.98	4.44	3.42	2.63	2.54	2.49	41.78	19.00	15.95	12.20
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
mean	**6.82**	**5.23**	**4.41**	**3.41**	**3.74**	**2.95**	**2.56**	**2.32**	**47.42**	**26.99**	**18.51**	**12.59**
sd	**1.57**	**1.31**	**1.69**	**1.05**	**0.64**	**0.45**	**0.35**	**0.34**	**5.26**	**6.80**	**5.30**	**3.27**
SEM	**0.56**	**0.46**	**0.60**	**0.37**	**0.23**	**0.14**	**0.10**	**0.12**	**1.86**	**2.40**	**1.87**	**1.16**

Table A10. The Variability In SR Measurements

Human muscle units:nmol.min^{-1}.mg muscle

Time	Name		Ca^{2+}uptake	Ca^{2+}release	Ca^{2+}ATPase
3/05/96	XN		0.047	0.046	0.353
		Rest			
		Rest	0.045	0.044	0.356
		Fatigue	0.029	0.017	0.268
		Fatigue	0.027	0.016	0.271
13/02/96	XN	**Rest**	0.048	0.045	0.361
		Rest	0.051	0.044	0.354
		Fatigue	0.027	0.018	0.267
		Fatigue	0.024	0.017	0.281

APPENDIX C

Methods for Crude Homogenate Sarcoplasmic Reticulum Vesicle Ca^{2+} Uptake and Ca^{2+}Release

Methods according to:
Ruell P.A., J. Booth, M.J. McKenna and J.R. Sutton. (1995) *Analytical Biochemistry.* 228, 194-201.
Based on previous methods:
O'Brien P.J. (1990) *J. Mol. Cell Biochem.* 94,113-119.
O'Brien, P.J.(1991) J.*Mol. Cell.Biochem.* 102: 1-12.
Simonides, W.S. and van Hardeveld C.(1990) *Anal.Biochem.*191,321-331.

Human Tissues:
Obtain human biopsy from vastus lateralis muscle. The SR assay requires usually 30-50 mg (wet weight). Rapidly blot muscle on filter paper to remove excess blood and weigh immediately. The muscle is then placed in a homogenate buffer and homogenised immediately.
Animal Tissue:
Rats obtained from Zoology Department at Melbourne University. Make a note of the age, sex, strain & weight. Rats killed by cervical dislocation and muscles exercised immediately, homogenised and either frozen in liquid N_2 or analysed fresh.

Homogenisation
Prepare the homogenate buffer as detailed below. This should be made fresh each 2 weeks and can be temporarily stored in the refridgerator.

Homogenising buffer

Reagants	Final [ ]	50ml	200ml
Sucrose (BDH)	300 mM	3.423 g	13.692 g
Sodium azide (NaN_3) (BDH)	5 mM	0.0325 g	0.130 g
EDTA (ANAL R,BDH)	1 mM	0.01681 g	0.0672 g
L-histidine (Calbiochem)	40 mM	0.31 g	1.24 g
Tris HCL (BDH)	40 mM	0.315 g	1.26 g

adjust to pH 7.9

Homogenisation
Keep sample on ice at all times during homogenisation

a) blot muscle and record wet weight of muscle = mg
amount of homog. buffer = wt muscle (mg)x 10 = µl (eg. 40 mg → 400 µl)
Approx 40mg tissue is homogenised in 400µl of buffer. For muscle < 40mg, use minimum volume of 400µl of homog. buffer.

b) place muscle + ice cold homogenate buffer in flat-bottomed glass tube. Homogenise at 0°C (on ice) using an Omni 2000 homogenizer with 5mm generator, at 18000 rpm, for 3 × 15 s bursts. Freeze the homogenate in liquid N_2 for later analysis.

SR Ca^{2+}uptake and release assay

Preparation:
Make up the following buffer to be used for CaU/CaR assay. Make up sufficient buffer for triplicate measures.

Reagants	Stock [ ]	Final [ ]	10 ml (4 runs)	20 ml (9 runs)
KCl Hepes (make together)	1.5 M 200 mM	150mM 20mM	1.1 ml	2.2 ml
oxalate	1 M	7.5mM	75 µl	150 µl
NaN3	2 M	10mM	55 µl	110 µl
TPEN	1 mM	5µM	55 µl	110 µl

adjust to pH 7.0

Equipment Set-up
Cuvette: use Plastic cuvettes of Kartell 1961 (these should not be washed):
Stir-bars: use small magnetic stirrer.
Waterbath: turn on water bath and allow at least one hour to heat to 37 C.

Fluorimeter Set up
Turn on the AB2, then the lamp, then the computer. The lamp should be on for ~30 min before any measurements are made. But, the lamp MUST be turned off when not in use. The manufacturer recommends replacement of lamp every 400 hours and lens every 800 hours.

Software Check
Go to: Setup/your name
Go to: Applications/Intracellular Probes/Probe Names/Indo-1
Check the following information is stored correctly:
Excitation wavelength = 349 nm
Emission wavelengths: Emission 1 = 410 nm and Emission 2 = 485 nm
Sensitivity=560 V
Speed=1 s
Duration = appropriate time – usually >300 seconds
Band Pass widths Excitation = 1 nm; Emission = 8 nm

Starting the Assay
1. Once the sample is ready, the assay itself can be run.
2. Add 2.2 ml assay buffer to the cuvette
3. At this time, add 200 µl of 50 mM ATP solution (See Analytical Reagent) to the cuvette
4. heat cuvettes for 5 min in the AB2.
5. prior to start assay add 3 µl of Indo-1 solution
6. Go to: Start application.

To Initiate Ca uptake:
1. To start CaU, add Homogenate to the 2.2 ml assay buffer.
   50 µl of human muscle
   15 µl of rat muscle (EDL) or 15-50 µl (SOL)

To Initiate Ca release:

1. To start CaR, add $AgNO_3$(BDH) 20 µl of 141 mM $AgNO_3$

For Calibration: (Figure 1-4)

1. Rmin. To chelate Ca and achieve a measurement of Rmin, add EGTA. Rmin is typically around 0.18. This should be very reproducible for your methods.
   EGTA (Boehringer Mannheim) 40 µl of 200mM EGTA

2. Rmax. To saturate all Indo-1 with Ca and achieve a measurement of Rmax, add $CaCl_2$. The Rmax is typically above 3.0 and should be around 3.1 or 3.2. This is more variable than Rmin and will be reduced by blood in the homogenate. However, this should be quite reproducible for your methods.
   $CaCl_2$ (UNIVAR) 120 µl of 100mM $CaCl_2$

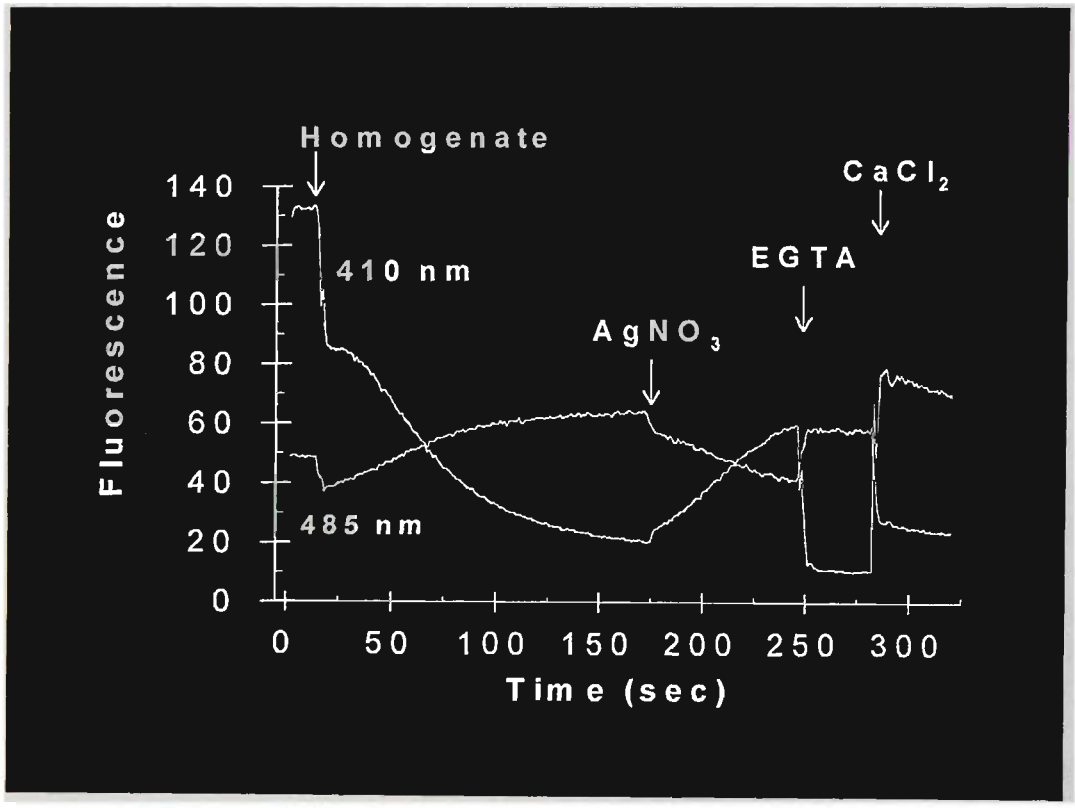

Figure. 1 One assay in the Fluorimeter (AB2)

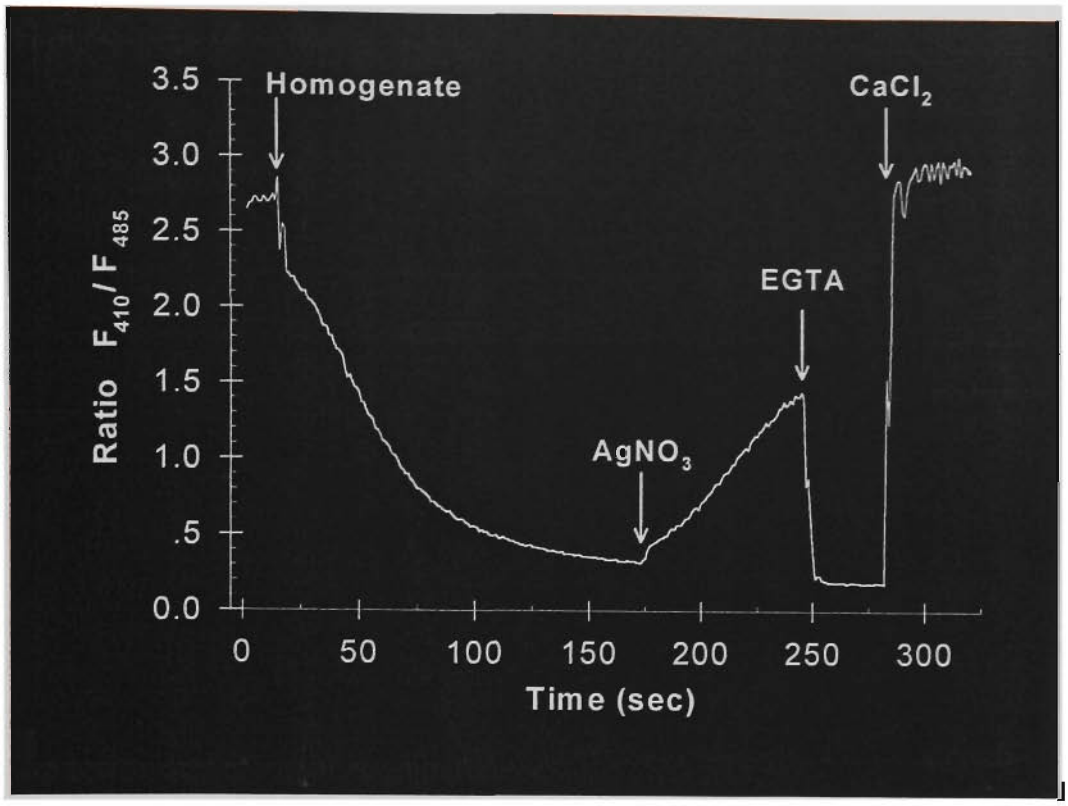

Figure. 2 Ratio F_{410}/F_{485}

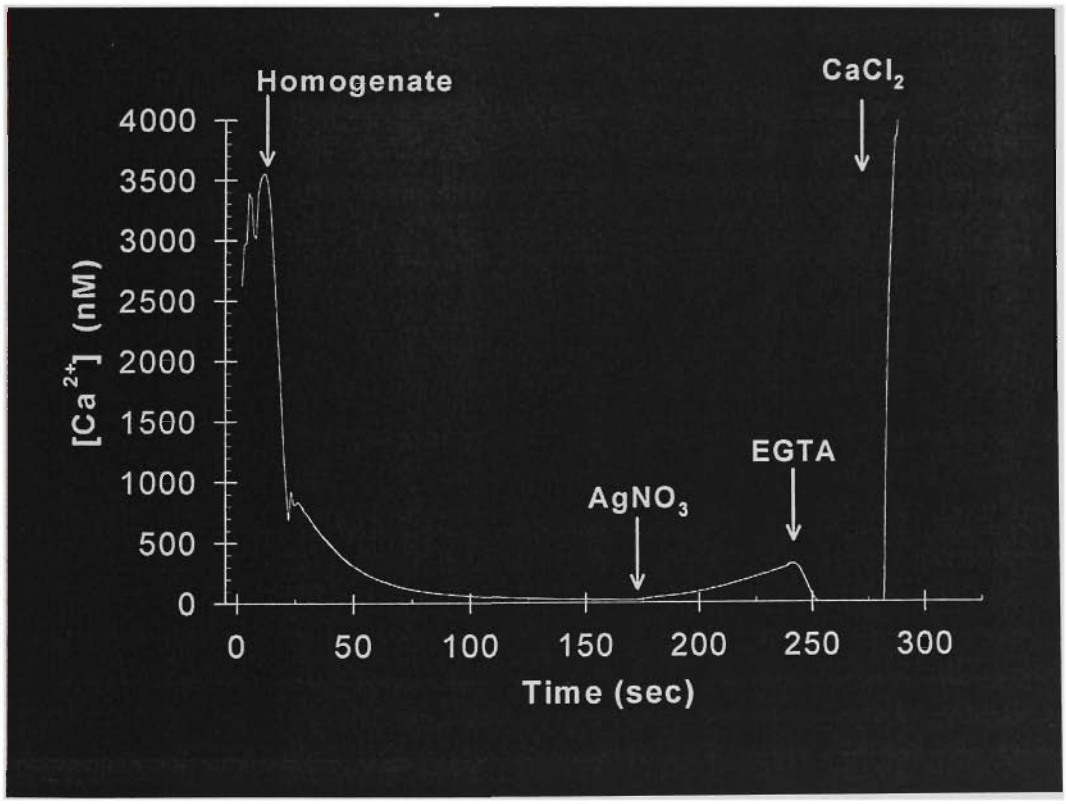

Figure. 3 Calcium concentration (nM).

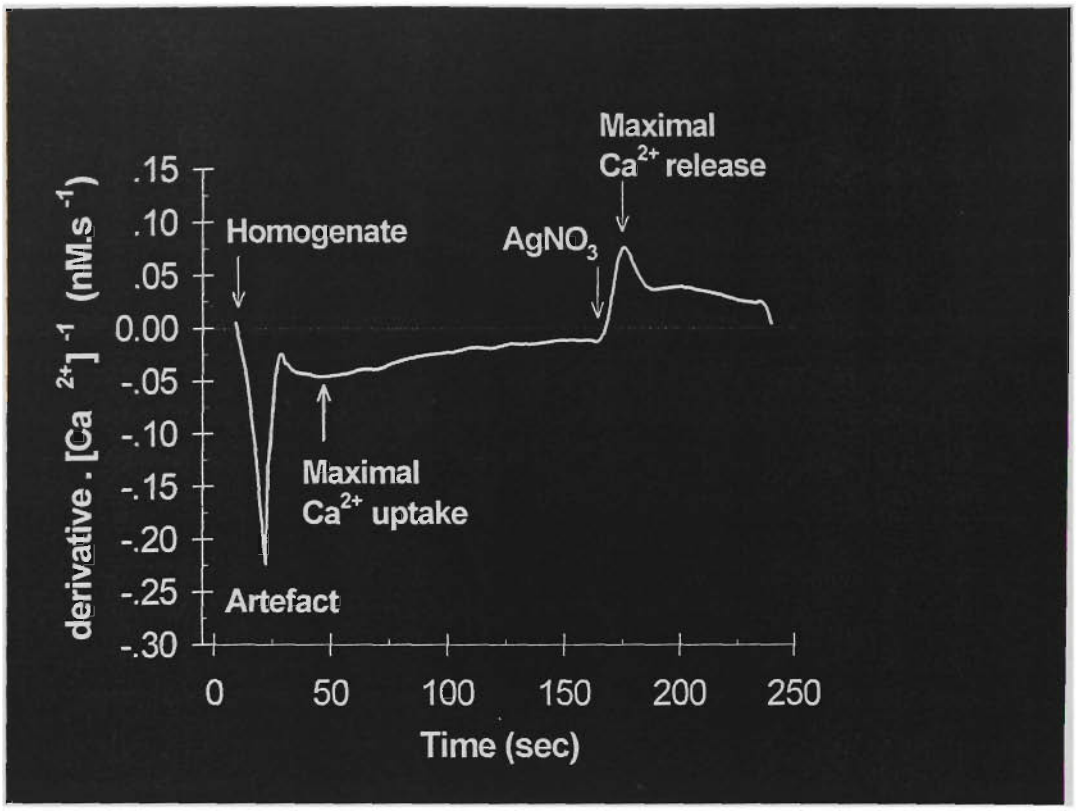

Figure. 4 Maximal SR Ca^{2+} uptake and Ca^{2+} release measurement.

ANALYTICAL REAGENTS
for Ca^{2+} UPTAKE and RELEASE assays

Working Buffer

Make up together:
1.5 M KCl (BDH #; Location: Benchtop)
200 mM Hepes (CALBIOCHEM Location: Benchtop)
pH 7.0
store at 4° C

1 M Oxalate (BDH Location: Benchtop)

This [oxalate] is difficult to dissolve. Run hot water (60-80 °C) on outside of Eppendorf tube to dissolve the oxalate. Aliquot into eppendorf tubes (500 µl each) and store at -20° C.

2 M NaN3 (BDH Location: Fume Cupboard)

aliquot into eppendorf tubes (500ul each)
store at -20° C

1 mM TPEN (CALBIOCHEM Location: Benchtop)

add 4.245 mg per 10ml must make up in DMSO to disolve
aliquot into eppendorf tubes (500 µl each)
store at -20° C

100 mM $CaCl_2$ (one of the $CaCl_2$ is no good!, use UNIVAR; Location: Benchtop)

add 1.4702g per 100 ml DDW
store at 4° C

*50 mM ATP Solution (pH=7.0)

Note: a solution is made with

- 50 mM Na_2ATP (BOEHRINGER MANNHEIM Location: Freezer)
- 50 mM $MgCl_2$ (SIGMA; use only non hydrated; Location: Benchtop)
- 100 mM Tris- HCL (BDH; Location: Benchtop)

To 20 ml DDW add:
0.7565 g Na_2ATP (MW =605.2)
0.119 $MgCl_2$ (MW= 95.21)
0.3940 Tris -HCl
adjust pH to 7.0 bring volume to 25 ml
aliquot into eppendorf tubes (500 l each)
store at -20°C

50 mM Glycine Buffer (pH=11.0) (For Indo-1)

Glycine (BDH Location: Benchtop)
add 0.3754 g glycine per 100 ml DDW
adjust pH to 11.0 (need pH=12.0 solution or cal. to pH 11 and be very careful!)
store at 4° C

1mM Indo-1

This can be obtained from several sources. Molecular Probes Inc. is preferred. It has a low $[Ca^{2+}]$ ~200 nM, although is more expensive, they also offer good technical advice. Calbiochem is cheaper, but has a high and somewhat variable Ca^{2+} contamination and $[Ca^{2+}] \sim 1$ µM)

Note: this chemical is photo- sensitive and must be kept in the dark while preparing and using it in the assay (ie. keep it in the original black plastic pouch orcover container with aluminum foil) (be quick-minimise light exposure)
1.chech Indo-1 vial for FW:
Molecular Probes=840.06
Calbiochem = 795.5
Sigma=840.1

2. for a 1mg vial, calculate the volume of buffer needed to make a 1mM solution
for calbiochem 1mM = 1mg per 1.257ml
add the appropriate volume of 50 mM Glycine Buffer (pH = 11)
ie for sigma product: if 1M = 840.1 g/l
1mM = 0.8401g/l
then 1mM =1mg per l/0.8401ml
= 1mg per 1.19ml

4.aliquot into mini-eppendorf tubes (50 µl each)
store in a dark container at 4° C,
Intruction of Molecular Probes say "don't freeze, although Jeronimo found no differences in Kd or function in Indo stored in firdge or freezer.

141 mM $AgNO_3$ (AJAX; Location: Benchtop)

MW= 169.87
add 0.0234 g per 10ml DDW
store at -20° C

Homogenising Buffer

Sucrose (BDH; Location: Benchtop)
Sodium azide (BDH; Location: Fume cupboard)
EDTA (BDH, AR; Location: Benchtop)
L-histidine (Calbiochem)
Tris HCL (BDH; Location: Benchtop)

The homogenising buffer used for the Ca^{2+} uptake assay consiseted of the following ingredients:
Sodium Azide (NaN_3):
Inhibits all mitochondrial ATPases and other mitochondrial proteins that may bind or sequester Ca^{2+} in the assay, which might contribute to the apparent calcium uptake rate. Stocks were stored at -20°C and used at a concentration of 10 mM (O'Brien et al.1991).
Oxalate:
Mimics calsequestrin, the natural calcium buffer of the SR, which precipitates calcium after uptake by the ATPase so as to increase the SR calcium capacity. In the vesicle preparation, however, the oxalate precipitates sequestered calcium to allow the uptake procedure to continue and thereby prevents back-inhibition, a phenomenon where increases in SR Ca^{2+} inhibits the Ca^{2+}-ATPase and thus calcium uptake. Stocks were stored at -20°C and used at a concentration of 7.5 mM (or 5 mM Martonosi 1984; O'Brien 1990).
TPEN:
Binds any contaminating heavy metals such as mercury or zinc which may interfere with the uptake process by inhibiting the ATPase protein and reducing the rate of uptake of calcium. This was stored at -20°C and used at a concentration of 5 µM (O'Brien et al. 1991).

KCL:

Increase the ionic strength of the buffer which will disrupt any myofibrillar ATPases which may bind Ca^{2+} and add to the apparent uptake rate observed. A stock solution was combined with Hepes buffer and sucrose solution, stored at 5°C and used at a working concentration of 40 mM.

Sucrose:

The major constituent which increased the osmolality of the buffer to enable a favourable environment for vesicle formation, by limiting passive osmotic fluxes of water into the vesicles which may cause disruption to the membrane. This was stored at 5°C, and used at a working concentration of 300 mM.

$AgNO_3$:

Addition of 141μM Ag^+ after the SR vesicles were loaded with Ca^{2+}, resulted in an increase in the $[Ca^{2+}]$. The increase in $[Ca^{2+}]$ after Ag^+ addition was blocked by 4mM DTT (Ruell et al. 1995).

Methods for Crude Homogenate Sarcoplasmic Reticulum Ca^{2+}ATPase activity

Methods according to:
Ruell P.A., J. Booth, M.J. McKenna and J.R. Sutton. (1995) *Analytical Biochemistry*. 228, 194-201.
Based on previous methods:
O'Brien P.J. (1990) *J. Mol. Cell Biochem.* 94,113-119.
O'Brien, P.J.(1991) J.*Mol. Cell.Biochem.* 102: 1-12.
Simonides, W.S. and van Hardeveld C.(1990) *Anal.Biochem.*191,321-331.

Measurement of Ca^{2+} and Basal ATPase
Ca^{2+}.ATPase and Mg^{2+} (basal) ATPase were measured in triplicate at 37°C spectrophotometrically using the ionophore A23187 (1uM) instead of Triton X-100. Activity was expressed as µmol of Ca^{2+}.g wet muscle^{-1}.min^{-1} or Ca^{2+}. mg^{-1}. min^{-1} protein.

1) Preparation of buffer for assay
a) Make up 1M $MgCl_2$ (MW 95.21): add 1ml DW to 0.0952g $MgCl_2$ (95.2mg).
b) See notes on following page for storage details.

Stock	Chemical Details	[Final] (mM)	50ml	20ml
40mM Hepes 400mM KCL make up Hepes and KCl together	Calbiochem, MW 238.3 BDH, MW 74.56	20 200	25 ml	10 ml
1M $MgCl_2$	Sigma Aldrich, MW 95.21	15	750 µl	300 µl
10mM NaN_3	MW 65.0	10	0.03125 g	0.0125 g
* 0.3 mM NADH	Boeheringer Mannh., MW 709.4	0.3	0.01065 g	0.00426 g
50mM EGTA	MW 380.4	1	1000 µl	400 µl

Bring to final volume
pH to 7.5
mix in beaker with a stirbar.
*Make up fresh NADH for each assay, don't make up a separate stock solution.

b) Make up 1 µM A23187 Ionophore solution
Location: in fridge door. Final [A23187] =1 µM
If make 50ml buffer : add 50 µl A23187

2) Prepation of cuvette
a) Add 1 ml of buffer to each cuvette
b) To 1ml buffer add the following:
50 µl of stock 200mM PEP (Boeheringer.Mannheim 128112, MW 206.1; 1ml to 0.093 g PEP powder, OR 500 µl to 0.0465 g), [final PEP] = 9 mM
25 µl of PK/LDH coupled enzyme solution (Sigma), [final]=3.6mM
16 µl of 250 mM stock solution of Na_2ATP (Boeheringer Mannheim, MW 605.2)

3) Addition of homogenate
a) To cuvette add 15 µl homogenate and mix by inversion

b) Assay at 37°C. Temperature control is critical for enzyme activity stability. Check function and temperature of water bath, cuvette before proceeding with assay. Keep cuvette in water bath at 37°C for 5 minutes then place in a spectrophotometer at 340nm and read absorbance for 30 sec. (rat 15 sec). Read at 5 sec intervals

4) Measure Total ATPase activity

Add 10µl of stock solution of 100mM $CaCl_2$ (final concentration = 0.6 mM $CaCl_2$). Mix by inversion. Follow absorbance for 1-2 min

5) Measure Background ATPase activity by Inhibiting SR Ca^{2+}ATPase activity

After measuring absorbance for 1-2 min for Total ATPase activity, then add 20µl of stock solution of 2M $CaCl_2$ to increase total [$CaCl_2$] to 40mM. Mix by inversion and read for a further 1-2 minutes.

6) Check assay working with rat muscle before proceeding.

ANALYTICAL REAGENTS
for Ca ATPase activity assay

Hepes/KCl Buffer

Make up together:
400 mM KCl (BDH #; Location: Benchtop)
40 mM Hepes (CALBIOCHEM Location: Benchtop)
To 200 ml DDW add 2.383 g Hepes and 7.456 g KCl
Adjust pH to 7.5.
Bring final volume to 250 ml
Make up fresh every 2 weeks. Store at 4° C.

1 M $MgCl_2$ (Sigma Aldrich, Location: Dessicator on Benchtop)
1M $MgCl_2$ (MW 95.21.): add 0.0952g $MgCl_2$ (95.2mg) to 1ml DW
Use only non-hydrated $MgCl_2$ (i.e. $MgCl_2$-$(H_2O)_6$ is no good).
Make up $MgCl_2$ fresh each day.

2 M NaN3 (BDH Location: Fume Cupboard)
10mM NaN_3 (MW 65.0)
normally make up fresh, but probably also quite OK to make up stock and freeze: aliquot into eppendorf tubes (500ul each), store at -20° C

0.3 mM NADH (Boehringer Mannheim. Location: Fridge)
Make up fresh NADH for each assay, don't use a stock solution.

50 mM EGTA (Boehringer Mannheim)
Make up fresh daily

100 mM $CaCl_2$ (one of the $CaCl_2$ is no good!, use UNIVAR; MW 147.02; Location: Benchtop)
add 1.4702g per 100 ml DDW
store at 4° C

2 M $CaCl_2$ (one of the $CaCl_2$ is no good!, use UNIVAR; Location: Benchtop)
add xxxx g per 100 ml DDW
store at 4° C

250 mM Na_2ATP Solution (pH=7.0)
250 mM Na_2ATP (BOEHRINGER MANNHEIM #519-987, MW 605.2; Location: Freezer)
To 20 ml DDW add:
CHECK = 5 x 0.7565 g Na_2ATP (MW =605.2)
adjust pH to 7.0 bring volume to 25 ml
aliquot into eppendorf tubes (500 l each)
store at -20°C