The Effects of Prolonged Exercise on Na^+, K^+- ATPase and SR Ca^{2+} Regulation in Humans.

Submitted by

James Alexander Leppik

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Supervisor: Associate Professor Michael McKenna

Department of Human Movement, Performance and Recreation

Victoria University

MELBOURNE, AUSTRALIA.

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Declaration

This thesis summarises original work conducted in the School of Human Movement, Recreation and Performance at Victoria University of Technology. This dissertation is the result of work performed by the author. However, considerable collaboration was also involved in the studies involving exercising humans. The muscle biopsies were conducted by Dr. Andrew Garnham. Associate Professor Michael McKenna, Associate Professor Michael Carey, Robert Aughey and Ivan Medved helped in conducting the exercise tests, drawing blood samples and processing both blood and muscle.

[redacted]

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Publication in support of this thesis

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Abbreviations

Subscript		Units
i	Intracellular	
e	extracellular	
E_m	Muscle membrane potential	mV
K^+	Potassium ion	$mmol.l^{-1}$
Na^+	Sodium ion	$mmol.l^{-1}$
Mg^{2+}	Magnesium ion	$mmol.l^{-1}$
Ca^{2+}	Calcium ion	$mmol.l^{-1}$
ΔPV	Changes in plasma volume	%.
$\dot{V}O_2$	Oxygen consumption	$l.min^{-1}$
RER	Respiratory exchange ratio	
Na^+, K^+-ATPase	Sodium-Potassium Adenosine Triphosphatase	
Na^+, K^+-pump	Sodium-Potassium Adenosine Triphosphatase	
3-*O*-MFP	3-*O*- methylfluorescein phosphate	
3-*O*-MF	3-*O*- methylfluorescein	
ATP	Adenosine 5' Triphosphate	
ADP	Adenosine Diphosphate	
PCr	Phosphocreatine	
P	Phosphate	
Cr	creatine	

ABSTRACT

Prolonged exhaustive submaximal exercise in humans induces marked metabolic changes, but little is known about effects on muscle Na^+, K^+ATPase activity and sarcoplasmic reticulum (SR) Ca^{2+} regulation. We therefore investigated whether these processes were impaired during cycling exercise at 74.3±1.2% $\dot{V}O_2$ peak (mean±SEM) continued until fatigue, in eight healthy subjects ($\dot{V}O_2$ peak 3.93±0.69 $l.min^{-1}$). A vastus lateralis muscle biopsy was taken at rest, at 10 and 45 min of exercise and at fatigue. Muscle was analysed for in-vitro Na^+,K^+ATPase activity (maximal K^+-stimulated 3-*O*-methylfluorescein phosphatase, 3-*O*-MFPase activity), Na^+K^+ATPase content (^{3}[H] ouabain binding sites), SR Ca^{2+} release rate induced by 4 chloro-*m*-cresol and Ca^{2+} uptake rate. Cycling time to fatigue was 72.18±6.46 min. Muscle 3-*O*-MFPase activity [$nmol.min^{-1}.(g\ protein)^{-1}$] fell from rest by 6.6±2.1% at 10 min ($P<0.05$), 10.7±2.3% at 45 min ($P<0.01$), and by 12.6±1.6% at fatigue ($P<0.01$), whereas ^{3}[H]ouabain binding site content was unchanged. Ca^{2+} release [$mmol.min^{-1}.(g\ protein)^{-1}$] declined from rest by 10.0±3.8% at 45 min ($P<0.05$) and by 17.9±4.1% at fatigue ($P<0.01$), whilst Ca^{2+} uptake rate fell from rest by 23.8±12.2% at fatigue ($P=0.05$). However, the decline in muscle 3-*O*-MFPase activity, Ca^{2+} uptake and Ca^{2+} release were variable and not significantly correlated with time to fatigue. Thus, prolonged exhaustive exercise impaired each of the maximal in-vitro Na^+,K^+ATPase activity, Ca^{2+} release and Ca^{2+} uptake rates. This suggests that acutely downregulated muscle Na^+, K^+ and Ca^{2+} transport processes may be important factors in fatigue during prolonged exercise in humans.

Chapter 1. Introduction

The regulation of Na^+, K^+ and Ca^{2+} is important for the production and maintenance of muscle force. The Na^+, K^+-ATPase enzyme regulates the concentration of Na^+ and K^+ within the muscle protecting against a rundown of K^+ in the muscle, which could lead to sarcolemma membrane inexcitability and depolarisation. Under those circumstances, Ca^{2+} cannot be released from the sarcoplasmic reticulum (SR) and therefore no muscle force can be produced.

Recently, disturbances of Na^+, K^+ and Ca^{2+} regulation during exercise have received attention. Although the majority of these studies involved the use of rat muscle, several studies have also been performed in human muscle. Most human studies have investigated disturbances during intense exercise. Most rat studies and all human studies have found changes in SR Ca^{2+} and Na^+, K^+ regulation during exercise. Two studies have investigated SR Ca^{2+}ATPase regulation and Na^+, K^+-ATPase activity during prolonged exercise in human muscle (Booth *et al.*, 1997; Sandiford *et al.*, 2004) although only the former was measured during exercise continued to fatigue (Booth *et al.*, 1997). No study though has investigated changes in SR Ca^{2+} release and Na^+, K^+-ATPase activity during prolonged exercise to fatigue. These, as well as SR Ca^{2+} uptake, were investigated in this thesis.

Chapter 2. Review of Literature

2.1 Overview of muscle fatigue

2.1.1 Definition of fatigue

Muscle fatigue has been described as "a failure to maintain the required force" (Edwards, 1981) and later described as the "failure to maintain the required or expected power output (Edwards, 1983). Power is the product of force development and the velocity of shortening. The definition of power recognises that the ability to sustain a given work capacity without decrement requires the maintenance of both force and velocity (Fitts, 1994). Muscle fatigue is not an "all-or-none" phenomenon, but develops progressively from the onset of exercise, as shown by a gradual reduction in maximal muscle force (Vollestad & Sejersted, 1988; Sahlin & Seger, 1995).

2.1.2 Overview of mechanisms of fatigue

The mechanisms of muscle fatigue are complex and not yet fully understood (Fitts, 1994). Fatigue is not caused by any single mechanism but rather may be caused by a number of mechanisms. These include central fatigue (fatigue of the central nervous system), failure of muscle membrane excitation; metabolic changes including a decline in muscle glycogen, ATP, PCr and pH or an increase in ADP, AMP, IMP; or muscle ionic imbalances with a decrease in intracellular/extracellular $[K^+]$ ratio, impairment of muscle Ca^{2+} regulation, or an accumulation of free radicals (Allen *et al.*, 1995); (Sjogaard *et al.*, 1985; Westerblad & Lannergren, 1991; Fitts, 1994; Stephenson *et al.*, 1998). The intensity of exercise, muscle fibre composition, and fitness levels can each influence which type of fatiguing mechanism occurs.

2.1.3Overview of metabolic disturbances with exercise

ATP contributes to muscle contraction by enabling binding of actin with myosin and thus crossbridge detachment and cycling. Muscle ATP declined during fatiguing prolonged exercise by 20% from 25.6 mmol.kg^{-1} dry wt at rest (Booth *et al.*, 1997), by 45% during intermittent exercise from 25.6 mmol.kg^{-1} dry wt at rest (Hargreaves *et al.*, 1998) and 24%, 20% and 14% from 25.7, 26.3 and 24.1 *u*mol.g^{-1} dry wt at rest in untrained, resistance trained and endurance trained subjects respectively during resistance exercise (Li *et al.*, 2002). These declines in bulk muscle ATP are unlikely to fall to the extent of impairing muscle force (Dawson *et al.*, 1978; Sahlin *et al.*, 1981). A decrease in bulk muscle ATP/ADP ratio though does have a negative effect on performance, resulting in slower shortening velocity. As changes in [ATP] affect SR Ca^{2+} release and Ca^{2+} uptake (Laver *et al.*, 2001), it is probably one important reason why SR function is impaired *in vivo* during exercise (see section 2.10.1).

Changes to ATP content may in part be due to reduced muscle glycogen content (Chin & Allen, 1997; Helander *et al.*, 2002). At submaximal intensities, such as those seen during prolonged exercise, fatigue is often associated with a large decline in bulk muscle glycogen (Bergstrom, 1962; Hermansen *et al.*, 1967) but not always (McKenna, 2003). A 90% reduction from 447 mmol glycosyl units^{-1}.kg^{-1} dry wt at rest was found in muscle glycogen after prolonged cycling at 70% $VO_{2\ peak}$ to fatigue (Booth *et al.*, 1997). It is possible a decline of that magnitude will impair SR function during exercise (see section 2.10.2).

During intense exercise ATP is rapidly hydrolysed, releasing ADP and H^+, which combine with creatine phosphate (PCr) to resynthesise ATP and release creatine in the creatine kinase reaction: PCr + ADP+ $H^+ \rightleftarrows$ ATP + Cr. Hence, PCr

declines very quickly during intense exercise. PCr declined by 70% from 87.7 mmol.kg^{-1} dry wt at rest after 30 s all out sprinting exercise (Cheetham *et al.*, 1986). During prolonged cycling to fatigue PCr content declined by 53% and 52% from it's initial content of 88.3 and 78.4 mmol.kg^{-1} dry wt respectively (Booth *et al.*, 1997; Sahlin *et al.*, 1997). Therefore reductions in PCr during both intense and prolonged exercise are substantial and these might inhibit muscular performance, as (1) increased [Pi] reduces sensitivity of the actin-myosin cross-bridges reducing muscle force; (2) increased [P_i] diminishes SR Ca^{2+} release through Ca^{2+}-P_i precipitation; and (3) the decline in PCr negatively affects the capacity to resynthesise local ATP at the SR Ca^{2+} release channels and SR Ca^{2+}-ATPase enzyme (see section 2.10.3).

2.1.4 Overview of Ionic disturbances in muscle fatigue

Impairments to ion regulation in skeletal muscle have been identified as important contributors to the fatigue process. Important sites of ion regulation in skeletal muscle include (a) Na^+, K^+ exchange across the muscle membrane via the Na^+, K^+-ATPase enzyme, which maintains cell excitability (see section 2.3), (b) Ca^{2+} release from the sarcoplasmic reticulum (SR) storage site which elevates cytosolic [Ca^{2+}] and thereby regulates muscle force development (section 2.8), and (c) Ca^{2+} re-uptake into the SR via the Ca^{2+}-ATPase enzyme, which lowers cytosolic Ca^{2+} concentration and thereby allows cross bridge dissociation and muscle relaxation (section 2.9).The focus of this literature review will be directed towards the contribution of disturbances in ion regulation to muscle fatigue.

2.2 Muscle Na^+ and K^+ regulation and membrane excitability

Action potentials are generated by an influx of Na^+, which is then followed by an efflux of K^+. Thus, during contractile activity, the muscle cell loses K^+ and

gains Na^+ with each action potential (Everts & Clausen, 1994). In skeletal muscle, the action potential propagates rapidly along the sarcolemma and down the transverse tubules, where it is detected by the voltage sensors, known as the dihydropyridine receptors (DHPR). These receptors couple mechanically with Ca^{2+} release channels, the ryanodine receptors (RyR), located within the SR terminal cisternae, which open releasing Ca^{2+} into the cell cytoplasm enabling muscle force. Therefore any factor that affects muscle sarcolemmal membrane potential (E_m) and excitability can have a direct effect on muscle force.

2.2.1 Effects of contraction on muscle [Na^+]

At rest, intracellular Na^+ concentration ($[Na^+]_i$) in human muscle is approximately 6-13 mM (Sjogaard, 1983; Sjogaard *et al.*, 1985). During heavy exercise in humans, muscle $[Na^+]_i$ doubled from rest, to ~21 mM at fatigue (Sjogaard, 1983; Sjogaard *et al.*, 1985).

2.2.2 Effects of contraction on muscle intracellular [K^+]

At rest intracellular K^+ concentration ($[K^+]_i$) in human muscle is approximately 161- 165 mM (Sjogaard, 1983; Sjogaard *et al.*, 1985). During heavy exercise, in humans, muscle $[K^+]_i$ fell from rest to 129 - 141 mM (Sjogaard, 1983; Sjogaard *et al.*, 1985). Numerous other studies have also confirmed an approximate 6-20% decline in muscle $[K^+]_i$ (Sreter, 1963; Costill, 1975; Sahlin *et al.*, 1978; Sjogaard & Saltin, 1982; Juel, 1986; Sjogaard, 1986; Lindinger & Heigenhauser, 1988).

2.2.3 Exercise effects on muscle interstitial [K^+]

During exercise K^+ ions are continually lost to the muscle interstitium (Nielsen *et al.*, 2004). Recent studies using microdialysis probes in muscle have demonstrated that the muscle interstitial $[K^+]$ may rise by 2-3 fold, from ~4.5 mM

from rest, to 6-9 mM during knee extensor exercise at 10, 30 and 50W (Juel *et al.*, 2000b) and to as much as ~10-15 mM during incremental exercise (Neilsen et al., 2004).

2.2.4 Effects of contraction on muscle intracellular/extracellular [K^+] ratio and Em

The combined effect of a large rise in muscle interstitial [K^+] and the decline in muscle $[K^+]_i$ is a large reduction in the intracellular/extracellular [K^+] ratio, which might be decreased by 50% or even more (Sjogaard, 1986). The action potential is dependent on membrane potential (E_m), which is controlled by changes in the $[K^+]_i/[K^+]_e$ (Costantin & Podolsky, 1967; Costantin, 1970). Thus the decline in $[K^+]_i/[K^+]_e$ ratio with exercise will reduce E_m from resting levels of approximately –88 mV, by as much as 10-20 mV. The 2-3-fold increase in $[K^+]_e$ will have a greater effect on E_m than the 6-20% decline in $[K^+]_i$ (Sjogaard *et al.*, 1985; Stephenson *et al.*, 1998). A reduction in E_m causes inactivation of the voltage-gated Na^+ channels causing membrane inexcitability (Costantin, 1970). A decline in the $[K^+]_i/[K^+]_e$ ratio depressed maximal force as well as endurance in the electrically stimulated isolated rat soleus and EDL muscles (Nielsen & Clausen, 1996; Verburg *et al.*, 1999). However, this decline in muscle $[K^+]_i$ and E_m during contractions is minimised *in-vivo* by an increased activity of the Na^+,K^+-ATPase enzyme. Thus Na^+,K^+-ATPase enzyme function constrains the run-down in trans-sarcolemmal [K^+] and [Na^+] gradients, thereby maintaining E_m, membrane excitability and force production.

2.2.5 Muscle K^+ loss and t-tubule inexcitability

Smaller relative Na^+,K^+-ATPase content in the t-tubules as compared to the sarcolemma membrane (Kirsch *et al.*, 1977; Clausen, 2003) suggests that increased

K^+ loss may occur within the t-tubules during heavy contractions, although this has not yet been determined. Increased K^+ loss across t-tubular membranes may depress t-tubular membrane excitability, especially in the centre of the fibre, thus accentuating loss of muscle force due to fatigue (Bezanilla *et al.*, 1972).

The inhibitory effects of an increased K^+ loss to the t-tubular network also depends on the rate of removal of K^+ from the t-tubule lumen. K^+ ions may leave the t-tubular fluid by diffusion, or be returned to the cells via the Na^+,K^+-ATPases located within the t-tubule walls. The t-tubule network is so extensive that diffusion seems to be an inefficient clearance mechanism (Clausen, 1996b). A smaller number of Na^+,K^+-ATPases present in the t-tubular membranes would delay the re-establishment of resting Na^+,K^+ levels in the t-tubular lumen (Nielsen & Overgaard, 1996). Thus loss of membrane excitability during exercise may be due to muscle K^+ loss, t-tubule network inexcitability, which might occur during both intense and prolonged exercise.

2.2.6 Exercise effects on plasma [K^+]

A consequence of muscle K^+ loss during exercise is a rise in plasma [K^+] (McKenna, 1992). When subjects cycled at an intensity at 110% maximal O_2 uptake, their venous plasma [K^+] reached 8.2 $mmol.l^{-1}$ from 4.4 $mmol.l^{-1}$ at rest (Vollestad *et al.*, 1994). The rise in plasma [K^+] also appears to depend on the activity of the Na^+,K^+-ATPase in skeletal muscle, since increased Na^+,K^+ATPase activity will return K^+ to the intracellular space, in active and inactive muscles.

During the first six min of cycling at 70% VO_2 peak, an increase in venous plasma [K^+] was found together with an increase in the arterio- venous plasma [K^+] concentration difference ($[K^+]_{A\text{-}V\ difference}$) (Vollestad *et al.*, 1994). This was suggested to be due to a lag in the activation of the Na^+,K^+-ATPase (Hallen *et al.*,

1994; Gullestad *et al.*, 1995). Beyond that time, there was no further increase in plasma $[K^+]$ or the $[K^+]_{A\text{-}V\ difference}$ until exercise ended at 20 min, indicating that Na^+,K^+-ATPase may have been functioning at the necessary capacity to prevent further increases (Vollestad *et al.*, 1994). Verburg *et al.* (1999) discovered that plasma $[K^+]$ reached a plateau after 20 min of two-legged knee extensor exercise at 30% MVC. This reflected both K^+ release with an associated accumulation of K^+ in the interstitium and blood extracellular space; as well as K^+ clearance from plasma, with K^+ uptake by active and non-exercising muscle (Hallen *et al.*, 1994; Gullestad *et al.*, 1995; Hallen, 1996; Verburg *et al.*, 1999). Importantly, Verburg et al. found that K^+ was continually lost from the muscle during prolonged muscle contractions (Verburg *et al.*, 1999). Several studies have estimated that the Na^+,K^+-ATPase functions below the capacity required to prevent a continuous loss of K^+ from the muscle, during moderate exercise intensity (Hallen *et al.*, 1994), incremental stepwise cycling (Hallen *et al.*, 1994), and double knee extension exercise at low power (Gullestad *et al.*, 1995).

Potassium is lost from the muscle during prolonged exercise and this might affect muscle performance. Increased arterialised-venous $[K^+]$ was found after 45 min prolonged cycling at 70 $VO_{2\ peak}$ and increased to 90% $VO_{2\ peak}$ to the cessation of exercise (Medved *et al.*, 2004). Venous plasma $[K^+]$ continued to increase during prolonged cycling at 67% VO_2 peak beyond 20 minutes of exercise (Sahlin & Broberg, 1989). Interestingly, a large decline in cycling exercise time to exhaustion at 70% $VO_{2\ peak}$ was found with subjects administered the ß-adrenoceptor antagonist propranolol (van Baak *et al.*, 1995). This might in part be due to an inhibitory effect of propranolol on muscle Na^+,K^+-ATPase (Clausen, 1986).

2.3 Muscle Na^+, K^+-ATPase Enzyme

The Na^+,K^+-ATPase enzyme, located in the sarcolemma and the transverse tubular membranes (Clausen, 2003), induces vectorial exchange of 3 Na^+ for 2 K^+ ions against their concentration gradients, at the expense of one ATP molecule, with a resultant electrogenic membrane depolarisation. Thus the Na^+,K^+ enzyme has a major effect on muscle membrane excitability.

2.3.1 Muscle Na^+, K^+-ATPase subunits and isoforms

The Na^+,K^+-ATPase is composed of two subunits, a catalytic α subunit involved in the splitting of ATP (molecular mass approx. 112 kDa) and a β subunit (approx. 35 kDa) (Blanco & Mercer, 1998). Like many other proteins, the Na^+,K^+-ATPase subunits are expressed in various isoforms, which can be detected using specific antibodies. Four isoforms of the Na, K^+-ATPase α and three isoforms of the β subunits have been identified. These are the α_1, α_2, α_3, α_4, β_1, β_2 and β_3 (Tsakiridis *et al.*, 1996; Juel *et al.*, 2000a; Murphy *et al.*, 2004a; Murphy *et al.*, 2004b). All seven isoforms are expressed in skeletal muscle (Hundal *et al.*, 1994; Tsakiridis *et al.*, 1996; Juel *et al.*, 2000a; Murphy *et al.*, 2004a; Murphy *et al.*, 2004b). Each of α_1, α_2, α_3, β_1, β_2 and β_3 isoforms have recently been identified in human muscle (Murphy *et al.*, 2004a; Murphy *et al.*, 2004b).

An αß complex is required for the Na^+,K^+ATPase to be functional (Sweadner, 1993). The functional significance of variations in the proportions of α_1 and α_2 isoforms among tissues is not yet clarified. A comparison of the affinities for Na^+ and K^+ showed no significant difference between α_1 and α_2, indicating that the transport functions of the Na^+,K^+-ATPase are independent of the relative abundance of the α_1 and α_2 isoforms (Munzer *et al.*, 1994). In contrast, Munzer et al., 1994)

found that the α_3 isoform seems to show a lower affinity for intracellular Na^+, and in cells containing mainly this version of the Na^+,K^+-ATPase the intracellular concentration of Na^+ seems to be higher. The β subunit appears to influence the conformation and ion sensitivity of the Na^+,K^+ATPase (Scheiner-Bobis, 2002). The abundance of β subunits appears to regulate overall Na^+-K^+-ATPase activity in subcellular membranes of rat skeletal muscle, such that membrane fractions with higher αβ ratio have higher enzyme activity (Lavoie *et al.*, 1996).

2.4 Factors affecting Na^+, K^+-ATPase activity

The muscle Na^+,K^+-ATPase activity is only at low levels under rest conditions *in-vivo* but increases rapidly and dramatically with muscle contraction (Everts & Clausen, 1994). The Na^+,K^+ATPase is activated by numerous mechanisms and plays a dynamic role in ongoing restoration and maintenance of excitability in contracting skeletal muscle. The mechanisms that activate the Na^+,K^+-ATPase include excitation of the muscle membrane, increase in $[Na^+]_i$, and increases in numerous hormones including catecholamines, insulin, adrenaline, and CGRP.

2.4.1 Excitation

There is overwhelming evidence that in working muscle, the excitation-induced K^+ efflux may exceed the functional capacity of the Na^+,K^+-ATPase, demonstrated by increases in both muscle $[Na^+]_i$ and $[K^+]_e$ (Clausen & Everts, 1989; Nielsen & Clausen, 1997; McKenna, 1998; Juel *et al.*, 2000b; Nielsen *et al.*, 2004). Any reduction in the functional capacity of the muscle Na^+,K^+-ATPase might then result in further decline in $[K^+]_i$, increase in $[K^+]_e$ decrease in E_m, reducing muscle membrane excitability, and thus inhibiting EC coupling and force production.

Exercise or muscle contraction induced by stimulation of the motor nerve leads to an efflux of K^+ and an influx of Na^+ into the muscle. The activation of the Na^+,K^+-ATPase, elicited by excitation, most likely reflects a rapid, but slowly reversible increase in the affinity of the Na^+,K^+-ATPase for $[Na^+]_i$ (Clausen, 1998). On opening of the Na^+ channels during electrical stimulation, the Na^+,K^+-ATPase undergoes activation within seconds allowing the cell to maintain lower Na^+ (Buchanan *et al.*, 2002). Excitation has been found to produce up to an 18- 22-fold increase in the active rate of Na^+ extrusion in skeletal muscle (Everts & Clausen, 1994; Nielsen & Clausen, 1997; McKenna *et al.*, 2003b).

Artificially increasing $[Na^+]_i$ without excitation of the muscle stimulated the Na^+,K^+-ATPase (Clausen, 1998). Although Na^+,K^+-ATPase activation can be elicited by an influx of Na^+ into the muscle, increased $[Na]_i$ is not necessary to induce or maintain the effects of excitation (Everts & Clausen, 1994; Nielsen & Clausen, 1997). Activation of the Na^+,K^+-ATPase is produced within a few seconds of stimulation, before an increase in $[Na^+]_i$ occurs (Nielsen & Clausen, 1997). Furthermore, during and after brief stimulation an undershoot of $[Na^+]_i$ occurs (Nielsen & Clausen, 1997). This indicated continued Na^+,K^+-ATPase activity, demonstrating an independence of Na^+,K^+-ATPase activation from increased $[Na^+]_i$. Although exercise causes K^+ to be lost from the muscle, increased muscle $[K^+]_e$ has little influence on Na^+, K^+-ATPase activation (Sejersted, 1987).

While stimulation of isolated rat soleus muscle increased activation of the Na^+,K^+-ATPase to maximal capacity, stimulation frequency needed to be approximately 120 Hz to achieve such an effect (Nielsen & Harrison, 1998). Human physiological motor unit discharge rate is far below that level and may not exceed even 50 Hz even during the most intense exercise (Jones, 1996; Roos *et al.*,

1999). At 50 Hz, in isolated rat soleus muscle, the Na^+,K^+-ATPase would only function at ~15-20% of its maximal capacity, well below the required capacity needed during such exercise (McKenna, 1998). This suggests that in human muscle, increased $[K^+]_e$ is consistent with submaximal Na^+,K^+-ATPase activity. Hence Na^+,K^+-ATPase activation may not keep pace with K^+ loss from the muscle, thereby enabling plasma $[K^+]$ to increase to ~8-9 mM, and muscle interstitial $[K^+]$ to 10-15 mM during fatiguing, intense exercise. During prolonged submaximal exercise, motor unit discharge rate may be as low as 10 Hz (Jones, 1996). Even at this discharge rate muscle K^+ loss still has a significant effect on fatigue and thus performance (Nielsen *et al.*, 2004). This further suggests the Na^+,K^+-ATPase activity would be less than the required capacity even at this level of work output, consistent with muscle K^+ loss.

2.4.2 Hormonal activation

Under physiological conditions the hormones calcitonin gene-related peptide (CGRP), adrenaline, noradrenaline and insulin are increased with muscle contraction and stimulate muscle increased Na^+,K^+-ATPase activity (Clausen, 2003). The slow time-course and small magnitude of these hormonal effects on Na^+,K^+-ATPase activation indicated that they were of limited impact during acute loss of muscle excitability (Clausen & Everts, 1991). Propranolol, which blocks the β-adrenoceptors, has been used to demonstrate that adrenaline and noradrenaline also have no extra cumulative effect on the level of Na^+,K^+-ATPase activation (Hallen *et al.*, 1994; Gullestad *et al.*, 1995). This indicates hormonal activation may work in parallel with the effects of excitation.

2.4.2.1 Calcitonin gene-related peptide

Excitation stimulates the release of calcitonin gene-related peptide (CGRP) from nerve endings during intense stimulation (Uchida *et al.*, 1990; Sakaguchi *et al.*, 1991) and therefore may contribute to the increase in Na^+,K^+ transport in electrically stimulated muscles and excitation-induced force recovery (Nielsen *et al.*, 1998). Reducing CGRP content by capsaicin pre-treatment or prior denervation prevented both the excitation-induced force recovery, the drop in $[Na^+]_i$ in rat soleus muscle (Nielsen *et al.*, 1998) as well as prevented an excitation-induced undershoot in $[Na^+]_i$ in rat EDL and soleus muscle (Nielsen & Clausen, 1997).

2.4.2.2 Adrenaline and noradrenaline

Both adrenaline and noradrenaline were found to have no additional effect to excitation on Na^+, K^+-ATPase activity during muscle contraction (Clausen, 1996a). However, an important role for catecholamines in the regulation of muscle Na^+,K^+-ATPase was suggested at the beginning of exercise, as the β-adrenoceptor blocker propranolol increased femoral venous $[K^+]$ (Hallen *et al.*, 1994; Gullestad *et al.*, 1995).

2.4.2.3 Insulin

Insulin also stimulates the Na^+,K^+-ATPase. Insulin decreased $[Na^+]_i$ in rat diaphragm muscle (Creese, 1968) and increased Na^+ uptake in rat soleus muscle (Clausen & Flatman, 1977; Clausen & Kohn, 1977). This effect was blocked by ouabain (Weil *et al.*, 1991) suggesting insulin activation of the Na^+, K^+-ATPase. In humans, hyperkalemia increases plasma insulin (Cox *et al.*, 1978; Clausen & Flatman, 1980; Clausen, 1986) and insulin-stimulated K^+ net uptake from the plasma in human forearm (Zierler & Rabinowitz, 1964).

2.4.3 Energetics of muscle Na^+,K^+-ATPase activity

The Na^+,K^+-ATPase requires ATP hydrolysis to provide energy for Na^+,K^+ transport. This ATP for Na^+,K^+-ATPase is synthesised preferentially by aerobic glycolysis (James *et al.*, 1999; Bundgaard *et al.*, 2003). Hence, a depression of local muscle glycogen with exercise may reduce local ATP available for the Na^+,K^+ATPase. A decline in muscle glycogen content was found at fatigue at the cessation of prolonged exercise (Booth *et al.*, 1997). Is it unknown whether these changes affected glycogen content localised at the Na^+,K^+ATPase, but this seems likely. It has been suggested that as Na^+,K^+ATPase activity is regulated by ankyrin-spectrin links to the cytoskeleton, subtle changes in muscle glycogen content may change the structural balance necessary for optimal function of the enzyme (Therien & Blostein, 2000).

2.4.4 Intracellular translocation of Na^+, K^+-ATPase

There is some evidence that both insulin and exercise may induce translocation of Na^+, K^+-ATPase subunits from an undefined intracellular pool to the sarcolemmal membrane. Insulin increased the α_2 content in the plasma membrane of rat skeletal muscle, with a parallel decrease in its abundance in intracellular membranes (Hundal *et al.*, 1992). The β_1 subunit isoform, but not β_2, was also elevated in the plasma membrane by insulin. Insulin-induced redistribution of the α_2 and β_1-isoforms of the Na^+,K^+-ATPase from an intracellular pool to the plasma membrane was restricted to oxidative fibre-type skeletal muscles (Lavoie *et al.*, 1996). However, in contrast to these findings, insulin failed to increase Na^+,K^+-ATPase content in cut rat soleus muscle, suggesting that any subunit translocation may be an artefact of membrane isolation or non-functional Na^+, K^+-ATPases (McKenna et al., 2003). This study confirmed previous findings that found no

change in Na^+, K^+ transport after stimulation of rat soleus muscle by insulin (Clausen & Hansen, 1977; Dorup & Clausen, 1995). These findings cast some doubt about the effects of insulin on Na^+,K^+-ATPase translocation.

Tsakaridis *et al.* (1996) found increased sarcolemmal abundance of α_1, α_2 and β_1 isoforms in muscle after 1hr treadmill exercise in rats. Low-intensity running increased the α_1, α_2, β_1, and β_2 abundance by 19-32% in the plasma membrane in oxidative muscle fibres, and in membranes from glycolytic muscle fibres the α_1, α_2, and β_2 contents were increased by 13-25% (Juel *et al.*, 2001). In human muscle, α_2 and β_1 isoform abundance in isolated sarcolemmal membranes was increased after 5 min knee extensor exercise (Juel *et al.*, 2000a). However, electrical stimulation of rat soleus and EDL muscle failed to detect any increase in ^{3}[H] ouabain binding, which fully quantifies functional Na^+,K^+-ATPase (McKenna *et al.*, 2003b). This therefore argues against any functional Na^+,K^+-ATPase translocation with exercise (McKenna *et al.*, 2003b).

2.5 Role of Na^+,K^+-ATPase in muscle fatigue and maintenance of muscle force

2.5.1 Force recovery, hormones and stimulation

In studies where isolated rat muscles were paralysed by exposure to high extracellular $[K^+]$, activation of Na^+,K^+-ATPase played an important role in force recovery. Whilst incubated in $[K^+]$ of 12.5 mM, stimulation of 30 Hz for 2 seconds every minute enabled tetanic force to recover from 13% to 97% within 10 min (Everts & Clausen, 1994), and from 16% to 62 % within 14 minutes (Nielsen *et al.*, 1998). In each case, force recovery was suppressible by ouabain, indicating force recovery was secondary to activation of the Na^+,K^+-ATPase. Thus excitation, which

markedly stimulated the Na^+,K^+-ATPase, led to restoration of force and excitability of skeletal muscle.

Previous work has shown that in muscles where contractile performance is depressed by exposure to high extracellular K^+, considerable force recovery can be elicited by acute stimulation of active transport with hormones such as adrenaline, noradrenaline, the ß-agonist salbutamol, insulin or CGRP (Clausen, 1998; Nielsen & Harrison, 1998; Nielsen *et al.*, 1998). Force recovery was closely related to stimulation of Na^+, K^+-ATPase-mediated K^+ uptake, and related to the restoration of membrane potential and the electrochemical gradient for Na^+ across the sarcolemma (Nielsen *et al.*, 1998).

2.5.2 Depressive effects of acute high intensity exercise

Fatigue induced by 50 maximal knee extension contractions resulted in a 17% fall in maximal *in-vitro* Na^+,K^+ATPase activity, as measured by K^+-stimulated 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity (Table 1) (Fraser *et al.*, 2002). This decline appeared to be independent of training status, with no difference in the extent of the decline in Na^+,K^+ATPase activity between endurance trained, strength trained and untrained subjects (Fraser *et al.*, 2002). Fowles *et al.* (2002b) found a decline in Na^+,K^+-ATPase activity following 30 min of isometric single leg exercise at 60% MVC, that could not be attributed to reduction in Na^+,K^+ATPase content. They also found that the decline in Na^+,K^+ATPase activity was correlated to a decline in M-wave area, and was thus associated with a decrease in muscle excitability (Fowles *et al.*, 2002a; Fowles *et al.*, 2002b).

2.5.3 Depressive effects of acute prolonged exercise

Few studies have investigated the effect of prolonged exercise on muscle Na^+,K^+-ATPase activity (Table 1). No change in Na^+,K^+-ATPase activity was found

after 2h of prolonged exercise in rats (Fowles *et al.*, 2002a). However, when exercise was continued for an additional 45 min beyond the end of exhaustive exercise at reduced speed, a 12% depression in Na^+,K^+-ATPase activity from 168 nmol·mg protein^{-1} h^{-1} at rest was found (Fowles *et al.*, 2002a). Na^+,K^+-ATPase activity was depressed by 12% and 13% from approximately 1680 and 1540 nmol.g protein^{-1}.min^{-1} at rest, respectively. after incremental exercise in trained subjects, either sleeping under hypoxic conditions but training under normoxic conditions, or living and training under normoxia respectively (Aughey *et al.*, 2004). No change was found in Na^+,K^+-ATPase content with isometric exercise (Fowles *et al.*, 2002a; Fowles *et al.*, 2004).

A 28% and 44% decline from 101 and 90.8 nmol.mg.protein^{-1}.h^{-1} at rest, respectively, was recently reported in Na^+,K^+-ATPase activity during prolonged exercise at 50% $VO_{2\ peak}$ in untrained subjects under both normal and hypoxic conditions, respectively (Sandiford *et al.*, 2004). The larger than usual depression found in Na^+,K^+-ATPase activity in the normoxic condition in this study may have been due to their subjects being untrained. Reductions in Na^+,K^+-ATPase activity were found after 30 and 90 min in both conditions. This exercise was not continued until fatigue so it is unclear whether a further decline would have occurred. Furthermore, these changes were reported in untrained subjects at low intensity.

Recent evidence found muscle fibre conduction velocity (MFCV) to be related to activation of the Na^+,K^+-ATPase. Increased MFCV in the human brachioradial muscle was found during each of four ischaemic isometric exercise bouts and during recovery from exercise (Rongen *et al.*, 2002). MFCV increase was completely prevented in the presence of ouabain indicating this was caused by rapid Na^+,K^+ATPase activation. This suggests that any decline in Na^+,K^+ATPase activity

during prolonged exercise may also have the added effect of reducing MFCV thus contributing to the decline in neuromuscular coordination and muscle force production. M-wave area declined after the fourth hour of exercise during 5h of prolonged cycling at 55% VO_2 peak (Lepers *et al.*, 2002). These provide evidence of a possible decline in both Na^+,K^+ATPase activity and excitability during prolonged exercise.

Table 1. Effects of exercise or muscle contraction on maximal rate of Na+,K+ ATPase activity

Ref	Species	Muscle	Mode	Protocol	Decline in Na^+,K^+ ATPase activity (%)
1	Human	vl	Knee extensor	50 knee extensions @180 deg s^{-1}	17%
2	Rat	sol, rvl, wvl, EDL	running	2 hr to fatigue.21m/min Additional 45 min at 10m/min	No change 12%
3	Human	vl	Leg extension	Isometric single leg, ~60% MVC 30 min	38%
4	Human	vl, UT	cycling	90 min @ 50 $VO_{2\ peak}$- mean VO_2 max 42 ml/kg/min	27.9%

References: 1 Fraser et al. 2002; 2 Fowles et al. 2002a; 3 Fowles et al. 2002b; 4. Sandiford et al., 2004
Abbreviations: vl=vastus lateralis; rvl=red vastus lateralis; wvl=white vastus lateralis; sol=soleus; EDL=extensor digitorum longus; UT=untrained

2.5.4 Possible mechanisms for depressed Na^+,K^+-ATPase activity

Several factors are implicated in the exercise-induced depression of maximal in-vitro Na^+,K^+-ATPase activity, including increased free radical production, reduced muscle glycogen content, phosphorylation of α subunits and elevated cytosolic [Ca^{2+}] (Fraser *et al.*, 2002).

2.5.4.1 Free radical production

Increased free-radical activity may play some role in muscle damage caused by extensive muscular activity (Jackson *et al.*, 1985). Interaction with reactive oxygen species reduced Na^+,K^+-ATPase activity in cardiac muscle (Kato *et al.*, 1997). Na^+,K^+-ATPase may be affected by free radicals since the α-subunit has a number of disulphide bonds that are susceptible to oxidation (Kourie, 1998). Thus, it is possible that any depression in Na^+,K^+-ATPase activity with exercise may be due in part to elevated free radical production.

2.5.4.2 Elevated [Ca^{2+}]

Elevated [Ca^{2+}], even at nanomolar concentrations, was found to decrease Na^+,K^+-ATPase activity in mouse diaphragm, indicating possible effects of proteolysis on Na^+,K^+-ATPase isoforms (Sulova *et al.*, 1998). Increased cytosolic [Ca^{2+}] during contractions occurs due to both SR release and inactivation of the Ca^{2+}ATPase (Westerblad & Allen, 1991; Westerblad & Allen, 1993; Westerblad *et al.*, 1993). Thus over the time course of prolonged exercise, cytosolic [Ca^{2+}] may be elevated for a considerable period of time, producing a proteolytic degradation of the Na^+,K^+-ATPase.

2.5.5 Exercise effects on Na^+,K^+-ATPase content

2.5.5.1 Acute exercise

Even brief exercise results in an increase in muscle Na^+, K^+-ATPase mRNA for each of six isoforms (Murphy *et al.*, 2004a; Murphy *et al.*, 2004b). Cellular adaptations to high-intensity exercise training may, in part, be induced by transcriptional regulation (Nordsborg *et al.*, 2003). This suggests that acute exercise may initiate the process of upregulation of Na^+, K^+-ATPase in human muscle. An upregulation in Na^+, K^+-ATPase was reported in human muscle following a 100km run lasting 10-11 h (Overgaard *et al.*, 2002) . It remains to be seen whether this also occurs in a more conventional bout of prolonged exercise of 60-90 min duration, given the smaller time course for Na^+, K^+-ATPase resynthesis.

2.5.5.2 Chronic exercise

Training has been shown to produce an upregulation of the Na^+, K^+-ATPase by similar magnitude with both prolonged and intense exercise training (Green *et al.*, 1993; McKenna *et al.*, 1993; Evertsen *et al.*, 1997). These studies indicate that

Na^+, K^+-ATPase upregulation in human muscle occurs with all forms of chronic training.

A recent study found reduced accumulation of interstitial [K^+] during incremental exercise after 7 weeks training in the one leg compared to a control leg (Nielsen *et al.*, 2004). This was associated with delayed fatigue (28%) during intense incremental exercise. Although muscle release of K^+ was the same under both conditions, interstitial [K^+] was lower in the trained leg at submaximal workrates, with similar concentration at fatigue (Trained Leg 9.9 from 4.8 mmol l^{-1} at rest, Control Leg 9.1 from 5.0 mmol l^{-1} at rest) (Nielsen *et al.*, 2004). Intense intermittent training reduced K^+ accumulation in human skeletal muscle interstitium during exercise, probably through a larger re-uptake of potassium due to greater muscle Na^+,K^+-ATPase activity (Nielsen *et al.*, 2004). Both endurance and sprint training reduced the exercise-induced rise in plasma [K^+] at the same absolute exercise work rate and duration (McKenna, 1995).

2.5.6 Relationships between Na^+,K^+-ATPase and performance during exercise

A relationship between Na^+,K^+-ATPase content and muscle performance in exercising humans has rarely been found. No relationship was found between increased Na^+,K^+-ATPase content with sprint training and time to fatigue during intense exercise, despite a significant improvement in performance and reduced exercise hyperkalemia relative to work performed (McKenna *et al.*, 1993). No relationship was found either between Na^+,K^+-ATPase content and performance with endurance exercise (Madsen *et al.*, 1994). However, a significant correlation was reported between maximal isometric strength and Na^+,K^+-ATPase content, but no relationship between Na^+,K^+-ATPase content and isometric endurance with long-term swim and run training (Klitgaard & Clausen, 1989). There was no

relationship either between Na^+,K^+-ATPase content and performance during a 100-km run (Overgaard *et al.*, 2002). By contrast though, a significant relationship was found between Na^+,K^+-ATPase content, distance completed during a 20-minute treadmill run and performance ranking in elite skiers, possibly due to these subjects being a homogenous group (Evertsen *et al.*, 1997). However, they reported no relationship between the actual increase in Na^+,K^+-ATPase content found during training and improved treadmill performance. These studies suggest that small changes in Na^+,K^+-ATPase content may have little effect on performance during exercise.

2.6 Excitation-Contraction (EC) coupling in skeletal muscle

Excitation-Contraction (EC) coupling in skeletal muscle comprises all processes from membrane excitation up to and including the activation of contractile proteins, and thus culminating in force production. Stephenson *et al.* (1998) suggests a six step model of force production: (1) initiation of an action potential along the sarcolemma and down the transverse t-tubule system; (2) detection of the T-system depolarization and signal transmission from the t-tubules to the sarcoplasmic reticulum (SR) membrane resulting in coupling of the voltage sensing protein, the dihydropyridine receptors (DPHR), with the Ca^{2+} release channel, known as the ryanodine receptor (RyR); (3) Ca^{2+} release from the SR; (4) transient rise in myoplasmic Ca^{2+}; (5) transient activation of the Ca^{2+} regulatory system; (6) Ca^{2+}-ATPase and Ca^{2+}binding to myoplasmic sites. Each of these is a potential site for fatigue.

2.7 Mechanisms of EC Coupling

Two vital proteins that are involved in the link between membrane excitation and the increase in cytosolic $[Ca^{2+}]$ that enables muscle contraction, are

the DHPR, which is located in the t-tubular membrane, and the RyR, which is located in the terminal cisternae of the SR. Whilst numerous proteins are essential in EC coupling, the regulation of these two proteins is briefly discussed due to their role in Ca^{2+} release.

2.7.1 Dihydropyridine Receptors (DHPR): voltage sensors

The DHPR, although in itself a Ca^{2+} channel, mainly acts as a voltage sensor, detecting change in voltage as the action potential propagates down the t-tubular membrane (Lamb, 1992). The DHPR complex contains four subunits and each subunit binds dihydropyridine, and each DHPR complex has a voltage-sensing component. The DHPR complexes are present in the junctional region of the t-tubular membrane and are arranged together in groups of four, known as the DHPR tetrad. Each DHPR tetrad is directly opposite a RyR, although every second RyR is not opposed to a DHPR tetrad (Dulhunty *et al.*, 1996). After the charge is carried by the DHPR, these loops are thought to communicate directly with the opposing RyR in the SR (Hille, 1992; Stephenson *et al.*, 1998). This is the mechanical model of SR Ca^{2+} release and explains most of the SR Ca^{2+} release in skeletal muscle.

Calcium Induced Calcium Release (CICR). Increased cytosolic [Ca^{2+}] also induces Ca^{2+} release from the RyR, known as "Calcium Induced Calcium Release (CICR)". This is thought to induce Ca^{2+} release from the RyR's that are not directly opposed to a DHPR tetrad (Wasserstrom, 1998). Changes in [Ca^{2+}] that occur that induce CICR may include normal Ca^{2+} release from the RyR, Ca^{2+} entry from extracellular spaces via sarcolemmal Ca^{2+} channels, or via the sarcolemmal Na^{+}/Ca^{2+} exchanger. However, the contribution of CICR to the total release of Ca^{2+} from RyR is only small (Bers, 1991).

2.7.2 Ryanodine Receptors (RyR): Physiological Ca^{2+} release channels

The Ryanodine receptor is the channel responsible for the release of Ca^{2+} from the SR in muscle cells. The RyR exists as a homotetramer and is predicted to have a short cytoplasmic C-terminus and 4-10 transmembrane domains, with the remainder of the protein termed the "foot" region, located in the cytoplasm between the T-tubule and the SR. The function of the RyR is to communicate between transverse-tubules and SR, with contraction of skeletal muscle triggered by release of Ca^{2+} ions from SR RyR following depolarization of t-tubules (Lamb, 1992; Stephenson *et al.*, 1998). The RyR is an extremely large protein and made up of four identical subunits. Several proteins are closely associated within the RyR and are thought to regulate its function. These include the protein calsequestrin, which is located within the SR lumen and binds Ca^{2+}, triadin and the FK506-binding protein known as FKBP. The FKBP is associated with the RyR in a 1:4 ratio and is thought to be important in triggering Ca^{2+} release from the RyR (Timerman *et al.*, 1993). The opening of the RyR and thus SR Ca^{2+} release is dependent on many factors. In the intact cell, the mechanical link between the DHPR and the RyR is essential for RyR opening. However, many ions and other compounds may also regulate RyR opening and these include Ca^{2+}, Mg^{2+}, inorganic phosphate, ATP and caffeine (Fryer *et al.*, 1995; Owen *et al.*, 1996; Chin & Allen, 1997; Blazev & Lamb, 1999; Laver *et al.*, 2001). Three major pharmacological activators used to induce in-vitro opening of Ca^{2+} release channels are caffeine, silver (Ag^{+}), and 4-chloro-*m*-cresol (4-CmC). The compound 4-CmC is a potent and specific activator, with 5mM 4-CmC having a 2-4-fold greater effect than Ag^{+} (Ward *et al.*, 1998; Williams *et al.*, 1998).

2.7.3 SR and Muscle fibre types

Important differences exist between fast and slow-twitch muscle fibres with relation to SR characteristics and contractile function. Time to peak tension, half relaxation time are 3-4 times longer, and peak rate of force development and relaxation rate are all lesser in slow-twitch than in fast-twitch muscle fibres (Fitts *et al.*, 1982). Fast-twitch muscle fibres also exhibit a greater rate of fatigability (Thorstensson & Karlsson, 1976). Fast-twitch fibres have approximately a 1.7 fold larger terminal cisternae, two times larger t-system volume and 1.5 fold larger SR volume, which allows more rapid action potential propagation, higher Ca^{2+} release rate and force production (Eisenberg *et al.*, 1974; Eisenberg & Kuda, 1975). The relationship between force production and the rate of SR Ca^{2+} release suggests fast-twitch fibres should produce a greater rate of Ca^{2+} release. In human muscle the rate of Ca^{2+} release is 2 fold higher in muscle, rich in fast-twitch muscle fibre than that of slow-twitch fibre (Li *et al.*, 2002). The rate of SR Ca^{2+} uptake and Ca^{2+}ATPase activity were also estimated to be 2-3 fold higher in fast-twitch muscle fibres (Li *et al.*, 2002).

2.7.4 Advantage of single fibre muscle preparations

Three models have been used to measure the effects of SR Ca^{2+} release with muscle. These include the use of intact single muscle fibres (Westerblad & Allen, 1991; Chin & Allen, 1997; Chin *et al.*, 1997), skinned muscle fibre preparations (Lamb, 1992) and muscle homogenates (Ward *et al.*, 1998; Williams *et al.*, 1998). Of these, only the intact and skinned single muscle fibre preparations allow measurement of SR Ca^{2+} release, together with $[Ca^{2+}]$, in relation to changes in muscle force.

Studies with intact single fibres have demonstrated a direct link between changes in $[Ca^{2+}]_i$ and muscle force with fatigue (Westerblad & Allen, 1991; Chin & Allen, 1997; Chin *et al.*, 1997). Fatigue produced by repeated tetani is caused by a combination of reduced maximum tension-generating capacity, reduced myofibrillar Ca^{2+} sensitivity, and reduced Ca^{2+} release from the sarcoplasmic reticulum (Westerblad & Allen, 1991). The depression of maximum tension-generating capacity developed early during fatiguing stimulation and was of greatest importance for the force decline at early stages of fatigue (Westerblad & Allen, 1991). As fatigue became more severe, reduced Ca^{2+} sensitivity and reduced Ca^{2+} release become quantitatively more important for the tension decline. Application of caffeine at the end of fatiguing stimulation induced a marked increase of tetanic $[Ca^{2+}]_i$, such that tension was almost completely restored. The decline in $[Ca^{2+}]_i$ in single mouse fibres stimulated at 100Hz to 30% muscle force, was associated with depressed muscle force and fatigue (Chin & Allen, 1997).

2.8 Effects of Exercise on muscle SR Ca^{2+} Release

2.8.1 Effect of intense muscle contraction on Ca^{2+} Release

A number of studies have recently investigated the effects of intense exercise or tetanic stimulation on *in-vitro* SR Ca^{2+} release in human, rodent and other mammalian fibres (Table 2). Many of these studies employed different protocols, muscle types, species, as well as differing chemical reagents for initiating SR Ca^{2+} release. For the sake of simplicity the relevant studies in this thesis will cite only the frequency and duration of stmulation procedures within the text. These procedures are produced in more detail in tables 2 and 3.

Animal Studies. In rat EDL muscle stimulated at 60 Hz for 4 min, SR Ca^{2+} release rate initiated by 5 mM 4-CmC in a homogenate preparation declined by 34%

(Ortenblad *et al.*, 2000b). SR Ca^{2+} release rate declined when measured in both skinned fibres and in homogenates from semitendinosus frog muscle after stimulation at 80 Hz to fatigue (Williams *et al.*, 1998). SR Ca^{2+} release rate in skinned fibres declined by 40% (as assessed by the rate of force increase in response to 8 mM caffeine), similar to that in a homogenate preparation, which declined by 38% from approximately 10.5 $nmol.mg^{-1}$ min^{-1}, when release was initiated by $AgNO_3$ (Williams *et al.*, 1998). In frog sartorius muscles, stimulated at 100 Hz, Ca^{2+} release rate initiated by 4-CmC was decreased by 45% from approximately 3.0 $nmol.mg^{-1}.min^{-1}$ at rest in muscle homogenates (Ward *et al.*, 1998). Interestingly, when muscle force was only reduced to 91% of initial force by 100 Hz stimulation, no change was found in SR Ca^{2+} release, further suggesting a link between SR Ca^{2+} release and muscle force (Ward *et al.*, 1998).

Human Studies. Fifty maximal knee extensor contractions depressed $AgNO_3$ induced SR Ca^{2+} release rate in vastus lateralis muscle homogenates, in each of endurance trained, resistance trained and untrained subjects (Li *et al.*, 2002). Following knee extensor exercise to fatigue, a similar 35% depression from 20.9 $nmol\ min^{-1}$ mg $protein^{-1}$ in $AgNO_3$–induced SR Ca^{2+} release rate was found in vastus lateralis muscle homogenate (Hill *et al.*, 2001). Depression in SR Ca^{2+} release rate was similar in all these studies irrespective of differing exercise protocols, which included voluntary exercise of humans, or stimulation of animals, time course of exercise, stimulation employing different duty cycles, use of muscle homogenate or skinned muscle fibres, initiated by $AgNO_3$ or 4-CmC. Thus, intense muscle stimulation or physical exercise consistently results in a 34-45% depression in SR Ca^{2+} release rate.

2.8.2 Effect of prolonged exercise on Ca^{2+} Release

A number of studies have investigated the effects of prolonged exercise on SR Ca^{2+} release in rodent muscles, using homogenate preparations, but there are no studies in humans (Table 2). After a treadmill run to fatigue, SR Ca^{2+} release rate in red gastrocnemius muscle declined by 20-30%, from approximately 5.5, 7.0 and 10.0 $nmol.mg^{-1}.sec^{-1}$ at rest when initiated by 5, 10 and 20 μM $AgNO_3$ respectively (Favero *et al.*, 1993). Similar exercise also produced a 29% from approximately 1.8 $umol.mg^{-1}.min^{-1}$ at rest depression in SR Ca^{2+} release in red gastrocnemius muscle (Stavrianeas et al., 2003). Inashima *et al.* (2003) found an approximate 35% from approximately 0.8 $umol.min^{-1}.g^{-1}$ at rest depression in SR Ca^{2+} release rate in both trained and untrained rat soleus muscle. Prolonged treadmill running at 100% $VO_{2\ peak}$ also depressed SR Ca^{2+} release rate in diaphragm by 22% from approximately 18.0 $nmol.min^{-1}.mg.protein^{-1}$ at rest (Matsunaga *et al.*, 2002). In contrast, no change in SR Ca^{2+} release rate was found in fast-twitch gastrocnemius muscle (Schertzer *et al.*, 2003), or in rat diaphragm at fatigue after prolonged running (Stavrianeas et al., (2003).

Table 2. Effects of exercise or muscle contraction on maximal rate of SR Ca^{2+} release.

Species	Ref	Muscle	Mode	Protocol	Force Decline (%)	Decline in SR Ca^{2+} Release (%)
Rat	1	EDL	ES	60Hz, 150ms/s, 5 min	~80	34 ±2
Rat	2	EDL	ES	6s@5 Hz /10s, 60 min,	62	30 ±4
		sol		10Hz,1.5s/2.5s, 10 min, 61% initial force		23 ±6
Frog	3	sart	ES	100Hz, 100ms/0.5s, 5 sec, 17% initial force		45
				1 min, 15% initial force		45
				100ms/5s, 1 min, 91% initial force		No change
Frog	4	semiten	ES	80 Hz, 100ms/0.5s, 5min, 5% initial force ($AgNo_3$ v 4-CmC)-$AgNo_3$		38
				4-CmC		45
rat	5	RG	TM run	To exhaustion (115 min) 21m/min^{-1}, 10% grade		20-30
		WG				No change
rat	6	Sol, trained	TM run	To exhaustion (559.4 min) 21m/min^{-1}, 10% grade		~35%
		sol, UT		(253 min)		~30%
		Plant, trained		(559.4 min)		No change
		Plant, UT		(253 min)		No change
Rat	7	RG	running	To exhaustion (81 min)		28
rat	8	Diaphragm	running	Run to exhaustion @ 100% VO_2 max (4.79 min)		22
Human	9	vl	Knee extensor	50 knee extension at 180 dg/s^{-1}		42.1±3.8
		UT				
		RT		50 knee extension at 180 dg/s^{-1}		43.4 ±3.9
		ET		50 knee extension at 180 dg/s^{-1}		31.3 ±6.1
Human	10	vl	Knee extensor	90 x 2 knee extension at 240 deg/s^{-1}		34.9

References: 1 Ortenblad et al 2000; 2 Verburg et al. 1999; 3 Ward et al. 1998; 4 Williams et al. 1998; 5 Favero et al. 1993; 6 Inashima et al. 2003; 7 Stavianeas et al. 2003; 8 Matsunga et al., 2003; 9 Li et al. 2002; 10 Hill et al. 2001.

Abbreviations: vl=vastus lateralis; rvl=red vastus lateralis; wvl=vastus lateralis; sol=soleus; EDL=extensor digitorum longus; RG= red gastrocnemius; WG= white gastrocnemius; Plant.=plantaris; sart=sartorius; semiten.=semitendinosus UT=untrained; RT=restistance trained; ET=endurance trained.; TM=treadmill; ES=electrical stimulation

2.8.3 Role of muscle fibre type in depressed SR Ca^{2+} release

One reason why not all studies found a depression of SR Ca^{2+} release rate during prolonged exercise may be the choice of fibre types analysed. A reduction in SR Ca^{2+} release rate was found in red, but not in white gastrocnemius muscle in rats (Favero *et al.*, 1993; Schertzer *et al.*, 2003). Similarly, depressed Ca^{2+} release was found in the slow-twitch soleus muscle, but not in the fast-twitch plantaris muscle, in either trained or untrained rats (Inashima *et al.*, 2003). These studies indicate that *in-vitro* SR Ca^{2+} release rate in muscle homogenates is consistently depressed by 20-35% during prolonged fatiguing exercise, but mostly in the slow-twitch muscle fibre or muscle appropriate to the exercise involved. All these studies, taken together, suggest that fatiguing intense or prolonged exercise, will depress SR Ca^{2+} release rate and therefore impair muscle force and precipitate fatigue.

2.8.4 Possible causes of depression in SR Ca^{2+} release during exercise

Numerous mechanisms have been proposed for failure of SR Ca^{2+} release with fatigue. These include (1) reduced action potential amplitude in the t-tubules affecting EC coupling; (2) increased cytosolic [Mg^{2+}], which inactivates the SR RyR; (3) decreased muscle glycogen (Byrd *et al.*, 1989a; Chin & Allen, 1997; Stephenson *et al.*, 1999); (4) decreased local ATP concentration (Korge & Campbell, 1995; Westerblad *et al.*, 1998); (5) precipitation of $Ca^{2+}P_i$ in the SR lumen (Fryer *et al.*, 1995); (6) a rise in free radical compounds (Favero *et al.*, 1998); (7) increased muscle temperature (Schertzer *et al.*, 2002; van der Poel & Stephenson, 2002); and (8) activation of calpain-neutral activated proteases (Belcastro, 1993; Beaton *et al.*, 2002).

2.9 Effects of Exercise on muscle SR Ca^{2+} Uptake

SR Ca^{2+} release may also be adversely affected by a depression in SR Ca^{2+} uptake, as less Ca^{2+} would be available from within the SR for release. This would then have a direct effect on muscle force production. Alternately, the Ca^{2+} binding proteins within the SR, such as calsequestin, suggest that free SR Ca^{2+} may not be a limiting factor in SR Ca^{2+} release, as most of the Ca^{2+} for release dissociates from these binding proteins, and SR Ca^{2+} does not become depleted. While a decline in SR Ca^{2+} uptake may initially increase force production due to increased resting cytosolic [Ca^{2+}] (Westerblad & Lannergren, 1991; Beaton *et al.*, 2002), it may induce fatigue later due to the onset of proteolysis and low frequency fatigue (Chin *et al.*, 1997). Reduced SR Ca^{2+} uptake rate could also contribute to slowed muscle relaxation due to increased cytosolic [Ca^{2+}] (Westerblad & Lannergren, 1991). The following sections demonstrate depressed SR Ca^{2+} uptake with all forms of fatiguing exercise, whether intense or prolonged duration. Thus, a depression in SR Ca^{2+} uptake may play a significant role in muscle fatigue.

2.9.1 Intense exercise effects on SR Ca^{2+} uptake

Numerous studies utilising many varying protocols have investigated the effects of fatiguing exercise on *in-vitro* SR Ca^{2+}-ATPase activity and/or Ca^{2+} uptake, in animal and human muscle with varying results (Table 3).

Animal muscle stimulation studies. In frog semitendinosus muscle, stimulated at 80 Hz, SR Ca^{2+} uptake declined by 46% and 55% from rest when measured in skinned fibres and homogenate preparations, respectively (Williams *et al.*, 1998). In frog sartorius muscles stimulated at 100 Hz, a 43% depression was found in Ca^{2+} uptake from approximately 12.5 $nmol.mg^{-1}.min^{-1}$ from rest in a homogenate preparation (Ward *et al.*, 1998). In contrast, no change in homogenate Ca^{2+} uptake rate was

found following 60 Hz tetanic stimulation of rat EDL (Ortenblad *et al.*, 2000b). Similarly, white gastrocnemius and red gastrocnemius muscle stimulated at 100 Hz did not alter SR Ca^{2+} uptake measured in homogenates (Dossett-Mercer *et al.*, 1995).

Animal exercise studies. In horses, a treadmill run to exhaustion at 100% VO_2 max reduced SR Ca^{2+} uptake measured in gluteal muscle homogenates and isolated SR membranes, by 43% and 51%, respectively (Byrd *et al.*, 1989b). In contrast, where fatigue was induced by 15-20 min of running in rats, no change was found in SR Ca^{2+} uptake in homogenates from soleus, or red and white gastrocnemius (Dossett-Mercer *et al.*, 1994; Dossett-Mercer *et al.*, 1995). It is possible that some *in-vitro* studies may not detect changes in SR Ca^{2+} uptake given that they are analysed under optimal conditions (Chin *et al.*, 1995). Studies performed under optimal conditions eliminate effects such as changes in metabolism and excitation-contraction coupling on SR Ca^{2+} release, Ca^{2+} uptake and Na^{+}, K^{+}-ATPase activity.

Human exercise studies. Reductions in human muscle SR Ca^{2+} uptake were found after intense fatiguing exercise, when measured in homogenate preparations. SR Ca^{2+} uptake rate was depressed by 26% from 12.3 $nmol.min^{-1}.mg\ protein^{-1}$ at rest in vastus lateralis muscle after 2 min knee extensor exercise (Hill *et al.*, 2001). Similarly, 50 fatiguing knee extensions induced 28, 34 and 43% declines from 11.9, 10.0 and 9.4 $umol.min^{-1}.g.\ protein^{-1}$ in vastus lateralis muscle in endurance-trained, resistance-trained and untrained subjects, respectively (Li *et al.*, 2002). Three, 30s all out bouts of cycling induced a 29% decline from 23.4 $nmol.min^{-1}.mg.wet\ wt^{-1}$ in SR Ca^{2+} uptake in vastus lateralis muscle when measured per g wet weight, and fell only marginally short of significance (P=0.06) when measured per mg protein (Hargreaves *et al.*, 1998). Despite varying results these studies seem to suggest that

intense fatiguing protocols depress muscle SR Ca^{2+} uptake during intense exercise or stimulation, irrespective whether measured in muscle homogenate, or in the purified SR membrane.

2.9.2 Prolonged exercise effects on SR Ca^{2+} uptake

Several studies have shown that *in-vitro* SR Ca^{2+} uptake measured in muscle homogenates is depressed during prolonged exercise (Table 3).

Animal muscle stimulation study. The only prolonged muscle stimulation study found that 90 min intermittent 10-Hz stimulation in red gastrocnemius muscle depressed Ca^{2+} uptake measured in crude SR fractions, but not in a muscle homogenate (Chin *et al.*, 1995).

Animal exercise studies. Fatiguing prolonged exercise depressed homogenate SR Ca^{2+} uptake rate by 40% from approximately 0.8 $u$mol.min^{-1}.g^{-1} at rest in soleus muscle in both trained and untrained rats, and by 17% from approximately 2.1 umol.min^{-1}.g^{-1} at rest in plantaris muscle in untrained rats, but not in trained rats (Inashima *et al.*, 2003). A 40% decline in Ca^{2+} uptake from 0.4 $u$mol.mg^{-1} min^{-1} at rest was found in rat deep gastrocnemius and vastus lateralis muscle homogenate after 20 min of prolonged treadmill exercise and this continued to decline marginally to 45 min and to fatigue at 140 min (Byrd *et al.*, 1989a). Ca^{2+} uptake in purified vesicles of the same combined muscles were depressed only at fatigue, which was approximately 5-8% larger than that in muscle homogenates (Byrd *et al.*, 1989a). In contrast, no change was found at the end of exercise in superficial gastrocnemius and vastus lateralis muscle. The declines in SR Ca^{2+} uptake and Ca^{2+}-ATPase activity were related to a reduction in muscle glycogen (Byrd *et al.*, 1989a).

Human studies. The only prolonged exercise study in human muscle found that exercise to fatigue at 70% $\dot{V}O_2$ peak induced a 17% decline from 10.42 nmol.min^{-1}.mg.protein^{-1} at rest in SR maximal Ca^{2+} uptake and 21% reduction from 88.83 mol.min^{-1}.mg.protein^{-1} from rest in Ca^{2+}-ATPase activity, measured in homogenates from vastus lateralis (Booth *et al.*, 1997). After 20 min of recovery, Ca^{2+} uptake remained 22% depressed, with no recovery evident at 60 min post-exercise. When measured in one subject after 6 h recovery, Ca^{2+} uptake was 18 % below resting levels (Booth *et al.*, 1997). The time course of changes during exercise was not determined.

Table 3. Effects of exercise or muscle contraction on maximal rate of SR Ca^{2+} uptake.

Ref	Species	Muscle	Mode	Protocol	Force Decline (%)	Decline in SR Ca^{2+} Uptake (%)
1	Human	vl	cycling	3 x 30sec all out-4 min rec,		19.7
				4^{th} bout following 30 min@30-35% $Vo_{2\ peak}$,then 60 min rec.		Increase 10.3
2	Rat	WG	TM run	90 min		No change
3	Rat	RG	TM runn	To exhaustion (81 min)		37
4	Human	vl-UT	Knee extensors	50 knee extension at 180 dg/s^{-1}		43.0 ±5.2
		RT				34.1 ±4.6
		ET				28.4 ±2.8
5	Human	vl	Knee extensor	90 x 2 knee extension at 240 deg/s^{-1}		26
6	Rat	EDL	ES	60Hz, 150ms/s, 5 min	~80	No change
7	Rat	WG	ES	10Hz, 100ms/s, 90 min	50	No change
		RG			50	No change
8	Rat	WG	TM run	Treadmill run to exhaustion 140 min		No change
		RG				No change
9	Rat	WG	ES	100Hz 200ms/s, 20 min	74	No change
		RG			74	No change
10	Rat	WG	TM run	Treadmill run to exhaustion, 20 min		No change
		RG				No change
		sol				No change
11	Frog	semiten.	ES	80 Hz, 100ms/0.5s, 5min ($AgNo_3$)	95	46
12	Frog	sart	ES	100Hz, 100ms/0.5s, 5 sec	83	43
				1 min	85	43
				100ms/5s, 1 min	9	No change
13	Human	vl	cycling	70% $VO_{2\ peak}$ to exhaustion		17 ±4
14	Rat	RG	TM run	20 min		40
		WG				No change
		RG		To exhaustion 140 min		55
		WG				No change
15	Horse	gluteal	TM run	100 $VO_{2\ max}$ to exhaustion 4.65 ±1.06 min		55
16	Rat	sol trained	TM run	To exhaustion (559.4 min)		40
		sol UT		(253 min)		40
		plant		(559.4 min)		No change
		trained				
		plant UT		(253 min)		17
17	Rat	diaphragm	TM run	To exhaustion @ 100% VO_2 max (4.79 min)		24

References: 1 Hargreaves et al. 2004; 2 Schertzer et al. 2003; 3 Stavianeas et al. 2003; 4 Li et al. 2002; 5 Hill et al. 2001; 6 Ortenblad et al 2000; 7 Chin et al. 1995; 8 Chin et al. 1995; 9 Dossett Mercer et al. 1995; 10 Dossett Mercer et al. 1994; 11 Williams et al. 1998; 12 Ward et al. 1998; 13 Booth et al. 1997; 14 Byrd et al. 1989; 15 Byrd et al. 1989; 16 Inashima et al. 2003; 17 Matsunga et al. 2003.

Abbreviations: vl=vastus lateralis; rvl=red vastus lateralis; wvl=vastus lateralis; sol=soleus; EDL=extensor digitorum longus; RG= red gastrocnemius; semiten=semitendinosus WG= white gastrocnemius; Plant.=plantaris; sart=sartorius; UT=untrained; RT=restistance trained; ET=endurance trained; TM=treadmill run; ES=electrical stimulation

2.9.3 Exercise effects on SR Ca^{2+} uptake and Ca^{2+}ATPase activity Coupling Ratio

Many studies have shown that the decline in Ca^{2+} uptake with exercise or muscle contraction is associated with a depression in Ca^{2+}ATPase activity (Byrd *et al.*, 1989a; Byrd *et al.*, 1989b; Booth *et al.*, 1997; Li *et al.*, 2002; Inashima *et al.*, 2003). This indicates possible structural alterations in the ATP binding or phosphorylation sites, rather than a direct impairment of Ca^{2+} binding or Ca^{2+} translocation. No change in the coupling ratio between SR Ca^{2+} uptake and SR Ca^{2+}-ATPase activity was found during prolonged running in rats (Byrd *et al.*, 1989a; Inashima *et al.*, 2003). Similarly, a 21% depression from 88.83 $nmol.min^{-1}$ .mg $protein^{-1}$ from rest in Ca^{2+}ATPase activity was found (17% reduction in SR Ca^{2+} uptake) after prolonged cycling to fatigue (Booth *et al.*, 1997). SR Ca^{2+}ATPase activity was depressed 30-49% (28-43% reduction in SR Ca^{2+} uptake) in untrained, resistance trained and endurance trained subjects at the end of fatiguing knee extensor exercise (Li *et al.*, 2002). A dissociation between SR Ca^{2+} uptake and SR Ca^{2+}-ATPase was shown in red gastrocnemius muscle during 90 min intermittent 10 Hz stimulation when measured in both homogenate and SR purified fraction (Chin *et al.*, 1995). No change in SR Ca^{2+}-ATPase activity was found after fatiguing knee extensor exercise, despite a depression in SR Ca^{2+} uptake (Hill *et al.*, 2001). This is most likely to reflect a Type II error due to the small sample size. Uncoupling of SR Ca^{2+} uptake and SR Ca^{2+}-ATPase activity has also been found to occur at high temperatures (Inesi *et al.*, 1973; Byrd *et al.*, 1989a; Luckin *et al.*, 1991; Davidson & Berman, 1996; Schertzer *et al.*, 2002). Exposure of homogenates from white gastrocnemius muscle to 41°C, a muscle

temperature that might be experienced in exercise, resulted in a reduction in the coupling ratio (Schertzer *et al.*, 2002). This was mediated primarily by lower Ca^{2+} uptake and occurred due to increased membrane permeability to Ca^{2+} (Schertzer *et al.*, 2002).

2.9.4 Role of muscle fibre type in depressed SR Ca^{2+} uptake

Findings from studies investigating SR Ca^{2+} uptake during prolonged exercise may also have been affected by the fibre composition of the muscle analysed, and whether the muscles were likely to be recruited by the exercise protocol. Byrd *et al.* (1989a) found depressed Ca^{2+} uptake in red muscles but not in white muscle. No change was found in Ca^{2+} uptake during prolonged exercise with rat fast-twitch gastrocnemius muscle, which may not have been recruited (Schertzer *et al.*, 2003). Inashima *et al.* (2003) only found a decline in plantaris muscle in untrained rats but not trained rats. Thus, it is possible that the 17% decline in human muscle may have underestimated the actual depression in Ca^{2+} uptake rate in slow-twitch muscle fibres (Booth *et al.*, 1997).

2.9.5 Effects of Training on SR Ca^{2+} release

Human Studies. Few studies have investigated training effects on SR Ca^{2+} release. Training studies may show some relationship to muscle fibre types. Five weeks intermittent sprint training enhanced SR Ca^{2+} release in human vastus lateralis muscle (Ortenblad *et al.*, 2000a). Ortenblad *et al.* (2000a) found enhanced total volume of SR and although the relative density of functional RyR channels did not increase, there was a 48% increase in the total number of RyR channels. By contrast, endurance training resulted in a 26% lower Ca^{2+} release in vastus lateralis muscle in untrained subjects (Green *et al.*, 2003). The increase in SR Ca^{2+} release with sprint and corresponding decrease with endurance training may have been

reflective of changes in fibres types with training (Baumann *et al.*, 1987) as fast-twitch muscle fibre produces greater Ca^{2+} release and Ca^{2+} uptake than that of slow-twitch fibre (Li *et al.*, 2002).

2.9.6 Effects of Training on SR Ca^{2+} uptake

Animal studies. Early studies in animal preparations generally found a reduction in SR Ca^{2+} uptake or Ca^{2+} ATPase activity, consistent with a shift in muscle fibre phenotype from fast towards slow-oxidative fibres (see review by McKenna *et al.*, 1996). More recently, a 14% increase in SR Ca^{2+} uptake rate from 0.18 $u$mol.min^{-1} mg.protein^{-1} at rest was found in horse middle gluteal muscle after 12 wks of sprint conditioning with horses (Wilson *et al.*, 1998).

Human studies. Endurance training induced an 18% lower SR Ca^{2+} uptake (Green *et al.*, 2003). This was explained by reduction in Ca^{2+}-ATPase activity (Ortenblad *et al.*, 2000a) and SERCA1 protein levels (Green *et al.*, 2003). Interestingly Ortenblad *et al.* (2000a) found no change in Ca^{2+} uptake rate despite an increase in density of SERCA 1 and 2 by 41 and 55%, respectively. Similarly Green *et al.* (1998) did not find a change in Ca^{2+}-ATPase activity with high resistance training despite an increase in muscle hypertrophy.

Effect of fibre type changes with training on SR function. These changes in both SR Ca^{2+} release and uptake rate with training may reflect the type of muscle used. Ortenblad *et al.* (2000a) and Green *et al. (1998)* both used mixed vastus lateralis muscle, which may have obscured any fibre specific change in SR function. No studies though have studied the effects of training on SR function at fatigue.

2.10 Role Of Muscle Metabolism On SR Ca^{2+} Regulation In Fatigue

2.10.1 Local depletion of ATP

Changes to bulk muscle ATP may not necessarily reflect changes in ATP content in the intracellular microenvironments (Korge & Campbell, 1995). Local SR ion channels have their own local supply of ATP, generated mostly through creatine kinase or glycolytic enzyme reactions (Korge & Campbell, 1995; Westerblad *et al.*, 1998; Duke & Steele, 1999). ATP is bound with two Mg^{+} ions to form Mg_2ATP at the SR Ca^{2+} release channel. The glycolytic and creatine kinase enzymes are localized in the vicinity of the myofibrils and the SR-ATPase reactions (Korge & Campbell, 1995; Westerblad *et al.*, 1998; Duke & Steele, 1999). A decline in glycogen stores or PCr could therefore potentially affect local supply of ATP (Stephenson *et al.*, 1998; Duke & Steele, 1999).

A decline in local ATP causes a depression in SR Ca^{2+} release (Chin & Allen, 1997; Stephenson *et al.*, 1999; Laver *et al.*, 2001). Adenosine compounds exert a deleterious effect on function of the SR Ca^{2+} release channels in rabbit skeletal muscle (Laver *et al.*, 2001). Increased ADP, AMP and IMP concentrations reduced SR Ca^{2+} release rate, as these compounds competed with ATP for its binding sites on the RyR (Laver *et al.*, 2001). Mg^{2+} is a potent inhibitor of the Ca^{2+} release channel. Activation of the voltage sensor via action potential propagation opens the Ca^{2+} release channels, thus releasing Ca^{2+} (Owen *et al.*, 1996; Blazev & Lamb, 1999). This activity lowers local ATP content, thereby releasing free Mg^{+} creating a dual effect in lowering the Ca^{2+} release rate (Blazev & Lamb, 1999).

2.10.2 Glycogen depletion

Muscle glycogen content has been associated with changes in $[Ca^{2+}]_i$ in single mouse flexor brevis fibres (Chin & Allen, 1997). A reduction in muscle

glycogen in single fibres to 27% of initial values coincided with $[Ca^{2+}]_i$ falling to 47% of initial values and a rapid force decline. Repletion of glycogen content allowed both $[Ca^{2+}]_i$ and force production to be re-established, whilst impairing glycogen recovery via the absence of glucose also prevented the recovery of force and $[Ca^{2+}]_i$ (Chin & Allen, 1997). Fatigue developed more rapidly in the absence of glucose, and was slower in the presence of a high glucose solution (Helander *et al.*, 2002). These findings were linked to changes in SR Ca^{2+} release. The higher glycogen content and fatigue resistance were associated with a slower decline in the tetanic $[Ca^{2+}]_i$ (Helander *et al.*, 2002). Thus, decreases in SR Ca^{2+} release contribute to the loss of force during fatigue (Westerblad & Allen, 1991), and higher glycogen content could delay fatigue by maintaining adequate SR Ca^{2+} release. This finding strongly suggests that the pronounced muscle glycogen depletion found at exhaustion during prolonged exercise (Coyle *et al.*, 1986; Coggan & Coyle, 1987; Booth *et al.*, 1997) may also contribute to an accelerated fall in SR Ca^{2+} release, $[Ca^{2+}]_i$, and thus, force production.

Under conditions where high and constant concentrations of ATP (8 mM) and creatine phosphate (10 mM) were maintained, the capacity of the cane toad skinned muscle fibre to respond to T-system depolarization was strongly correlated with the glycogen pool in the fibre (Stephenson *et al.*, 1999). Moreover, successive force responses induced by T-system depolarizations were shown to be the major factor responsible for the depletion of the glycogen pool in the skinned fibre preparation. In turn, the depolarization-induced depletion of fibre glycogen markedly diminished the capacity of the skinned fibre preparation to respond to T-system depolarization. These results suggest that glycogen exerts a protective effect on SR Ca^{2+} release, which is not based on the role of glycogen as an energy store.

2.10.3 Effect of PCr on SR regulation

2.10.3.1 Effect of creatine kinase on SR function

Depressed PCr appears to affect local ATP supply for SR regulation. When creatine kinase was inhibited by 2,4-dinitro-1-fluorbenzene (DNFB), a creatine kinase inhibitor, the descending phase of the Ca^{2+} transient in skinned rat EDL muscle was prolonged (Duke & Steele, 1999). Prolongation of the descending phase of Ca^{2+} transients is consistent with a reduced rate of SR Ca^{2+} uptake (Duke & Steele, 1999). Thus, this was prolonged due to a reduction in ATP resynthesis via PCr system

Actual PCr withdrawal also produced a marked decrease in the amplitude of Ca^{2+} transients, indicating a decline in Ca^{2+} release. The descending phase of the Ca^{2+} transient was substantially prolonged when PCr was halved (Duke & Steele, 1999). This prolongation became more pronounced at PCr below 5mM. These data suggest that Ca^{2+} regulation is strongly dependent on the supply of ATP via CK and PCr and indicates a probable reliance of both the Ca^{2+}-ATPase and Ca^{2+} release channels on local ATP content.

It has been suggested that during the early stages of exercise, PCr and anaerobic glycolysis would meet local ATP consumption (Westerblad *et al.*, 1998). At fatigue when PCr becomes depleted, glycolysis becomes the only form of ATP production in the microenvironment and cannot match ATP consumption requirements, as judged by a sudden increase in $[Mg^{2+}]_i$. This will lead to an increase in the ADP/ATP ratio in the microenvironment, which would in turn lead to reduced Ca^{2+} release (Westerblad *et al.*, 1998)

2.10.3.2 Ca^{2+}-P_i precipitation

A fall in PCr also increases inorganic [P_i], adversely affecting SR Ca^{2+} regulation via Ca^{2+}-P_i precipitation within the SR, thereby depressing SR Ca^{2+} release (Fryer *et al.*, 1995; Duke & Steele, 1999, 2000, 2001). P_i enters the SR via a transporter inhibited by ATP (Posterino & Fryer, 1998). Thus, the opening probability of these channels increases at low ATP. Therefore, Ca^{2+}-P_i is more likely to occur late in exercise despite P_i increasing from the onset of exercise.

When P_i content in the SR lumen was high enough to exceed the solubility, $CaHPO_4$ forms preventing [Ca^{2+}] in the SR lumen from increasing beyond a certain value, thus reducing the amount of releasable Ca^{2+} (Fryer *et al.*, 1995). Ca^{2+}-P_i precipitation is important in stimulating Ca^{2+} uptake (Fryer *et al.*, 1995). Precipitate formation prevents the rapid increase in the [Ca^{2+}] in the SR lumen that would normally inhibit further SR Ca^{2+} loading, thus prolonging steady-state SR Ca^{2+} uptake. The large declines found in PCr after both intense and prolonged exhaustive exercise (Cheetham *et al.*, 1986; Booth *et al.*, 1997; Sahlin *et al.*, 1997) suggests that SR Ca^{2+} release and thus performance may be compromised by Ca^{2+}-P_i precipitation.

2.10.3.3 Effects of Mg^{2+} on Ca^{2+}-P_i

The effects of Ca^{2+}-P_i precipitation were dependent on both the content of P_i and free [Mg^{2+}], each of which are bi-products of the decline of Mg_2ATP (Duke & Steele, 2001). A decline in local ATP would also have the effect of increasing free [Mg^{2+}], which inhibits SR Ca^{2+} release. At the physiological [Mg^{2+}] of about 1 mM at rest, greater than 5mM P_i is required before the SR is affected by Ca^{2+}-P_i precipitation. Increases in free [Mg^{2+}] produce additive inhibitory effects. A [Mg^{2+}] of 3mM inhibited Ca^{2+} release rate even at 2 mM P_i (Duke and Steele, 2001).

2.10.4 Effect of ADP on SR

Elevated P_i content at the site of the Ca^{2+}-ATPase reduces Ca^{2+}ATPase-mediated transport of Ca^{2+} into the SR lumen, thus reducing the availability of Ca^{2+} in the SR for release (Duke & Steele, 2001). This was due to increased ADP content (Duke & Steele, 2001). Increased ADP content causes a reversal of Ca^{2+}-ATPase function, thereby creating a Ca^{2+} leak or efflux back into the cytosol; hence reducing Ca^{2+} re-uptake into the lumen (Duke & Steele, 2000). This also has the effect of increasing resting $[Ca^{2+}]_i$. The decline in SR $[Ca^{2+}]$ would also reduce the rate of SR Ca^{2+} release.

2.11 Effects of Free Radicals on SR Ca^{2+} Regulation

2.11.1 Effects of free radicals on SR Ca^{2+} Regulation

Skeletal muscles generate reactive oxygen species (ROS), primarily as a by-product of aerobic metabolism (Chance *et al.*, 1979; O'Neill *et al.*, 1996; Kolbeck *et al.*, 1997; Duval *et al.*, 2002). Increased free radicals may impact adversely on the regulation of muscle ions, force production and thus performance (Favero *et al.*, 1995; Brotto & Nosek, 1996; Andrade *et al.*, 1998; Favero *et al.*, 1998). The presence of anti-oxidants within skeletal muscle cells tightly regulates cellular redox balance. Despite protective mechanisms against oxidation, oxidants also react with membranes and proteins within skeletal muscles when produced in excessive amounts, inducing damage or structural alteration, which has the potential to disrupt or modulate normal function (Abramson & Salama, 1989).

Free radicals have been have been suggested as one possible link to impaired SR regulation in fatigue, due to their effect on EC coupling, Ca^{2+} release and force production (Favero, 1999). One such example of oxidant-induced alteration of function is the increased activity of the sarcoplasmic reticulum SR

Ca^{2+}-release channel and decreased activity of the SR Ca^{2+}-ATPase, in isolated membrane vesicle preparations following equilibration with hydrogen peroxide (H_2O_2) (Favero *et al.*, 1995; Oba *et al.*, 1996; Oba *et al.*, 1998). In mechanically and chemically skinned fibres from the rat EDL muscle hydrogen peroxide reduces free SH groups to disulfides, impaired normal EC coupling and thus Ca^{2+} release during oxidative stress (Brotto & Nosek, 1996). Redox states of critical sulfhydryls, located on the cytoplasmic side of the RyR, may alter both gating properties of the channel and responsiveness to channel modulators (Oba *et al.*, 2002). Initially Andrade *et al.* (1998) found brief exposure to non-physiologically high 100-300 μM H_2O_2 enabled an increase in force production, without any change in myoplasmic Ca^{2+}. This was due to a Ca^{2+} leak and decreased Ca^{2+} uptake rate which thereby increased tetanic $[Ca^{2+}]_i$. However, prolonged exposure to H_2O_2 reduced force generation without change in myoplasmic $[Ca^{2+}]$. This force decline was mainly due to reduced myofibrillar Ca^{2+} sensitivity. The elements of the contractile machinery are differentially responsive to changes in the oxidation-reduction balance of the muscle fibres. Myofibrillar Ca^{2+} sensitivity appears to be especially susceptible, while the SR functions (Ca^{2+} leak and uptake) are less so (Andrade *et al.*, 1998).

It was later found that both force and $[Ca^{2+}]_i$ were changed by H_2O_2 concentrations within the physiological range of 10^{-9}–10^{-7} M, where myofibrillar function was modified independent of myoplasmic Ca^{2+} (Andrade *et al.*, 2001). Concentrations of H_2O_2 as low as 10 μM increased resting $[Ca^{2+}]$ and slowed Ca^{2+} uptake; exposure to H_2O_2 and *t*-BOOH at 10 μM for 30 min increased mean resting $[Ca^{2+}]_i$ by 78% and 74%, respectively. Moreover, 10 μM of either peroxide slowed the decline of $[Ca^{2+}]_i$ to resting levels after stimulation, with the rate of SR Ca^{2+} re-

uptake reduced by more than 70%. Micromolar levels of hypochlorous acid (HOCL) altered the RyR in a biphasic manner, similar to that of H_2O_2 (Favero *et al.*, 2003). The Ca^{2+} release channel was activated by 20 μM HOCL but inhibited at 40 μM. They suggest that thiols critical for normal channel function were targets of HOCL-induced oxidation. Favero *et al.* (1998) also found that HOCL inhibited Ca^{2+} uptake. Ca^{2+} uptake was dramatically reduced to 10% of the control value of 95 $nmol.mg^{-1}$ at rest with 200 μM HOCL indicating that the Ca^{2+}-ATPase protein was also inactivated by oxidation of the thiols group. These studies suggest that SR Ca^{2+} release, SR Ca^{2+} uptake, myofibrillar Ca^{2+} sensitivity and cross-bridge kinetics are all influenced by oxidant concentrations that approach those found physiologically. These findings suggest an important role for endogenous oxidants in the regulation of skeletal muscle function.

2.11.2 Effects of Temperature on SR Ca^{2+} Regulation

Elevated temperature has been implicated as a major reason for the decline in Ca^{2+} regulation. Exposure of the muscles to a raised temperature of 40°C reduced the rate of SR Ca^{2+} uptake in both the EDL and soleus muscle homogenates (Warmington *et al.*, 1996). Elevated free radical production may have been in part due to temperature effects during exercise. At 43.5°C, Ca^{2+} was uncoupled from force production in rat skinned EDL muscle fibres due to elevated hydrogen peroxide bi-product, superoxide ($0_2^{\cdot-}$) concentration (van der Poel & Stephenson, 2002). Beyond 43.5°C, Ca^{2+} activated force could not be produced (van der Poel & Stephenson, 2002). In whole gastrocnemius muscle homogenates, treatment with DTT, an SH-reducing agent, demonstrated that the decline and uncoupling of Ca^{2+} uptake and Ca^{2+}-ATPase that occurred at elevated temperatures was due to SH oxidation (Schertzer *et al.*, 2002). SH oxidation was elevated at higher temperatures

and was not found at 37°C (Schertzer *et al.*, 2002). Therefore, it is possible that increased free radical production during exercise may in part be due to elevated temperature. This may be especially so during prolonged exercise given the prolonged elevation of muscle temperature (Parkin *et al.*, 1999; Febbraio, 2001).

2.12 Role of Ca^{2+} and of Ca^{2+}-activated proteases

Some aspect of the period of intense activity leads to a prolonged reduction in Ca^{2+} release. Chin and Allen (1997) found that despite a post-contraction recovery of metabolites and muscle glycogen content, muscle fibre tetanic force and $[Ca^{2+}]_i$ remained depressed, indicating an independent effect. While SR function was affected by metabolic causes (Byrd *et al.*, 1989a; Chin & Allen, 1997; Duke & Steele, 1999, 2000, 2001; Laver *et al.*, 2001), a decline in muscle force still existed hours or even days after exercise, despite a recover of metabolites (Edwards *et al.*, 1977; Westerblad *et al.*, 1993; Booth *et al.*, 1997; Chin & Allen, 1997; Ortenblad *et al.*, 2000b; Hill *et al.*, 2001). The slow rate of recovery after exercise therefore suggests that non-metabolic causes are involved as well.

Chin *et al.* (1997) found that an increased $[Ca^{2+}]_i$ was responsible for the decline in force in single mouse fibres that exhibited low frequency fatigue. They depressed force by only 30% to minimise metabolite alterations. Of the changes in the peak $[Ca^{2+}]_i$; resting $[Ca^{2+}]_i$; and the $[Ca^{2+}]_i$-time integral, the $[Ca^{2+}]_i$-time integral had the greatest effect in reflecting changes in force. Application of the protease inhibitor calpeptin did not prevent the failure of the Ca^{2+} release channel suggesting that low frequency fatigue was not due to the effect of protease.

Others have suggested that activation of Ca^{2+} neutral-calpain activated proteases caused by elevated resting $[Ca^{2+}]_i$ degrades muscle and membrane tissue, and possibly affects either an unknown point between the DHPR and the Ca^{2+}

release channel or the SR itself (Belcastro, 1993). It is possible that prolonged exposure to elevated resting $[Ca^{2+}]_i$ during prolonged exercise, may initiate a Ca^{2+} activated protease effect, causing SR protein degradation, thereby affecting force production and fatigue. Given the importance of duration of elevated $[Ca^{2+}]$ to low frequency fatigue, and the expected large $[Ca^{2+}]_i$-time integral during prolonged exercise, impaired Ca^{2+} release may be an important contributor to muscle fatigue during prolonged exercise.

2.13 Relationship of force production to performance

2.13.1 Relationship of altered muscle cytosolic Ca^{2+} to force decline

Considerable evidence suggests that the decline in force production with fatigue is largely due to failure of the SR Ca^{2+} release mechanism (Westerblad & Allen, 1991; Westerblad *et al.*, 1993; Allen *et al.*, 1995). Single mouse muscle fibres stimulated at high frequency (100 Hz) reduced maximal force by 70%, coincident with a decline in $[Ca^{2+}]_i$ by 53% from resting values (Chin & Allen, 1997). The depression of muscle force was significantly correlated with the depression in Ca^{2+} release rate during recovery after tetanic stimulation at 60 Hz (Ortenblad *et al.*, 2000b). The depression in SR Ca^{2+} release was also significantly related to the rate of muscle force development after human voluntary knee extensor exercise (Hill *et al.*, 2001). Ward *et al.* (1998) found that when muscle force was reduced to the same level, depression in SR Ca^{2+} release and uptake were similar and independent of the exercise protocol. Ward *et al.* (1998) also found that when force was reduced by only 9% there was no change in SR Ca^{2+} release, despite a muscle stimulation protocol as high as 100 Hz. Thus a lack of decline in SR Ca^{2+} release was consistent with minimal decline in muscle force. Therefore any

depression found in SR regulation during prolonged exercise would be expected to adversely impact on muscle force production and performance.

2.13.2 Decline in Voluntary Force Production During Prolonged Exercise

Repeated muscle contractions are usually accompanied by a progressive loss of muscle force (Vollestad & Sejersted, 1988). Several studies reported that prolonged exercise impairs muscle force production. One-legged maximal voluntary contractions (MVC) for 30 min produced a 40% reduction in maximal force generating capacity (Vollestad *et al.*, 1988). Sahlin and Seger (1995) measured the time course of the decline in muscle force throughout prolonged cycling exercise to fatigue at 75% $\dot{V}O_2$ peak (mean duration 85 minutes). They found that the isometric MVC had decreased by 9 % from resting values after 5 minutes, by 18% after 40 minutes and by 34% at fatigue, with force recovering to only 80% of pre-exercise values after 30 minutes. Maximal voluntary concentric muscle force fell by 26% at fatigue compared to a 20% decline in maximal voluntary eccentric muscle force (Sahlin & Seger, 1995). After prolonged cycling to fatigue at 70% $\dot{V}O_2$ peak, peak quadriceps force elicited during both a maximal twitch and 10 Hz contraction were depressed by 65% and 45% from 96 and 166 N at rest respectively (Booth *et al.*, 1997). In recovery, both twitch and 10 Hz force remained depressed at 10 and 20 min after the cessation of exercise, whilst at 60 min after the cessation of exercise, 10 Hz force had still only recovered to 84% (Booth *et al.*, 1997). In one subject, muscle force elicited at 10 Hz was still depressed by 24% at 6 h post-exercise (Booth *et al.*, 1997).

Two hours of prolonged running reduced each of concentric (11-14%) and eccentric (18-21%) knee extensor force in the quadriceps muscle (Lepers *et al.*, 2000). The height of a counter movement jump also declined by 10% after exercise

(Lepers *et al.*, 2000). Five hours of cycling at 55% $\dot{V}O_2$ peak depressed quadriceps MVC by 18% from 319 N.m at rest at the end of exercise (Lepers *et al.*, 2002). Peak twitch torque, contraction time, and total area of mechanical response were decreased after the first hour of exercise (Lepers *et al.*, 2002). Thus prolonged exercise depresses muscle force generating capacity, suggesting a possible impairment in muscle ion regulatory processes.

2.14 General Aims

Few studies have investigated SR function during prolonged exercise, and no studies in humans have measured each of (1) Na^+, K^+-ATPase activity, (2) SR Ca^{2+} release and Ca^{2+} uptake, and (3) the time course of any of changes in these measurements during prolonged exercise to fatigue. This thesis therefore investigates the effects of prolonged exercise on the magnitude and time-course of the decline in muscle ion regulatory protein function to determine their importance to muscle fatigue.

2.15 Hypotheses

This thesis tested the hypotheses that: (1) prolonged exercise in humans would depress each of the maximal Na^+, K^+-ATPase activity, SR Ca^{2+} release and SR Ca^{2+} uptake rates in skeletal muscle; (2) that these changes would develop progressively with exercise duration; and (3) that the magnitude of these changes at fatigue would be correlated with endurance exercise performance.

Chapter 3. Methods

3.1 Subjects

A total of eleven healthy volunteers participated in the study. Eight subjects, seven male and one female (age 27±9 yr, height 177.9 ± 9.0 cm, body mass 74.1 ± 9.8 kg, mean±SD), participated in the prolonged exercise trials. A further three healthy males (age 37.3±7.4 yr, height 179±7.5 cm, body mass 75.7±9.5 kg, mean±SD), participated in separate trials to determine intra-subject variability, and the effects of a protease inhibitor, on muscle sarcoplasmic reticulum variables. All subjects gave written informed consent, and all procedures were approved by the Victoria University of Technology Human Research Ethics Committee.

3.2 Exercise Tests

Subjects underwent three exercise test sessions, 3-7 d apart, at least 2 h after a light meal. They abstained from strenuous physical activity in the previous 24 h, and avoided caffeine, nicotine, or any medications in the previous 12 h. All tests were conducted on an electronically braked cycle ergometer at approximately 80 rev. min^{-1} (Lode N.V. Groningen, Netherlands). During all tests expired gases were collected to determine oxygen consumption ($\dot{V}O_2$).

Incremental exercise trial. The initial trial comprised a maximal incremental test (Li *et al.*, 2002). Subjects breathed through a Hans-Rudolph 3-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 O_2 and CO_2 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 and

rechecked after each test, using commercially prepared gas mixtures. The ventilometer was calibrated prior to each test using a standard 3 l syringe. Subjects cycled for 3 min at each of 60, 90 and 120 W, followed by a 25 W increment each min until fatigue, defined as an inability to maintain pedal cadence above 55 $rev.min^{-1}$. The highest $\dot{V}O_2$ over a 30 s interval was termed $\dot{V}O_{2\ peak}$. The workrate corresponding to 75% $\dot{V}O_{2peak}$ was then calculated from the linear regression of submaximal $\dot{V}O_2$ versus power output, for use in all subsequent exercise sessions.

Prolonged exercise fatigue trial. In the second visit subjects performed a familiarisation trial, comprising submaximal cycling exercise at a workrate corresponding to 75% $\dot{V}O_{2peak}$, continued to the point of fatigue, defined as the inability to maintain cadence above 55 $rev.min^{-1}$. In the final laboratory visit the subjects performed an identical prolonged cycling exercise test, with inclusion of muscle biopsy and blood sampling procedures. Pulmonary $\dot{V}O_2$, carbon dioxide output and respiratory exchange ratio (RER) were measured during the periods 4-9, 25-30 and 39-44 min and then continuously from 50 min until fatigue. A muscle biopsy was taken at rest, at 10 and 45 min of exercise and at the point of fatigue. Arterialised venous blood samples were taken at rest, at 9, 30 and 44 min of exercise, and at fatigue.

Muscle torque measurements. To verify earlier observations of a decline in quadriceps maximal muscle torque with fatigue (Booth *et al.*, 1997), two subjects returned for an additional prolonged exercise trial, with measurement of the quadriceps muscle maximal isometric torque before, during and after exercise. Subjects were familiarised with isometric knee extensor exercise, performed on a Cybex dynamometer (Cybex Norm 770, Henley Health Care, Sugar Land, Texas,

USA), using standard procedures (Li *et al.*, 2002). Subjects warmed up for 10 min at 50% $\dot{V}O_2$ peak and then performed three maximal voluntary isometric contractions (MVC). Subjects then commenced the prolonged exercise trial with three further MVC conducted at the same time points as muscle biopsies were taken in the previous invasive trial.

Repeat biopsy and intense exercise trial. To determine the intra-subject and inter-assay variability of SR Ca^{2+} uptake and Ca^{2+} release, three healthy males underwent two vastus lateralis muscle biopsies at rest. A third muscle biopsy was taken immediately following 50 maximal isokinetic knee extensor contractions performed at a cadence of $180°s^{-1}$ and at 0.5 Hz, to test the effects of addition of the Ca^{2+}-activated protease inhibitor leupeptin to the SR homogenising buffers (detailed below). This exercise protocol was used since this depressed muscle SR Ca^{2+} uptake, Ag^{+}-induced SR Ca^{2+} release and Na^{+},K^{+}ATPase activity (Fraser *et al.*, 2002; Li *et al.*, 2002). Each biopsy sample was immediately separated into two portions, with one piece homogenised in the standard homogenising buffer and a separate portion in the standard buffer plus 1 mM leupeptin (Sigma Aldrich).

3.3 Blood Sampling and Analyses.

Prior to commencing the prolonged exercise trial, an indwelling catheter (Jelco 20-22G) was inserted into a dorsal hand vein and kept patent by periodic infusions of isotonic heparinised saline. To obtain arterialised blood samples, the hand was sheathed by a waterproof glove and heated in a 45° C water bath for 10 min prior to all sampling. Arterialised venous blood samples (2.5 ml) were drawn into heparinised syringes (Rapidlyte, Ciba Corning, Diagnostic Corporation, Medfield, USA), well mixed and then divided into two separate tubes for measurement of plasma potassium concentration ($[K^{+}]$), and whole blood

haematocrit (Hct) and haemoglobin concentration ([Hb]). For plasma [K^+] determinations (n=6), an aliquot of whole blood was centrifuged at 4000 rpm for 4 min, plasma was then separated, stored in liquid N_2 and later analysed in triplicate using a K^+-selective electrode, housed in an automated blood gas electrolyte analyser (Ciba Corning 865, Bayer, Medfield, USA). Blood Hct and [Hb] (n=4) were measured in triplicate using an automated haematology analyser (Sysmex K800, Roche Diagnostics, Australia). The decline in plasma volume from rest (ΔPV, n=4) was calculated from changes in Hct and [Hb], as previously described (McKenna *et al.*, 1993; Fraser *et al.*, 2002).

3.4 Muscle Biopsy Sampling, Processing and Analyses

Upon arrival at the laboratory subjects rested on a laboratory bed and four small incisions (2 per leg) were made under local anaesthesia (1% Xylocaine) in the skin overlying the middle third of the vastus lateralis. A muscle biopsy was taken by the percutaneous biopsy technique modified for suction, at rest, 10 and 45 min of exercise and at fatigue. Approximately 100-120 mg of muscle tissue was removed, rapidly divided into portions with one immediately frozen and stored in liquid N_2 for later analysis of Na^+,K^+-ATPase content. The remaining two portions were blotted on filter paper, rapidly weighed, immediately homogenised in the respective buffer and then stored in liquid N_2 for later analysis of Na^+,K^+-ATPase activity and for SR Ca^{2+} release and Ca^{2+} uptake rates.

3.5 Na^+,K^+ATPase activity

Muscle homogenates for Na^+,K^+ATPase activity measurements were prepared as previously described (Fraser & McKenna, 1998; Fraser *et al.*, 2002). Muscle samples (30 mg) were immediately blotted on filter paper, weighed, then homogenized (5% w/v) at 0°C for 2 x 20s, 15000 rpm (Omni 1000, Omni

International, Warrenton, USA) in an homogenate buffer containing 250 mM sucrose, 2 mM EDTA, and 10 mM Tris (pH 7.40). Muscle homogenates were rapidly frozen and stored in liquid nitrogen for later determination of activity. Before analysis, homogenates were freeze-thawed four times and then diluted 1/5 in cold homogenate buffer. Muscle Na^+,K^+ATPase activity was determined in quadruplicate using the K^+-stimulated 3-*O*-methyl fluorescein phosphatase (3-*O*-MFPase) activity assay (Fraser & McKenna, 1998; Fraser *et al.*, 2002). The assay medium in which 3-*O*-MFPase activity was measured contained 5 mM $MgCl_2$, 1.25 mM EDTA, 100 mM Tris, and an 80 nM 3-*O*-methyl fluorescein standard (pH 7.40). The freeze-thawed, diluted homogenate (30 µl) was incubated in 2.5 ml of assay medium at 37°C for 5 min before addition of 40 µl of 10 mM 3-*O*-MFP to initiate the reaction. After 60 s, 10 µl of 2.58 M KCl (final concentration 10 mM) was added to stimulate K^+-dependent phosphatase activity and the reaction was measured for a further 60 s. All assays were performed at 37°C, using continuous stirring, with data sampled at 1 Hz, on a spectrofluorimeter (Aminco Bowman AB2 SLM, Thermospectronic, Madison, WI, USA). Excitation wavelength was 475 nm and emission wavelength 515 nm, with 4 nm slit widths. The 3-*O*-MFPase activity was calculated from the slope after addition of 10 µM KCl minus the slope prior to KCL addition (the latter comprises unspecific-ATPase activity and any spontaneous hydrolysis of 3-*O*-MFP). All slopes were measured over 20-50 s. A least squares linear regression was also calculated for each slope, and any results with $r<0.97$ were excluded from the analyses.

3.6 Na^+,K^+ pump content

Muscle Na^+,K^+ pump content was determined by the vanadate-facilitated [^{3}H]-ouabain binding content (McKenna *et al.*, 2003b). Vanadate facilitates the

binding of [^{3}H]-ouabain and allows quantification of the Na^+,K^+ pump content in biopsies of skeletal muscle (Norgaard *et al.*, 1983). Samples were cut into 2-4 mg pieces. In all experiments freshly made vanadate solution was used. The binding of [^{3}H]-ouabain was determined by incubating cut muscle specimens for 120 min at 37°C in a buffer containing 10^{-6} M [^{3}H]-ouabain (0.6 μCi ml^{-1}), tris chloride (10 mM), $MgSO_4$ (3 mM), tris vanadate (1 mM) and sucrose (250 mM), pH 7.3. Following washout for 4 x 30 min at 0 °C the tissue samples were soaked in 0.3 M trichloroacetic acid (TCA) and taken for counting of ^{3}H-activity using a beta counter. The content of ^{3}H-ouabain binding sites was determined and expressed as pmol (g wet wt.)$^{-1}$ (Clausen & Hansen, 1977).

3.7 SR Homogenisation, Ca^{2+}Release and Ca^{2+}uptake measurements

Assay Procedures and Modifications. Muscle homogenates for SR Ca^{2+} release and Ca^{2+} uptake measurements were prepared as earlier described (Li *et al.*, 2002). Approximately 30 mg of muscle was weighed, diluted 1:11 (wt/vol) in a cold buffer containing Tris-HCl (40 mM, pH 7.9), sucrose (0.3 M), L-histidine (10 μM), EDTA (10 mM), sodium azide (10 mM), and then homogenized on ice at 15,000 rpm for 3x15 s (Omni 1000, Omni International, Warrenton, USA). The homogenate was then rapidly frozen in liquid nitrogen for later analyses of SR Ca^{2+} release and Ca^{2+} uptake. All assays were conducted in triplicate, using magnetic stirring and at 37 °C. The Ca^{2+} release and Ca^{2+} uptake rates were measured in triplicate 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). The assay medium utilized several important procedural and analytical improvements, compared to previous

methods (Ruell *et al.*, 1995; Booth *et al.*, 1997; Li *et al.*, 2002; McKenna *et al.*, 2003a). The reaction was initiated by addition of 40 μL homogenate (Fig 1). After the SR Ca^{2+}-ATPase mediated reduction in the F_{410}/F_{485} ratio had reached a plateau, the Ca^{2+}ATPase specific inhibitor cyclopiazonic acid (CPA) was added to the cuvette at a final concentration of 20 μM, to inhibit further vesicular Ca^{2+} uptake (Ruell *et al.*, 1995). The addition of CPA produced a small rise in [Ca^{2+}] (Fig 1), consistent with the initial Ca^{2+} leak via the inhibited Ca^{2+}ATPase (Tupling & Green, 2002). Initiation of SR Ca^{2+} release was then induced by the addition of the specific pharmacologic activator of the SR Ca^{2+} release channels, 4-chloro-*m*-cresol (4-CmC, (Herrmann-Frank *et al.*, 1996)) at a final concentration of 5 mM (Fig 1) (Ortenblad *et al.*, 2000b; Tupling & Green, 2002). This was followed by determination of Rmin and Rmax with sequential addition of 3.5mM EGTA and 5mM $CaCl_2$, respectively (Fig 1). The sample was excited by a Xenon lamp at 349 nm with a bandpass of 1 nm; emission was measured at 410 nm for Ca^{2+}-bound and at 485 nm for Ca^{2+}-free forms of Indo, with 8 nm bandpasses, using a spectrofluorimeter with an additional external photomultiplier tube (Aminco Bowman AB2, Thermospectronic, Madison, WI, USA). All 410/485 nm fluorescent ratiometric data were sampled at 10 Hz, which was the limit of the Aminco Bowman data acquisition software. The maximal rates of Ca^{2+} uptake and Ca^{2+} release were determined from individual curves applied to smoothed data portions, using custom-made software (LabView, Austin, Texas, USA). Curve fits were used to reduce variability resulting from noisy data points, and thus enhance precision of the method.

Determination of SR Ca^{2+} uptake rate. The Ca^{2+} uptake rate was calculated from the entire Ca^{2+} uptake curve data, which typically comprised 200-300 s. This

excluded data artefacts with homogenate addition (Fig. 1), which most likely reflects Ca^{2+} binding by haemoglobin (Ruell *et al.*, 1995), EGTA and myoglobin in the homogenate, and by oxalate in the assay medium. The raw F_{410} and F_{485} data were each initially smoothed using a 15-point running average. A higher order polynomial curve was then fitted to each of the smoothed F_{410} and F_{485} data sets, with the best curve fit determined on the basis of the lowest mean square error. The F_{410}/F_{485} ratio (R) was then derived and $[Ca^{2+}]$ calculated using standard equation: $[Ca^{2+}] = K_d \times [(R-R_{min})/(R_{max}-R)] \times (S_{f2}/S_{b2})$, where S_{f2} and S_{b2} represent the fluorescence at 485 nm for Ca^{2+}-free Indo and for bound-Indo (saturating $[Ca^{2+}]$), respectively (Grynkiewicz *et al.*, 1985) and where K_d for Indo-1 was 167 nm (Li *et al.*, 2002). The maximal Ca^{2+} uptake rate was then measured as the minimum $d[Ca^{2+}].dt^{-1}$, expressed in $nmol.l^{-1}.s^{-1}$ and then corrected to both $mmol.min^{-1}.(g$ muscle wet weight$)^{-1}$ and after determination of homogenate protein content, to $mmol.min^{-1}.(g$ protein$)^{-1}$ (Li *et al.*, 2002). Free $[Ca^{2+}]$ was calculated from ratiometric data throughout the Ca^{2+} uptake and release curves, and is reported prior to the addition of the homogenate (pre-homogenate), immediately after the homogenate artefact (post-homogenate), at the $[Ca^{2+}]$ plateau prior to addition of CPA (end-uptake), during the plateau immediately prior to 4-CmC addition (pre-release) and at 10 s after 4-CmC addition (post-release) (Table 6).

Determination of SR Ca^{2+} release rate. The SR Ca^{2+} release rate was determined using data from a duration of 10 s following addition of 4-CmC, to ensure detection of the initial fast phase of SR Ca^{2+} release. Any artefact associated with 4-CmC addition was excluded from the analysis. The F_{410} and F_{485} raw data were first smoothed using a 5-point running average. Smoothing was conducted to minimize

the effects of any aberrant points and was less than the 15-point smoothing for Ca^{2+} uptake analyses due to the fewer number of data points in the Ca^{2+} release phase.

To ascertain the most appropriate analysis period to reflect the fast initial phase of Ca^{2+} release under our measurement conditions, Ca^{2+} release rates were calculated for 14 analyses, using curve fits of data of between 3 - 20 s durations, yielding a total of between 28-198 data points. The best curve fitting duration for Ca^{2+} release analysis was required to meet each of three criteria: (i) yielded a good curve fit, evidenced by visual inspection and by a low mean square error, (ii) detected the fast phase of the Ca^{2+} release rate, and (iii) had a low variability between triplicate measures on the same homogenate, thus reflecting minimal disturbances due to any small number of aberrant or noisy points.

The Ca^{2+} release rates determined from curve fitting over 3 s and 5 s were higher than for 10 s and longer durations ($P<0.01$, Table 4). This does not reflect any physiological differences, since all analyses were conducted on the same 14 data sets. Rather, this difference reflected the bias of the curve fit to the initial steeper component of the Ca^{2+} release curve when analysed over a short time period, since this initial portion of the data represented a higher proportion of the total data incorporated into the curve fit. The curve fit over longer durations, which included more data points, were biased towards the less steep portion of the Ca^{2+} release curve and thus yielded lesser maximal Ca^{2+} release rates (Table 4). Although the mean square error for data fits declined with longer analysis durations beyond 10 s due to the increased number of data points, these analyses failed to meet the criterion of detecting the fast phase of Ca^{2+} release, and were therefore excluded. The Ca^{2+} release data for the 3 s (i.e. only 28 points) and 5 s curve fit analyses were each found to be highly variable with respect to both the magnitude

of Ca^{2+} release, and the time point at which the maximum $d[Ca^{2+}].dt^{-1}$ was detected. During these analyses, even a single aberrant data point during the fast initial phase of Ca^{2+} release measurement induced considerable variability and in some instances delayed the highest measured release rate to a time point up to 1.9 sec after addition of 4-CmC. Thus the highest rate of Ca^{2+} release was not found in the 3 s analysis in 7 out of 14 data sets, and the 3 s analysis under these conditions was therefore excluded. Analysis of Ca^{2+} release using curve fitting over a 10 s duration (i.e. 98 smoothed data points), was minimally influenced by aberrant data points, produced much more consistent results, and the greatest Ca^{2+} release always occurred within the first few smoothed data points following application of 4-CmC. Consequently, a duration of 10 s with 5 s data smoothing was chosen for curve fitting for all Ca^{2+} release analyses. Whilst the 10 s analysis period underestimates the maximal in vitro Ca^{2+} release rate (Table 4), these results were reproducible, allowing testing of our hypotheses. Whilst these *in-vitro* rates are clearly much less than *in-vivo* release rates, this underestimation is not disadvantageous for this study. The maximal Ca^{2+} release rate was measured as the maximum $d[Ca^{2+}].dt^{-1}$, expressed in $nmol.l^{-1}.s^{-1}$ and then corrected to $mmol.min^{-1}$.(g wet weight)$^{-1}$, or $mmol.min^{-1}$.(g $protein^{-1}$).

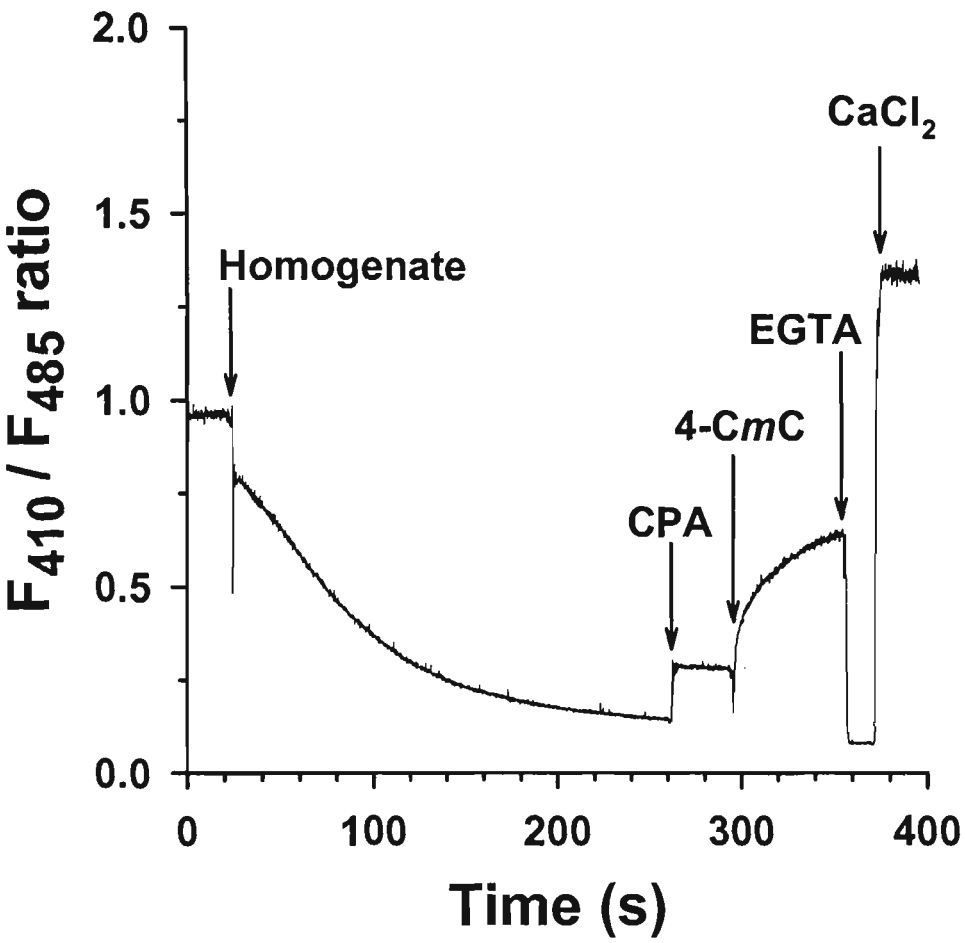

Figure 1. A typical SR Ca^{2+} uptake and Ca^{2+} release curve in human muscle homogenate.

Abbreviations (final concentration): CPA, cyclopiazonic acid (20 μM); 4-CmC, 4 chloro-*m*-cresol (5 mM); EGTA, ethlenebis (oxyethlenenitrib)-tetraacetic acid (3.5 mM), $CaCl_2$, calcium chloride (5 mM).

Table 4. Effect of curve fit durations on SR Ca^{2+} release rates ($d[Ca^{2+}].dt^{-1}$, $nmol.l^{-1}.s^{-1}$).

	Time duration (s)						
	3	5	7.5	10	12.5	15	20
Ca^{2+} release	65.4±5.4	60.1±5.3*	44.8±4.9†	32.1±2.9†	28.1±2.8†	25.6±2.3†	17.9±1.7†

Different from 3 s analysis * $P< 0.05$; † $P< 0.01$

Data expressed as mean±SEM, n=14.

3.8 Statistical analyses

All data are reported as mean±SEM and were analysed using a one-way analysis of variance, with repeated measures for time. Post hoc analyses were determined using the Newman-Kuels test. Correlations between muscle variables and exercise performance were determined by least-square linear regression. Significance was accepted at $P< 0.05$.

Chapter 4. Results

4.1 Exercise Responses

The incremental exercise $\dot{V}O_2$ peak was 3.93 ± 0.69 L.min^{-1} and the mean $\dot{V}O_2$ during prolonged exercise was 2.92 ± 0.49 L.min^{-1} (74.3 ± 1.2 % $\dot{V}O_{2peak}$, Table 5). Time to fatigue was 72.18 ± 6.46 min. The RER declined from 9 min to fatigue (P<0.01, Table 5), plasma [K^+] increased throughout exercise and at fatigue (P<0.001), whilst plasma volume declined with exercise (Table 5).

Table 5. Respiratory variables, plasma potassium concentration and fluid shifts during prolonged cycling exercise at 74% $\dot{V}O_{2peak}$ to fatigue.

	Exercise time (min)				
	Rest	9	30	44	Fatigue
VO_2	-	2.77	2.92	2.96	3.06
(L.min^{-1})		± 0.46	± 0.51	± 0.53	± 0.52
RER	-	1.01	0.98	0.97	0.94
		± 0.01	± 0.01 †	± 0.01 †	± 0.01 ‡
Plasma	4.17	4.95	5.27	5.42	5.43
[K^+]	± 0.05	± 0.18*	± 0.14*	± 0.05*	± 0.16 *
(mmol.l^{-1})					
ΔPV (%)	-	-9.6	-12.4	-12.5	-13.1
		± 2.1 *	± 1.6 *	± 1.7 *	± 1.6 *

Data expressed as mean ±SEM, n=8 for VO_2 and RER, n=6 for [K^+] and n=4 for ΔPV. * Different from rest P< 0.05; Different from 9 min † P< 0.05; ‡ P< 0.01

4.6 Muscle Na^+,K^+ATPase

Maximal in-vitro 3-O-MFPase activity. Muscle 3-*O*-MFPase activity (per g wet weight) was not significantly lower than rest at 10 min ($P<0.10$), but was decreased at 45 min (-9.0 ± 3.9%, $P<0.05$) and at fatigue (-14.0 ± 3.1%, $P< 0.01$, Figure 2A). These changes did not reflect fluid shifts into muscle, with similar reductions in 3-*O*-MFPase activity expressed per g protein, at 10 min (-6.6 ± 2.1%, $P<0.05$), 45 min (-10.7 ± 2.3%, $P<0.01$) and at fatigue (-12.6 ± 1.6 %, $P<0.001$, Figure 2B). Furthermore, 3-*O*-MFPase activity per g protein at 45 min and fatigue were less than at 10 min ($P<0.05$). The inter-assay (CV 2.7%, n=8) and intra-assay (CV 3.7%, n=31) variability for 3-*O*-MFPase were low.

[^{3}H]-ouabain binding site content. Despite reduced 3-*O*-MFPase activity with exercise, no significant differences in the [^{3}H]-ouabain binding site content were found between rest, 10 min, 45 min and fatigue [332.9±19.2, 350.0±12.8, 336.7±22.8, and 316.6±18.9 pmol.(g wet weight)$^{-1}$], respectively, $P=0.21$, NS).

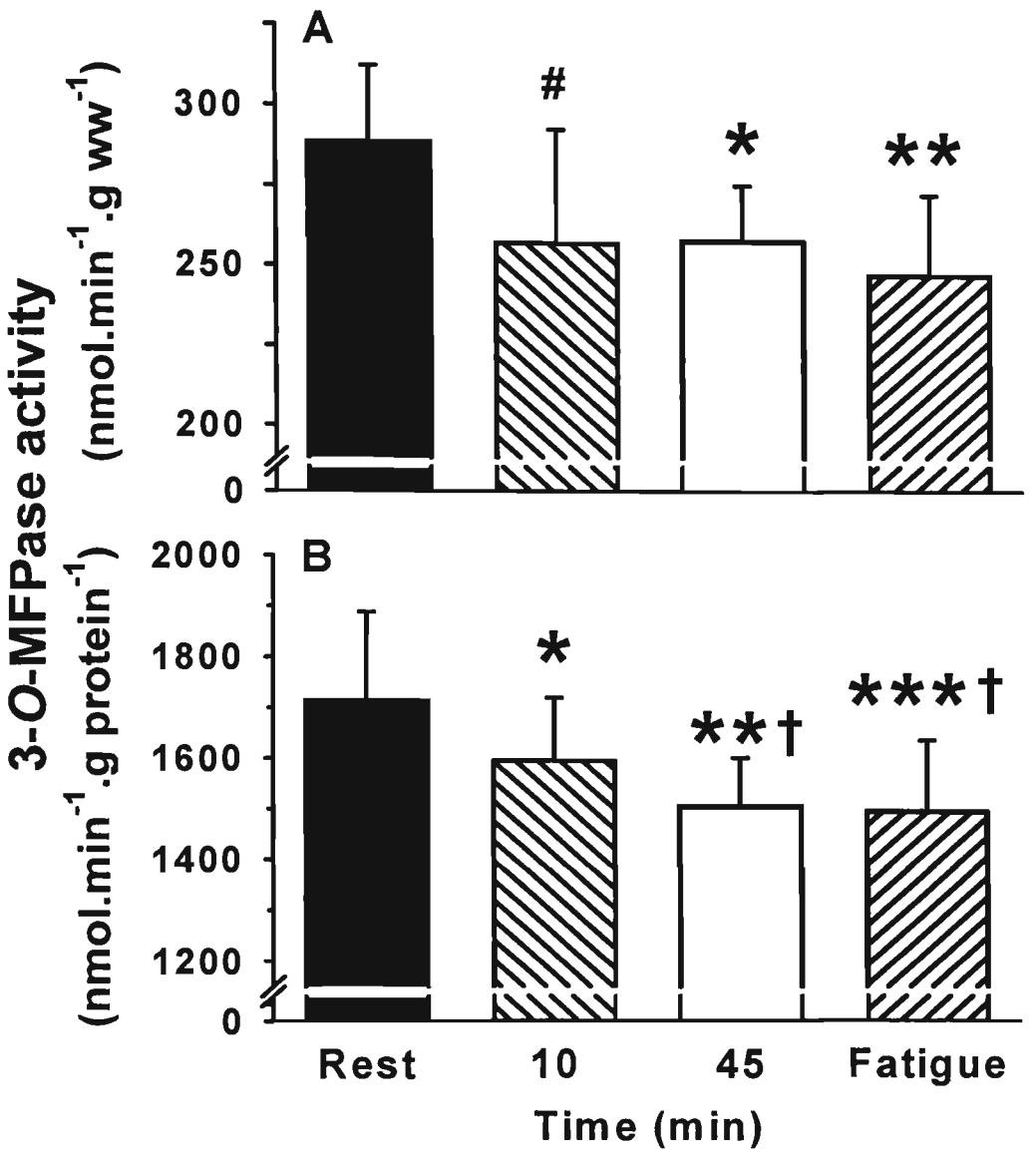

Figure 2. Depressed maximal 3-*O*-MFPase (Na^+,K^+ATPase) activity in skeletal muscle during prolonged cycling exercise to fatigue at 75% $\dot{V}O_{2peak}$.

Activity expressed as (A) nmol.min^{-1}.(g wet weight)$^{-1}$, (B) nmol.min^{-1}.(g protein)$^{-1}$ Data is Mean ±SEM, n=8, except at 45 min where n=7. Less than Rest, # $P< 0.10$, * $P<0.05$; ** $P<0.01$; *** $P<0.001$; Less than 10min † $P< 0.05$

4.7 Muscle SR Ca^{2+} Regulation

Assay [Ca^{2+}]. Starting [Ca^{2+}] prior to homogenate addition was 1734±202 nM (overall mean±SEM) and did not differ significantly between time points (Table 6). The post-homogenate [Ca^{2+}] fell to 568±21 nM, and was higher in the 10 min than in the rest and fatigue assays (P<0.05, Table 6). The end-uptake [Ca^{2+}] was low and similar between assay times, as was the pre-release [Ca^{2+}] (Table 6). The post-release [Ca^{2+}] did not differ between the first three assay times, but was less in the 45 min than in the fatigue assays (P<0.05, Table 6).

Table 6. Assay medium [Ca^{2+}] at selected assay time points (nM).

	Biopsy Sample Time (min)			
	Rest	10	45	Fatigue
Pre-homogenate [Ca^{2+}]	1478± 1231	2395± 1554	1512± 747	1546± 806
Post-homogenate [Ca^{2+}]	537± 27	664± 54 *	556± 36	516± 33 †
End-uptake [Ca^{2+}]	29± 3	44± 8	31± 4	42± 9
Pre-release [Ca^{2+}]	70± 5	107± 18	73± 5	92± 12
Post-release [Ca^{2+}]	150± 12	180± 19	139± 4 ‡	169± 13

Data expressed as Mean ±SEM, n=8.

* Different from rest assay P<0.05; † Different from 10 min assay P<0.05; ‡ Different from fatigue assay P<0.05

Maximal in-vitro SR Ca^{2+} uptake rate. SR Ca^{2+} uptake expressed per g wet weight was not significantly reduced from rest to fatigue (-25.3±11.6 %, P=0.09, Fig 3A). However, Ca^{2+} uptake expressed per g protein fell by 23.8±12.2% from rest to fatigue (P=0.05) and declined between 10 min and fatigue (P<0.05, Fig 3B). The intra-assay CV for SR Ca^{2+} uptake was 15.1% (n=31), whilst the inter-assay CV was not determined due to insufficient biopsy sample obtained. The SR Ca^{2+} uptake for the two resting biopsies (n=3) were 0.90±0.21 and 1.16±0.32 $\mu mol.min^{-1}$.(g wet weight)$^{-1}$.

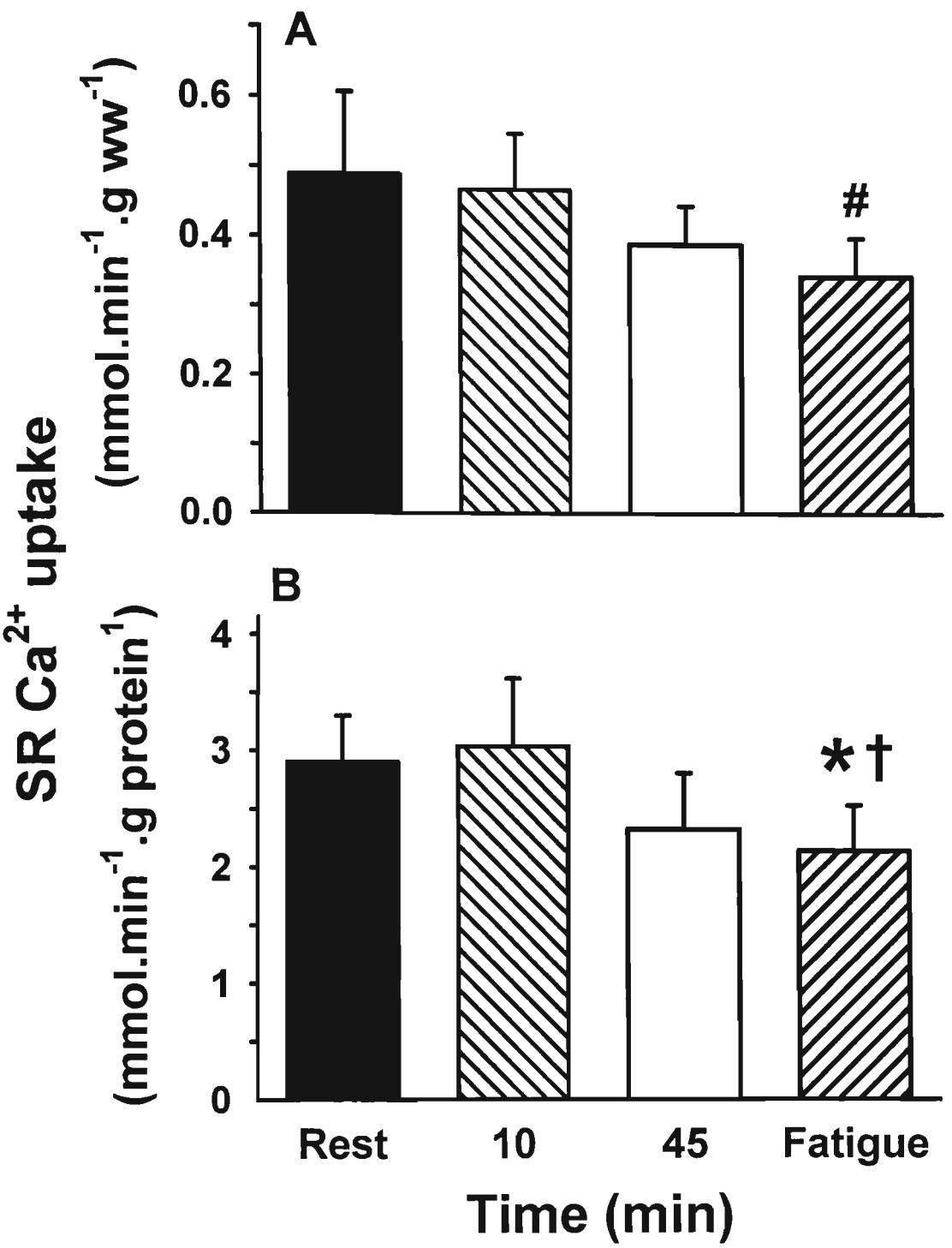

Figure 3. Depressed maximal SR Ca^{2+} uptake rate in skeletal muscle during prolonged cycling exercise to fatigue at 75% $\dot{V}O_{2peak}$.

Mean ±SEM, n=8 except at 45 where min n=7. Ca^{2+} uptake expressed as (A) $mmol.min^{-1}$.(g wet weight)$^{-1}$, (B) $mmol.min^{-1}$.(g protein)$^{-1}$.

Less than Rest, # $P< 0.10$, * $P<0.05$; Less than 10min † $P<0.05$

Maximal in-vitro SR Ca^{2+} release rate. SR Ca^{2+} release expressed per g wet weight (Figure 4A) was unchanged from rest to 10 min, but then fell below 10 min levels at 45 min ($P<0.05$) although this was not significantly less than at rest ($P<0.10$). A clear reduction in SR Ca^{2+}release was evident at fatigue, where Ca^{2+}release had decreased by 19.4±5.1% from rest ($P<0.01$) and was also less than at 10 min ($P<0.05$). When expressed per g protein (Figure 4B), SR Ca^{2+} release was less than rest at 45 min (-10.0±3.8%, $P<0.05$) and at fatigue (-17.9±4.1%, $P<0.01$). The SR Ca^{2+} release at both 45 min ($P<0.01$) and at fatigue ($P<0.05$) were less than at 10 min (Fig 4B). The intra-assay CV for SR Ca^{2+} release was 15.8% (n=28), whilst the inter-assay CV was not determined due to insufficient sample. The SR Ca^{2+} release for the two resting biopsies (n=3, measured at 1 Hz) were 0.61±0.10 and 0.56±0.02 $\mu mol.min^{-1}.(g\ wet\ weight)^{-1}$.

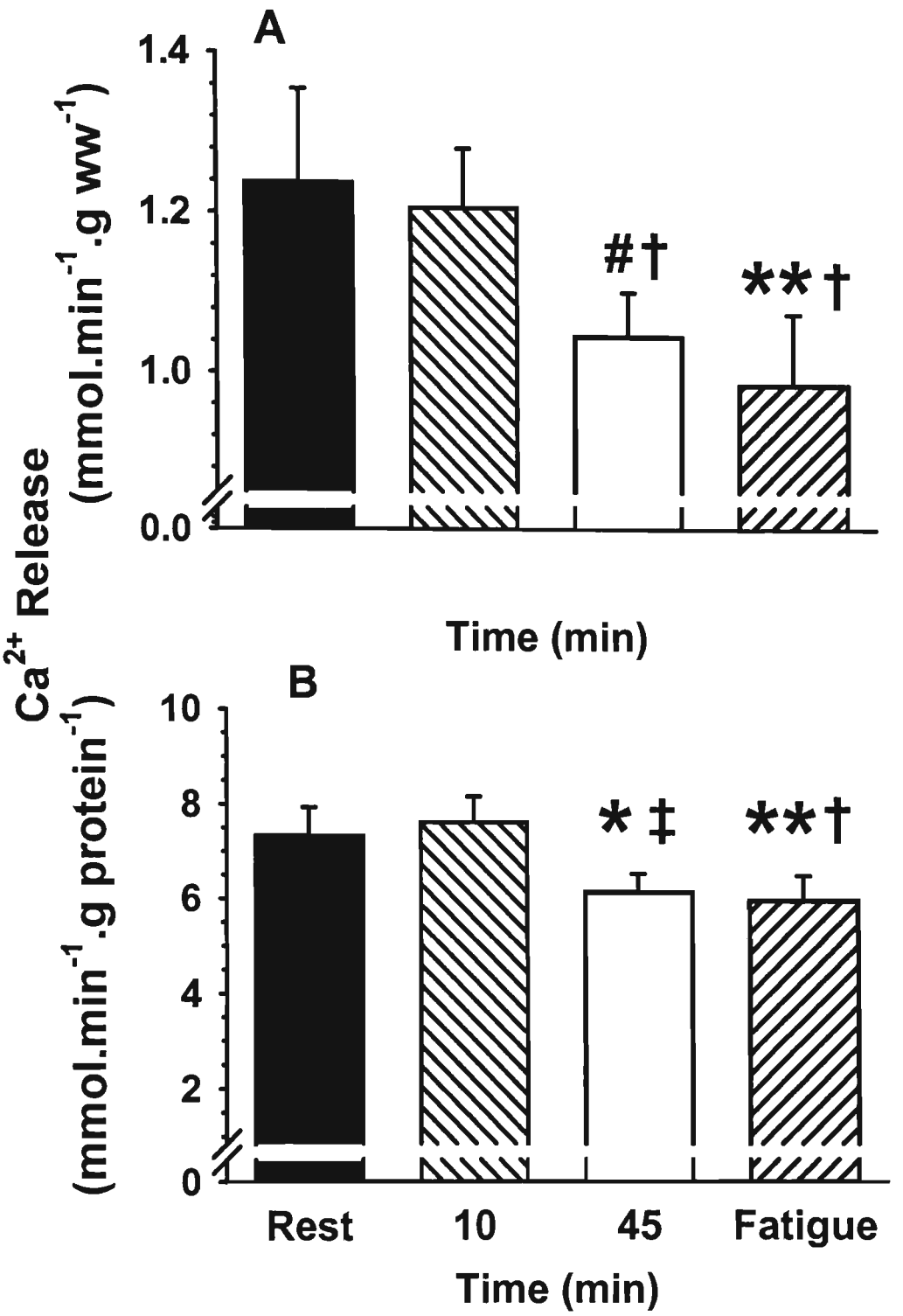

Figure 4. Depressed maximal SR Ca^{2+} release rate in skeletal muscle during prolonged cycling exercise to fatigue at 75% $\dot{V}O_{2peak}$.

Mean ±SEM, n=8 except at 45 min where n=7.units. Ca^{2+} release expressed as (A) mmol.min^{-1}.(g wet weight)$^{-1}$, (B) mmol.min^{-1}.(g protein)$^{-1}$. Less than Rest, # $P< 0.10$, * $P<0.05$, ** $P<0.01$; Less than 10min † $P<0.05$, ‡ $P<0.01$

Leupeptin effects on SR variables. SR Ca^{2+} uptake and release were compared in two resting biopsies and in a biopsy taken after 50 fatiguing maximal contractions (n=3), between muscle homogenised either in normal buffer, or in homogenising buffer plus 1mM leupeptin. *Ca^{2+} uptake.* Addition of leupeptin to the homogenising buffer lowered Ca^{2+} uptake [control homogenising buffer 1.07±0.12 versus buffer+leupeptin 0.60±0.07 μmol.min^{-1}.(g wet weight)$^{-1}$], $P<0.05$, n=9 rest and exercise pooled observations). No difference was found between rest and exercise (each n=3) for either the control [Mean rest, 1.03±0.25 vs fatigue 1.16±0.11 μmol.min^{-1}.(g wet weight)$^{-1}$] or buffer+leupeptin [Mean rest, 0.64±0.08 vs fatigue 0.53±0.13 μmol.min^{-1}.(g wet weight)$^{-1}$]. *Ca^{2+} release.* Addition of leupeptin to the homogenising buffer did not affect Ca^{2+} release (control buffer 0.54±0.04 versus buffer+leupeptin 0.51±0.06 μmol.min^{-1}.(g wet weight)$^{-1}$, n=9 pooled rest and exercise observations) For this experiment only, Ca^{2+} release was measured on data sampled at 1 Hz, thereby explaining the lesser release values than in the main study. Furthermore, Ca^{2+} release was depressed at fatigue ($P<0.05$, n=3) in both the control homogenising buffer [mean rest, 0.59±0.04 vs fatigue 0.45±0.03 μmol.min^{-1}.(g wet weight)$^{-1}$] and the buffer plus leupeptin [mean rest, 0.60±0.04 vs fatigue 0.32±0.05 μmol.min^{-1}.(g wet weight)$^{-1}$].

4.8 Maximal Isometric Voluntary Contraction Muscle Torque

The quadriceps maximal isometric torque was depressed at fatigue by 26% in these two subjects, similar to previous findings (Booth *et al.*, 1997). The peak isometric quadriceps torque at rest, 10 min, 45 min and at fatigue for the two

subjects were 120, 127, 109 and 88 Nm; and 169, 171, 155, 126 Nm, respectively. SR Ca^{2+} release for these two subjects was 9.13, 10.28, 7.67 and 8.07 μmol.min^{-1}. g $protein^{-1}$ and 6.51, 7.34, 5.00 and 4.59 mmol.min^{-1}.(g protein)$^{-1}$, respectively.

4.9 Relationships amongst muscle Na^+,K^+ATPase and SR variables

For resting muscle (n=8), 3-*O*-MFPase activity was correlated with Ca^{2+} uptake (r=0.79, $P<0.05$) but not with Ca^{2+} release (r=0.49) or [^{3}H]-ouabain binding (r=0.22), whilst Ca^{2+} release and Ca^{2+} uptake correlations were close to significance (r=0.69, $P<0.06$). With resting and exercise muscle data pooled (n=31), Ca^{2+} release and Ca^{2+} uptake were correlated (r=0.52, $P<0.01$) and each was also correlated with 3-*O*-MFPase activity (Ca^{2+} release, r=0.46, $P<0.05$; Ca^{2+} uptake, r=0.70, $P<0.01$).

4.10 Relationships between Na^+,K^+ATPase and SR variables and Exercise Performance

Resting muscle (n=8) 3-*O*-MFPase activity (r= -0.70, $P<0.05$), Ca^{2+} uptake (r= -0.80, $P<0.05$) and Ca^{2+} release (r=-0.68, $P<0.07$) were inversely related to time to fatigue during prolonged exercise. No significant correlations were found with $\dot{V}O_2$ peak. Fatigued muscle (n=8), 3-*O*-MFPase activity was inversely related with time to fatigue (r= -0.71, $P<0.05$), with no significant relationships found with Ca^{2+} release and Ca^{2+} uptake (r=-0.45, r=-0.50, respectively). The decline in Ca^{2+} release at fatigue tended towards a negative relationship with time to fatigue (r=-0.61, $P=0.11$), but no significant relationship was found between time to fatigue and decline in either 3-*O*-MFPase activity (r=-0.24, $P=0.56$) or Ca^{2+} uptake (r=-0.39, $P=0.35$).

Chapter 5. Discussion

This thesis shows for the first time in exercising humans, that skeletal muscle in-vitro maximal Na^+,K^+ATPase activity and sarcoplasmic reticulum Ca^{2+} release rates were significantly depressed during prolonged exhaustive exercise, thus confirming the first hypothesis. Furthermore, these changes were progressive when variables were expressed relative to muscle protein, therefore confirming the second hypothesis for these two variables. It is not known whether these impaired *in-vitro* muscle cation transport properties also reflect impairment *in-vivo*. If so, this would implicate membrane inexcitability and disruption to excitation-contraction coupling as important factors in muscle fatigue during prolonged exhaustive exercise in humans. Although there was a relationship between Na^+,K^+ATPase activity at fatigue and time to fatigue, this thesis was unable to demonstrate significant correlations between changes in *in-vitro* Na^+,K^+ATPase activity, SR Ca^{2+} release and SR Ca^{2+} uptake during prolonged exercise and time to fatigue and must therefore reject our third hypothesis. However, the absence of significant correlations may simply reflect the small sample size in this study, the variability in each of our assays and the likely multiplicity of factors contributing to fatigue.

5.1 Depressed maximal Na^+,K^+ATPase activity in skeletal muscle with fatigue

The maximal 3-*O*-MFPase (Na^+,K^+ATPase) activity was depressed by ~12% at fatigue. The decline with exercise also appeared to be progressive, being greatest at cessation of exercise due to fatigue. These findings confirm the first and second hypotheses in relation to Na^+,K^+ATPase activity, and are consistent with the importance of this decline in the progressive development of muscle fatigue (see

below). The validity of these findings is demonstrated since the K^+-stimulated 3-*O*-MFPase activity is fully inhibited by ouabain and is therefore specific to the Na^+,K^+ATPase enzyme (Fraser & McKenna, 1998); and since the variability of the 3-*O*-MFPase assay was low and less than the observed percentage depression with exercise. The finding of depressed maximal 3-*O*-MFPase activity during prolonged exercise is also consistent with other human studies with repeated maximal dynamic (Fraser *et al.*, 2002) and isometric contractions (Fowles *et al.*, 2002b). This finding contrasts the lack of reduction in rat muscle after 2 h running (Fowles *et al.*, 2002a); in that study a reduction was only observed after an additional 45 min of running occurred (Fowles *et al.*, 2002a). Thus, fatiguing exercise in humans reduces maximal 3-*O*-MFPase activity in muscle, across a diverse range of exercise types, durations and intensities, further suggesting that this is an obligatory response to exercise, as earlier suggested (Fraser *et al.*, 2002).

The decline in Na^+,K^+ATPase activity with prolonged exercise could not be attributed to a loss of Na^+,K^+ pumps, since no reduction in [^{3}H]-ouabain binding site content occurred. This is consistent with unchanged [^{3}H]-ouabain binding site content in human muscle after repeated isometric contractions (Fowles *et al.*, 2002b) and in rat soleus and EDL muscles after either brief, high-frequency, or prolonged, low-frequency electrical stimulation-induced muscle contractions (McKenna *et al.*, 2003b). A recent study did find a 10% increase in [^{3}H]-ouabain binding site content with prolonged exercise (Overgaard *et al.*, 2002). However, this occurred after 10 h of running, during which time Na^+,K^+ATPase synthesis is probable (Wolitzky & Fambrough, 1986).

5.2 Functional implications of impaired maximal Na^+,K^+ATPase activity

The progressive decline observed in Na^+,K^+ATPase activity during prolonged exercise is consistent with a role in fatigue. However, an important limitation in interpreting the importance of our findings is that depressed in vitro maximal activity does not directly reflect the functionally important in-vivo activity. Furthermore, the magnitude and the time-dependent pattern of depression in Na^+, K^+ ATPase activity might also differ from that observed in-vitro, due to additional effects of any localized decline in glycogen, phosphocreatine and ATP. Nonetheless, the marked K^+ fluxes in contracting muscle are consistent with a possible depressed maximal Na^+,K^+ATPase activity also occurring in-vivo. Muscle K^+ content is decreased during prolonged exercise [see references in (McKenna, 1992)] and muscles continually lose K^+ during submaximal contractions (Hallen, 1996; Verburg *et al.*, 1999), indicating a reduction in intracellular $[K^+]$. A widening of the arterio-venous $[K^+]$ difference also occurs across contracting leg muscles during fatiguing isometric contractions (Verburg *et al.*, 1999) and during cycling exercise at 67% $\dot{V}O_{2peak}$ (Sahlin & Broberg, 1989). Large increases in interstitial $[K^+]$ also occur in contracting muscle (Green *et al.*, 2000; Juel *et al.*, 2000b; Nielsen *et al.*, 2003). Whilst plasma $[K^+]$ reached only 5-6 mM during prolonged exercise in this study, a far greater increase in muscle interstitial $[K^+]$ is likely (Green *et al.*, 2000; Juel *et al.*, 2000b; Nielsen *et al.*, 2003).

Whilst the results do not allow us to conclude that depressed maximal Na^+,K^+-ATPase activity directly contributes to fatigue, it is nevertheless speculated that such a link exists. The combined effect of reduced intracellular and increased

interstitial [K^+] would be a greatly reduced intracellular-to-extracellular [K^+] ratio, which together with a possible decline in the Na^+,K^+ATPase-mediated electrogenic contribution, may then reduce membrane potential and excitability in some fibers (Sejersted & Sjogaard, 2000; Nielsen *et al.*, 2003). The functional significance of depressed Na^+,K^+ATPase activity with prolonged exercise can be inferred from studies in isolated rat muscles, in which Na^+,K^+ATPase inhibition by ouabain markedly enhanced fatigue development and retarded subsequent recovery (Everts & Clausen, 1994). The decline observed in Na^+,K^+ATPase activity at fatigue was also consistent with the decline in muscle isometric MVC in two subjects, which was similar to earlier studies (Sahlin & Seger, 1995; Booth *et al.*, 1997). The decline in maximal in-vitro Na^+,K^+ATPase activity and time to fatigue during exercise were non-significantly correlated, suggesting that depressed Na^+,K^+ATPase activity may have a limited role in muscle fatigue. However, correlational analyses are problematic with such a small sample size, especially when combined with the typical variability in these assays. Furthermore, it is highly probable that a multiplicity of factors contribute to fatigue, including impaired SR Ca^{2+} regulation (see below). Hence, the absence of a correlation between Na^+,K^+ATPase activity and performance time is not necessarily indicative of its contribution to impaired muscle function.

The relatively small depression in Na^+,K^+ATPase activity at fatigue with prolonged and intense exercise (~12-17%), may also question the functional significance of these findings. However, we do not know whether this depression is due to a similar, relatively small decline in Na^+,K^+ATPase activity in all muscle fibers, or reflects a more marked depression in Na^+,K^+ATPase activity in some fibers. Interestingly, the percentage decline in Na^+,K^+ATPase activity at fatigue is

similar to the percentage gain in total Na^+,K^+ pump content ([^{3}H]-ouabain binding) with intense exercise training in humans [see references in (McKenna, 1998)] and the percentage decline with inactivity (Leivseth & Reikeras, 1994). It is evident that the up- and down-regulation of Na^+,K^+ATPase in human muscle is much more restricted than observed in rat muscle (83%) with training and inactivity (Kjeldsen *et al.*, 1986). Hence a small relative decline in activity with fatigue might also be expected to have important adverse functional implications for human skeletal muscle.

Finally, Na^+,K^+ATPase inactivation could also be involved in the depression in muscle membrane excitability when under metabolic stress (Ortenblad & Stephenson, 2003). Hence, depressed maximal Na^+,K^+ATPase activity could then be an important contributory ATP conserving mechanism (Green, 1998) in a concerted downregulation of muscle function with fatigue. Further studies combining multiple in-vivo and in-vitro techniques are however, required to determine the functional importance of these findings.

5.3 Impaired SR Ca^{2+} Release and Ca^{2+} Uptake with Fatigue

This thesis reports for the first time in human skeletal muscle, that the maximal SR Ca^{2+} release rate, induced by 4-CmC, was depressed by 18% after prolonged exercise to fatigue. Greater reductions were evident at fatigue than after 10 minutes of exercise, suggesting a progressive decline in Ca^{2+} release. A major portion of the decline in SR Ca^{2+} release rate had already developed by 45 min of exercise with a further reduction at fatigue. These findings therefore confirm the first and second hypotheses in relation to SR Ca^{2+} release and are consistent with the proposed importance of depressed SR Ca^{2+} release as a causal factor in exercise cessation due to fatigue. The ~25% decline in SR Ca^{2+} uptake at fatigue with

prolonged exercise confirms the first hypothesis and also earlier findings (Booth *et al.*, 1997). Interestingly, there did not appear to be a progressive decline in SR Ca^{2+} uptake with exercise duration, with the fall being evident only at fatigue. Thus the second hypothesis is rejected with respect to SR Ca^{2+} uptake.

The methods used are valid for measurement of SR Ca^{2+} release and Ca^{2+} uptake rates. SR Ca^{2+} release was induced by 4-CmC, a potent agonist of the Ca^{2+} release channel (Herrmann-Frank *et al.*, 1996). As assays were performed under standardized in vitro conditions, the depressed SR Ca^{2+} release with fatigue most likely reflects structural alterations to the ryanodine receptor or to associated regulatory proteins. Reduced Ca^{2+} release with fatigue occurred in both the presence and absence of leupeptin in the homogenising buffer, suggesting that this reduction was not simply due to Ca^{2+}-activated protease degradation of the RyR during post-biopsy sampling and processing. It has previously been demonstrated that this measurement of SR Ca^{2+} uptake is mediated via SR Ca^{2+}ATPase activity, being inhibited by cyclopiazonic acid, and a close correspondence between SR Ca^{2+} uptake and Ca^{2+}ATPase activity has been reported (Ruell *et al.*, 1995; Booth *et al.*, 1997; Li *et al.*, 2002). Depressed SR Ca^{2+} uptake with fatigue in this study is therefore likely caused by inhibition of the SR Ca^{2+}ATPase enzyme (Gollnick *et al.*, 1991; Li *et al.*, 2002). The variability of the Ca^{2+} release and Ca^{2+} uptake assays (~15%) was greater than for measures of Na^{+},K^{+}-ATPase activity. This may explain the failure to detect significant differences in Ca^{2+} uptake results between rest and 45 min of exercise. Our maximal SR Ca^{2+} uptake and release rates are also higher than that previously reported in human muscle homogenates (Ruell *et al.*, 1995; Booth *et al.*, 1997; Li *et al.*, 2002; McKenna *et al.*, 2003a), due to the higher frequency of ratiometric data collection (10 vs 1 Hz), use of curve fitting techniques

to restricted time points and by not normalising the $d[Ca^{2+}]/dt/[Ca^{2+}]$ data to the corresponding assay free $[Ca^{2+}]$ (eg (Ruell *et al.*, 1995).

Reduced SR Ca^{2+} release channel opening, indicated by decreased ryanodine binding, was found with fatigue after prolonged exercise in rat soleus muscle (Favero *et al.*, 1993), consistent with the decreased SR Ca^{2+} release rate in rat red gastrocnemius and soleus muscles. This study measured Ca^{2+} release in vastus lateralis muscle, which has a mixed fiber composition; this may explain the smaller response than in rat oxidative muscles (Favero *et al.*, 1993; Inashima *et al.*, 2003). These results are also consistent with other human brief, intense exercise studies, which reported a larger 35-42% decline in SR Ca^{2+} release induced by Ag^{+} following fatiguing knee extensor exercise (Hill *et al.*, 2001; Li *et al.*, 2002). Studies that used electrical stimulation of isolated rat muscles to evoke fatigue also demonstrated a similar depression in SR Ca^{2+} release rate, together with depressed maximal force (Ward *et al.*, 1998; Williams *et al.*, 1998; Ortenblad *et al.*, 2000b). Thus depressed SR Ca^{2+} release appears to be a common factor with repeated muscle contractions, including intense and prolonged exercise, with the magnitude dependent on the intensity of contractions and on fiber composition, further suggesting an important role in fatigue.

5.4 Implications of impaired SR Ca^{2+} release and uptake for muscle fatigue

The depressed maximal SR Ca^{2+} release and Ca^{2+} uptake with prolonged exercise to fatigue were measured in-vitro and thus most likely reflect structural alterations to the ryanodine receptor, Ca^{2+} ATPase, and/or to associated regulatory proteins. These effects must presumably also occur in-vivo, suggesting important functional consequences. Whilst depressed in vivo Ca^{2+} uptake might initially act to

maintain cytosolic [Ca^{2+}] during contractions and thus preserve muscle force, continual depression in Ca^{2+} uptake may induce sustained exposure to elevated intracellular [Ca^{2+}], possibly initiating a protease-induced protein degradation, leading to myofibrillar disruptions (Lamb *et al.*, 1995) and reduced SR Ca^{2+} release (Chin *et al.*, 1997). Decreased Ca^{2+} uptake during exercise may also diminish SR Ca^{2+} loading, possibly leading to a decline in SR Ca^{2+} release in-vivo. Such a decline would be consistent with a declining maximal muscle force, evidenced here by the 26% decline in isometric MVC at fatigue in two subjects, consistent with earlier findings (Sahlin & Seger, 1995; Booth *et al.*, 1997). It is possible that both the magnitude and the time-dependent pattern of depression in SR Ca^{2+} regulation with prolonged exercise may have been different in-vivo, due to additional effects of any localized decline in glycogen, phosphocreatine and ATP. Reduced phosphocreatine (Booth *et al.*, 1997), elevated P_i and Ca^{2+}-P_i precipitation in the SR may have further depressed Ca^{2+} release (Lamb, 2002).

5.4.1 Relationship of SR and Na^+, K^+-ATPase changes with time to fatigue

Both SR Ca^{2+} release and Ca^{2+} uptake were depressed during prolonged submaximal exercise, suggesting that these may be important factors in muscle fatigue. However, no significant relationships were found between changes in either SR Ca^{2+} release or Ca^{2+} uptake with fatigue and time to fatigue during exercise. The lack of significance between these variables does not preclude their potential importance in fatigue, due to the small sample size, variability within each assay and in performance time. More mechanistic studies with a more homogenous and larger sample are required to resolve the in vivo functional significance of depressed SR Ca^{2+} release and Ca^{2+} uptake during exercise in human muscles.

The mechanisms causing depressed maximal Na^+,K^+ATPase activity, SR Ca^{2+} release and Ca^{2+} uptake with prolonged exercise are not known, but production of free radicals and cystolic Ca^{2+} accumulation might be responsible for degradation in maximal Na^+,K^+ATPase activity (Kukreja *et al.*, 1990; Sen *et al.*, 1995; Kourie, 1998; Sulova *et al.*, 1998) and decreased SR Ca^{2+} release (Chin & Allen, 1997; Chin *et al.*, 1997; Sulova *et al.*, 1998; Favero, 1999; Lamb, 2002; van der Poel & Stephenson, 2002) and most likely involve structural changes to the regulatory proteins.

5.5 Conclusion

In conclusion, this thesis shows a reduction in the function of major cation transport regulatory proteins in human skeletal muscle with fatigue induced by prolonged exercise, with depressed maximal Na^+,K^+ATPase activity and SR Ca^{2+} release and also confirm depressed Ca^{2+} uptake. The depression in Na^+,K^+ ATPase activity and SR Ca^{2+} release were also progressive, suggesting a role in muscle fatigue. It is possible these changes may reflect a concerted downregulation of Na^+, K^+ and Ca^{2+} transport properties with fatigue, as energetically conservative mechanisms for reduction in muscle function. This thesis was unable to demonstrate direct correlations between depressed *in-vitro* cation transport regulatory protein function and prolonged exercise performance. If these in-vitro changes are consistent with in-vivo impairments, these findings then suggest important roles for membrane excitability and excitation-contraction coupling in muscle fatigue during prolonged exercise in humans. Further studies are required to determine the direct functional significance of these in-vitro changes.

Chapter 6. Future Perspectives

To date, human exercise research studies have been limited to *in-vitro* measurements of SR Ca^{2+} release, SR Ca^{2+} uptake and Na^+, K^+ ATPase activity rates, usually within a muscle homogenate preparation. As such analyses are performed under optimal in-vitro conditions, changes in in-vitro SR Ca^{2+} release, Ca^{2+} uptake and Na^+, K^+ ATPase activity are most likely due to structural alterations to the channels and pumps themselves. Hence these measures do not take into account changes in metabolic factors surrounding the local microenvironment. The effect of such local metabolic perbutations, in addition to the structural changes that this thesis has identified, could augment the magnitude of depression in SR Ca^{2+} release, SR Ca^{2+} uptake and Na^+, K^+ ATPase activity compared to changes found in-vitro that might further precipitate fatigue. Thus far it has not been possible to measure in-vivo changes in these variables during exercise, due to methodological limitations.

Using force-pCa relationships in healthy human vastus lateralis muscle fibres (Lynch *et al.*, 1994), assuming proportionate reductions in SR Ca^{2+} release and tetanic cytosolic [Ca^{2+}] and physiological tetanic cytosolic [Ca^{2+}] (i.e. ~1 μM), we estimate that a 19.4% lower Ca^{2+} release at fatigue would reduce muscle force in Type I, IIA and Type IIB fibers by 10%, 16% and 19%, respectively. Although only measured in two subjects, isometric MVC declined by 26% at fatigue, comparable with these estimations and consistent with earlier findings (Sahlin & Seger, 1995; Booth *et al.*, 1997). Using these calculations the actual changes in muscle force found during prolonged exercise studies suggest a much greater decline in SR Ca^{2+} release in-vivo. Therefore we need to develop methodologies capable of

determining these changes in vivo, which would therefore allow a more accurate reflection of changes within the muscle during exercise.

An important future advance would be to measure changes in SR function and Na^+,K^+-ATPase activity in single individual muscles fibres. Such analysis may provide a more accurate reflection of changes that may occur within the muscle during a prolonged exercise bout.

Future studies should also involve both larger sample sizes and homogenous groups, such as either trained or untrained subjects, thereby producing greater consistency within the subject group and reducing the chances of a Type Π error.

Studies should also further investigate the importance of Ca^{2+} and reactive oxygen species in the decline of Na^+,K^+-ATPase activity, SR Ca^{2+} release and SR Ca^{2+} uptake.

Appendices

List of Appendices

Appendix #1 Subject information sheet.

Victoria University of Technology

Sample Consent Form for Subjects Involved in Research

INFORMATION TO PARTICIPANTS:

We would like to invite you to be a part of a study investigating possible causes of muscular fatigue during prolonged exercise.
CERTIFICATION BY SUBJECT

I,
of

certify that I am at least 17 years old* and that I am voluntarily giving my consent to participate in the experiment entitled:

"The effects of prolonged exercise on Na^+, K^+-ATPase and SR Ca^{2+} regulation in humans".

being conducted at Victoria University of Technology by: **Dr Michael McKenna and Jim Leppik**

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. **Maximal incremental exercise test on a cycle ergometer**
2. **Muscle function tests on a Cybex isokinetic dynamometer**
3. **Prolonged exercise test to fatigue on a cycle ergometer**
4. **Venous catheterisation and blood sampling during prolonged exercise test**
5. **Muscle biopsies at rest, and during prolonged exercise test.**

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

I have been informed that the information I provide will be kept confidential.

Signed: .....

Witness other than the experimenter: }

.....}

Any queries about your participation in this project may be directed to the researcher (Name: Dr M.McKenna ph. 9688-4499). If you have any queries or complaints about the way you have been treated, you may contact the Secretary, University Human Research Ethics Committee, Victoria University of Technology, PO Box 14428 MCMC, Melbourne, 8001 (telephone no: 03-9688 4710).

[*please note: where the subject/s is aged under 18, separate parental consent is required; where the subject is unable to answer for themselves due to mental illness or disability, parental or guardian consent may be required.]

Subject Information Sheet

"The effects of prolonged exercise on Na^+, K^+-ATPase and SR Ca^{2+} regulation in humans".

INVESTIGATORS:
Dr Michael J. McKenna and Jim Leppik,
Department of Human Movement, Recreation and Performance
Victoria University of Technology, Footscray, Vic.

Aim of the study:
This study will investigate the importance of several factors thought to contribute to muscle fatigue during prolonged exercise. These are the ability of your muscle to regulate changes in the concentrations of the electrolytes calcium, potassium and sodium in your muscles during exercise.

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. Your total time involvement will be from 3-4weeks.

Exercise Testing Procedures:
You will be required to attend the Exercise Physiology Laboratory (Building L), at the Footscray Campus of Victoria University of Technology on four separate 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 performance (VO_2 peak). This test involves continuous exercise with the exercise intensity (effort) becoming progressively greater. The test is completed when you have reached volitional exhaustion (wish to stop), or unless we stop the test due to you having an abnormal response to exercise. We will closely monitor you and your heart electrical activity (ECG) during exercise to ensure your safety.

The second visit will be to familiarise you with the techniques used to measure your maximal leg muscle strength and with the prolonged exercise test. This will involve performing maximal contractions on a special dynamometer in which the speed of your leg movement is controlled. These measures will be conducted before and at five minute intervals during exercise, which comprises riding a stationery cycle ergometer at 75% VO_2 peak for a total of 30 minutes.

The third test will involve prolonged exercise on a stationery cycle ergometer at 75% VO_2 peak continued until you fatigue. After 10 minutes, 45 minutes and at the point of fatigue you will perform maximal muscle strength measures. This is to determine whether your maximal muscle force is reduced with prolonged exercise.

The final test will involve an identical exercise trial to that undertaken in the previous test. The purpose of this test is to determine the reliability of the measures obtained in the previous trial.

Blood Samples:

At specific intervals throughout the exercise test a small blood sample (each 6 ml) will be taken via a catheter placed into a vein in the back of your hand. 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 that blood samples can be drawn from the catheter at specific times. This procedure allows the taking of multiple blood samples without the need for multiple venepunctures (puncturing of the vein). A total of less than one hundred (100) ml of blood will be taken during the test. (Normally 400 ml is taken when you donate at the Blood Bank). Each time a blood sample is taken, a small volume of sterile heparinised saline (1-2 ml) will be injected to clear the catheter and keep it patent. Catheterisation of subjects can cause slight discomfort and can lead to bruising and infection. However, 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. Blood samples will be analysed for metabolites, electrolytes and anti-oxidants.

Muscle Biopsies and Muscle Fatigue Testing:

On the fourth visit to the Exercise Physiology Laboratory, Footscray Campus muscle biopsies will be taken from your thigh muscle. These will be taken at rest, at 10 minutes, 45 minutes of exercise and at the point of fatigue. All four biopsies will be performed in the same exercise session and thus, all on the one day. Two biopsies will be performed on each leg. Muscle biopsies are routinely carried out in our laboratory, with no adverse effects.

The muscle biopsy procedure is used to obtain small samples of your muscle tissue for analysis of enzymes and energy sources. An injection of a local anaesthetic is made in the skin overlying the muscle in your thigh, and then a small incision (approx. 0.6 cm long) is made in the skin. The biopsy needle is then inserted into your muscle and a small piece of tissue removed from the muscle. During this part of the procedure you will feel pressure and this will be quite uncomfortable, but will last for only about 1-2 seconds. When the small piece of muscle is removed you may also experience a mild muscle cramp, but this only persists for a few seconds. Many people experience pain during this part of the procedure but this lasts only for 1-2 seconds. The size of muscle removed by the biopsy needle is similar to a medium sized grain of rice. This poses no long term effects for your muscle and will not be noticeable to others apart from a small scar on the skin for a few months. Following the biopsy the incision will be closed using a steri-strip and covered by a transparent waterproof dressing. Then 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. The soreness is due to slight bleeding within the muscle and is best treated by "ice, compression and elevation". An ice pack will be applied over the biopsy site after the biopsy procedure to minimise any bleeding and therefore

soreness. In some rare cases mild haematomas have been reported, but these symptoms disappear within a week. The whole procedure will be performed 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.

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.

Contact Numbers:

Dr Michael McKenna	**Work: (03) 9688-4499**	**H (03) 5422-6089**
Jim Leppik	**Mobile: 0407-905-366**	**H:(03) 5243-6908**

CARDIOVASCULAR RISK FACTOR QUESTIONNAIRE

In order to be eligible to participate in the experiment investigating:

"The effects of prolonged exercise on skeletal muscle ion regulation in humans."

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? Yes No

If 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: _____

Appendix #2. Subject exercise performance

Subject statistics				
Subject	**Time (min)**	**Power (W)**	**VO2 ($l.min^{-1}$)**	**$VO2_{peak}$ ($ml.kg^{-1}.min^{-1}$)**
1	57.13	205	3.89	54.6
2	94.06	250	4.02	54.7
3	82.09	213	3.91	58.8
4	78.23	243	4.67	57.0
5	65.10	287	4.93	67.3
6	85.57	140	2.67	45.8
7	37.13	202	3.77	53.9
8	78.12	210	3.55	48.1
n	8	8	8	8
mean	65.05	195.33	4.38	49.80
SD	18.59	46.17	0.74	7.11

Appendix #3 Subject physical characteristics

Subject	Age (years)	Body mass (kg)	Height (cm)
1	24	92.20	192
2	28	73.38	178
3	33	68.30	178
4	39	82.10	178
5	20	73.25	178
6	26	59.00	159
7	19	70.00	180
8	30	74.18	180
n	8	8	8
Mean	27.38	74.05	177.88
SD	6.67	9.79	8.98

Appendix #4. Plasma [K^+] at rest and during prolonged exercise at 74% $VO_{2\ peak}$.

PLASMA [K^+] ($mmol.l^{-1}$)					
Subjects	**rest**	**9 min**	**30 min**	**44 min**	**fatigue**
1	4.34	5.24	5.4	5.30	5.25
2	4.11	4.08	5.29	5.42	5.41
3	3.94	4.95	5.82	5.47	5.72
4	4.21	5.00	5.06	5.30	4.75
6	4.17	5.06	4.78		4.83
8	4.23	5.36	5.27	5.59	5.96
n	6	6	6	5	6
mean	4.17	4.95	5.27	5.42	5.32
SD	0.13	0.45	0.35	0.12	0.48
sem	0.05	0.18	0.14	0.05	0.20

Appendix #5. Respiratory Exchange Ratio (RER) during prolonged exercise at 74% $VO_{2\ peak}$.

	RER				
Subjects	**5 min**	**10 min**	**30 min**	**45 min**	**fatigue**
1	1.06	1.01	0.98	0.99	0.94
2	0.95	0.96	0.96	0.97	0.96
3	1.04	1.01	0.94	0.94	0.90
4	1.01	0.99	1.01	1.01	0.97
5	1.05	1.03	0.99	0.98	0.96
6	1.01	0.97	0.95	0.95	0.88
7	1.03	1.00	0.98		0.93
8	1.13	1.07	1.03	0.97	0.95
n	8	8	8	7	8
mean	1.04	1.01	0.98	0.97	0.94
SD	0.05	0.04	0.03	0.02	0.03
sem	0.02	0.01	0.01	0.01	0.01

Appendix #6. Hemoglobin and Hct at rest and during prolonged exercise at 74% $V0_{2\ peak}$

HEMOGLOBIN ($g.dl^{-1}$)					
Subject	**rest**	**9 min**	**30 min**	**44 min**	**fatigue**
2	15.2	15.4	16	16.4	16.3
3	14.5		15.8	15.7	15.9
6	12.9	13.8	13.6	13.5	13.6
8	15.6	16.8	16.7		16.9
n	4	4	4	4	4
mean	14.5	15.3	15.5	15.2	15.7
SD	1.2	1.5	1.3	1.5	1.5
sem	0.6	0.8	0.7	0.7	0.7

HCT (%)					
Subject	**rest**	**9 min**	**30 min**	**44 min**	**fatigue**
2	45.6	47	48.6	50.2	48.8
3	44.4		48.2	47.7	48.9
6	39.2	42.4	41.7	41.2	41.7
8	47.6	51.3	52.8		51.8
n	4	4	4	4	4
mean	44.2	46.9	47.8	46.3	47.8
SD	3.6	4.5	4.6	4.7	4.3
sem	1.8	2.2	2.3	2.3	2.2

Appendix #7. Skeletal muscle Na^+,K^+-ATPase activity at rest and during prolonged exercise at 74% $VO_{2\ peak}$.

	Na^+,K^+-ATPase activity ($nmol.g.ww^{-1}.min^{-1}$)					
Subject	**rest**	**10 min**	**45 min**	**fatigue**	**Δ rest-fatigue**	
					(%)	**($nmol^{-1}$)**
1	292.3	271.5	260.9	240.6	-17.7	-51.7
2	289.3	286.2	284.9	294.8	1.9	+5.5
3	313.1	190.9	249.3	240.0	-23.3	-73.1
4	264.6	218.3	248.1	219.4	-17.1	-45.2
5	281.5	246.1	225.5	234.4	-16.7	-47.1
6	306.4	282.2	268.2	233.6	-23.8	-72.8
7	315.2	276.8		276.8	-12.2	-38.4
8	248.1	284.4	268.7	236.0	-4.9	-12.1
n	8	8	7	8	8	8
mean	288.8	257.1	257.9	247.0	-14.2	-41.8
SD	23.6	35.5	17.6	25.3	8.9	27.3
sem	8.4	12.6	6.2	8.9	3.1	9.6

Appendix #8. Skeletal muscle Na^+,K^+-ATPase activity at rest and during prolonged exercise at 74% $VO_{2\ peak}$.

Na^+,K^+- ATPase (nmol.g.protein.min^{-1})						
Subject	**rest**	**10 min**	**45 min**	**fatigue**	**Δ rest-fatigue**	
					(%)	**(nmol^{-1})**
2	1554.4	1506.9	1491.3	1431.5	7.9	122.9
6	1604.3	1690.0	1498.2	1358.1	15.3	246.2
3	1665.4	1514.6	1529.1	1481.4	11.0	184.0
7	1854.3	1618.8		1667.7	10.1	186.6
1	2073.0	1835.1	1714.9	1748.8	15.6	324.2
4	1593.6	1537.4	1494.5	1496.8	6.1	96.8
8	1747.5	1615.8	1406.9	1404.6	19.6	342.9
5	1617.7	1439.5	1400.7	1371.4	15.2	246.3
n	8	8	7	8	8	8
mean	1713.8	1594.8	1505.1	1495.0	12.6	218.8
SD	174.6	125.0	96.7	141.7	4.6	88.1
SEM	61.7	44.2	34.2	50.1	1.6	31.2

Appendix #9. Skeletal muscle Na^+,K^+--pump content at rest and during prolonged exercise at 74% $VO_{2\ peak}$.

Na^+, K^+-pump content ($pmol.g.ww^{-1}$)				
Subject	**rest**	**10 min**	**45 min**	**fatigue**
5	405	391	380	401
6	311		259	260
7	319	348		307
3	318	339	395	
4	280	300	325	324
1	293		250	255
2		374	382	326
8	404	348	366	343
n	7	6	7	7
mean	332.9	350.0	336.7	316.6
SD	47.1	26.4	55.9	46.4
sem	16.7	9.3	19.8	16.4

Appendix #10 Skeletal muscle Ca^{2+} uptake at rest and during prolonged exercise at 74% $VO_{2\ peak.}$

Ca^{2+} uptake (mmol.g $ww^{-1}.min^{-1}$)					
Subject	**rest**	**10 min**	**45 min**	**fatigue**	**Δ rest-fatigue (%)**
1	0.64	0.82	0.77	0.48	25
2	0.40	0.32	0.57	0.36	10.4
3	0.62	0.49	0.43	0.51	17.2
4	0.28	0.56	0.36	0.35	-24.5
5	0.50	0.26	0.17	0.14	72.8
6	0.46	0.29	0.33	0.14	69.1
7	0.77	0.77		0.54	29.8
8	0.26	0.26	0.11	0.25	2.7
n	8	8	7	8	8
Mean	0.45	0.43	0.39	0.32	25.3
SD	0.15	0.21	0.23	0.15	32.7
sem	0.05	0.07	0.08	0.05	11.6

Appendix # 11. Skeletal muscle Ca^{2+} uptake at rest and during prolonged exercise at 74% $VO_{2\ peak}$.

Ca^{2+} uptake (mmol.g.protein^{-1}.min^{-1})					
Subject	**rest**	**10 min**	**45 min**	**fatigue**	**Δ rest-fatigue (%)**
1	4.50	5.66	5.08	3.61	19.9
2	2.13	1.66	2.97	1.72	19.1
3	3.27	3.87	2.62	3.14	4.0
4	1.70	3.93	2.18	2.33	-36.8
5	2.86	1.53	1.06	0.79	72.4
6	2.41	1.71	1.84	0.83	65.7
7	4.52	4.52		3.25	28.1
8	1.82	1.46	0.56	1.50	17.8
n	8	8	7	8	8
Mean	2.90	3.04	2.33	2.14	23.8
SD	1.12	1.65	1.37	1.11	34.4
sem	0.40	0.58	0.48	0.31	12.2

Appendix # 12. Skeletal muscle SR Ca^{2+} release at rest and during prolonged exercise at 74% $VO_{2\ peak}$.

	Ca^{2+} release ($mmol.g.ww^{-1}.min^{-1}$)				
Subject	**rest**	**10 min**	**45 min**	**fatigue**	**Δ rest-fatigue (%)**
1	1.07	1.23	1.17	0.71	34.1
2	1.21	1.39	0.95	0.95	21.9
3	1.72	1.30	1.25	1.31	23.9
4	1.00	1.14	0.95	0.71	29.4
5	1.21	1.17	0.90	0.91	24.8
6	0.88	0.78	0.85	0.81	7.9
7	1.78	1.16		1.34	24.6
8	1.04	1.48	1.25	1.15	-11.4
n	8	8	7	8	8
Mean	1.24	1.21	1.05	0.99	19.4
SD	0.33	0.21	0.16	0.25	14.5
sem	0.12	0.08	0.06	0.09	5.1

Appendix # 13. Skeletal muscle SR Ca^{2+} release at rest and during prolonged exercise at 74% $VO_{2\ peak.}$

Ca^{2+} release (mmol.g.protein^{-1}.min^{-1})					
Subject	**rest**	**10 min**	**45 min**	**fatigue**	**Δ rest-fatigue (%)**
1	7.62	8.55	7.68	5.36	29.6
2	6.51	7.34	5.00	4.59	29.5
3	9.13	10.28	7.69	8.07	11.7
4	6.02	8.06	5.71	4.68	22.4
5	6.94	6.83	5.60	5.31	23.5
6	4.62	4.65	4.76	4.72	-2.2
7	10.48	6.80		8.09	22.8
8	7.29	8.43	6.52	6.86	5.8
n	8	8	7	8	8
Mean	7.33	7.62	6.14	5.96	17.9
SD	1.72	1.55	1.11	1.50	11.6
Sem	0.61	0.55	0.39	0.53	4.1

Appendix #14. Effect of Leupeptin SR Ca^{2+}muscle homogenates at rest and 50 knee extensors exercise.

Leupeptin Study						
Ca2+ release (nmol .g.ww^{-1}.min^{-1})						
	rest 1		rest 2		fatigue	
Subject	control	leupeptin	control	leupeptin	control	leupeptin
9	0.43	0.52	0.61	0.67	0.41	0.23
10	0.77	0.64	0.55	0.72	0.51	0.36
11	0.62	0.41	0.54	0.66	0.41	0.37
n	3	3	3	3	3	3
mean	0.61	0.52	0.56	0.68	0.45	0.32
SD	0.17	0.11	0.04	0.04	0.06	0.08
SEM	0.10	0.08	0.02	0.02	0.03	0.05

Ca^{2+} uptake (nmol.g.ww^{-1}.min^{-1})						
Subject	rest 1		rest 2		fatigue	
	control	leupeptin	control	leupeptin	control	leupeptin
9	0.71	0.96	1.22	0.55	0.94	0.57
10	1.31	0.67	1.68	0.67	1.27	0.73
11	0.67	0.37	0.57	0.61	1.28	0.28
n	3	3	3	3	3	3
mean	0.90	0.67	1.16	0.61	1.16	0.52
SD	0.36	0.30	0.56	0.06	0.20	0.23
SEM	0.21	0.21	0.32	0.03	0.11	0.13

Appendix #15. Summary of effect of Leupeptin SR Ca^{2+} muscle homogenates at rest and 50 knee extensors exercise.

Summary-Leupeptin Study					
Ca2+ Release (nmol.g.ww.min^{-1})			Ca2+ Uptake (nmol.g.ww.min^{-1})		
	Control	Leupeptin		Control	Leupeptin
Rest 1	0.43	0.52	**Rest 1**	0.71	0.96
	0.77	0.64		1.31	0.67
	0.62	0.41		0.67	0.36
Rest 2	0.61	0.66	**Rest 2**	1.23	0.55
	0.55	0.72		1.68	0.67
	0.54	0.66		0.57	0.61
Fatigue	0.41	0.23	**Fatigue**	0.94	0.57
	0.51	0.36		1.27	0.73
	0.41	0.37		1.28	0.28
n	9	9		9	9
mean	0.54	0.51		1.07	0.60
SD	0.12	0.17		0.37	0.20
SEM	0.40	0.06		0.12	0.07

Appendix #16. $[Ca^{2+}]_i$ changes 10s after addition of 4-CmC at rest and during prolonged exercise at 74% $VO_{2\,peak}$.

Changes $[Ca^{2+}]_i$ ($nmol.l^{-1}$) 10s after addition of 4-CmC				
Subject	**rest**	**10 min**	**45 min**	**fatigue**
1	67.5	81.5	88	88
2	78	72	85	78
3	100	104.5	80	97.5
4	60	71.5	71.5	67.5
5	110.5	69	56	58.5
6	59	43.5	45.5	40.5
7	116	79.5		117
8	45	58.5	42	68
n	8	8	7	8
Mean	79.5	72.5	66.9	76.9
SD	26.3	17.7	17.6	23.9
sem	9.3	6.3	6.2	8.4

Appendix #17. $[Ca^{2+}]_i$ 10s after addition of 4-CmC at rest and during prolonged exercise to fatigue at 74% $VO_{2\ peak}$.

$[Ca^{2+}]_i$ (nmol.l^{-1}) 10s after addition of 4-CmC				
Subject	**rest**	**10 min**	**45 min**	**fatigue**
1	111	125	146	159
2	166	243	151	160
3	175	189	150	171
4	137	148	130	137
5	189	174	153	217
6	109	106	123	153
7	188	254		231
8	122	200	123	122
n	8	8	7	8
Mean	149.6	179.9	139.4	168.8
SD	33.8	52.8	13.6	37.5
sem	12.0	18.7	4.8	13.2

Appendix #18. Changes in $[Ca^{2+}]_i$ from addition of homogenate to addition of CPA at rest and during exercise at 74% $VO_{2\ peak}$ (Ca^{2+} uptake curve).

Changes $[Ca^{2+}]_i$ ($nmol.l^{-1}$) from addition of homogenate to addition of CPA				
Subject	**rest**	**10 min**	**45 min**	**fatigue**
1	550	602	699	501
2	449	606	466	531
3	518	449	462	510
4	490	727	510	459
5	394	481	357	362
6	558	438	589	371
7	631	787		656
8	471	702	589	656
n	8	8	7	8
Mean	512.9	584.3	513.8	484.3
SD	77.6	137.0	117.9	100.9
sem	27.4	48.4	41.7	35.7

Appendix #19. $[Ca^{2+}]_i$ prior to addition of 4-CmC at rest and during prolonged exercise to fatigue at 74% $VO_{2\ peak}$ (after addition of CPA).

$[Ca^{2+}]_i$ ($nmol.l^{-1}$) prior to addition of 4-CmC				
Subject	**rest**	**10 min**	**45 min**	**fatigue**
1	44	44	58	71
2	88	171	66	82
3	75	85	70	73
4	77	77	58	69
5	78	105	97	158
6	50	62	78	113
7	72	174		114
8	77	141	81	54
n	8	8	7	8
Mean	70.1	107.4	72.6	91.8
SD	15.1	49.5	12.9	34.1
sem	5.3	17.5	4.6	12.1

Appendix #20. Starting $[Ca^{2+}]_i$ at rest and during prolonged exercise at 74% VO_2 peak.

Starting $[Ca^{2+}]$ ($nmol.l^{-1}$)				
Subject	**rest**	**10 min**	**45 min**	**fatigue**
1	867	2032	1129	797
2	970	4347	1093	1013
3	1229	987	1067	2043
4	765	1927	1287	1101
5	847	978	684	1210
6	1294	805	2663	3178
7	1378	4669		2002
8	4471	3418	2663	2002
n	8	8	7	8
Mean	1477.6	2395.4	1512.3	1668.3
SD	1230.7	1553.7	747.0	789.5
sem	435.1	549.3	264.1	279.1

Appendix #21. $[Ca^{2+}]_i$ prior to addition of CPA at rest and during prolonged exercise at 74% $VO_{2\,peak}$ (end Ca^{2+} uptake).

$[Ca^{2+}]_i$ ($nmol.l^{-1}$) prior to addition of CPA (end Ca^{2+} uptake)				
Subject	**rest**	**10 min**	**45 min**	**fatigue**
1	25	29	20	31
2	27	73	22	26
3	19	14	21	19
4	38	33	24	26
5	19	61	47	85
6	30	31	42	75
7	31	44		55
8	45	70	42	55
n	8	8	7	8
Mean	29.3	44.4	31.1	46.5
SD	9.0	21.4	11.0	24.7
sem	3.2	7.6	3.9	8.7

Appendix #22. $[Ca^{2+}]_i$ after addition of homogenate at rest and during prolonged exercise at 74% $VO_{2\ peak}$.

$[Ca^{2+}]_i$ ($nmol.l^{-1}$) after addition of homogenate				
Subject	**rest**	**10 min**	**45 min**	**fatigue**
1	575	801	719	532
2	476	679	488	558
3	537	463	483	528
4	528	759	534	486
5	413	541	404	447
6	588	468	631	446
7	662	831		711
8	516	772	631	711
n	8	8	7	8
Mean	536.9	664.3	555.8	552.4
SD	74.9	151.9	101.0	105.7
sem	26.5	53.7	35.7	37.4

Appendix #23. Maximal isometric knee extensor strength at rest and during prolonged exercise at 74% $VO_{2\ peak}$.

CYBEX (isometric torque Nm)					
Subjects	**rest**	**10 min**	**45 min**	**fatigue**	**change from rest (%)**
2	169	171	155	126	25.4
3	120	127	109	88	26.7
n	2	2	2	2	2
mean	169	171	155	126	25.4
SD	34.6	31.1	32.5	26.9	0.9
sem	24.5	22.0	23.0	19.0	0.6

Maximal isometric knee extensor strength changes from rest during prolonged exercise at 74% $VO_{2\ peak}$

Subject	**10 min (% of rest)**	**45 min (% of rest)**	**fatigue (% of rest)**
2	101.2	91.7	74.6
3	105.8	90.8	73.3
n	2	2	2
mean	103.1	91.3	74.0

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